TRANSFORMATION OF LEUKOCYTES BY THEILERIA ANNULATA

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PhD

University of Edinburgh

1988
Dedicated to my grandfather, Thomas,

my parents Anne and Tom,

and to Patsy.
The experimental work described in this report was carried out in the Department of Animal Genetics, University of Edinburgh and at the Wellcome Unit of Molecular Parasitology, University of Glasgow, between October 1985 and August 1988. The research was supported by a studentship granted by the Medical Research Council.

This thesis and the results reported therein, are all my own work.

Michael Dyer

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PUBLICATIONS
When the protozoan parasite *Theileria annulata* invades its bovine host, one of the pathological states of the resultant disease (Tropical Theileriosis) is lymphoproliferation. This follows entry of the parasites into the leukocytes. The aim of this project was to characterise the transformed phenotype of *Theileria*-infected leukocytes using an approach involving molecular biology and protein chemistry, and thus to provide a better understanding of the mechanism by which the parasite interferes with the leukocyte growth regulatory pathways to induce proliferation. The results described in this thesis demonstrate a number of features unique to *T. annulata*-transformed cells:

1. A collection of oncogene probes, known to be homologous to genes encoding various components of growth regulatory pathways, were used in the detection of several such genes which were increased in expression as a result of *Theileria*-infection. Infected and uninfected leukocyte cell lines were used for comparison. The growth rate of infected cells was also formally shown to be increased relative to uninfected cells.

2. The same oncogene probes were also used to search for *Theileria*-encoded genes which, being homologous to oncogenes, would be candidates for giving rise to products which may interfere with the host growth control pathways, resulting in transformation. *T. annulata* genomic sequences were detected which were homologous to a known transforming oncogene, itself encoding a tyrosine-specific protein kinase. This same oncogene probe also hybridised to an RNA transcript specifically found in *Theileria*-infected leukocytes, and to a cloned DNA fragment which was isolated from a *T. annulata* genomic DNA library.

3. Infected and uninfected cell lines were compared for differences both in protein kinase activity and in the profile of phosphorylated substrates, due to the well-documented association between cell transformation and altered protein kinase activity. A number of phosphoproteins were detected both in vivo and under in vitro conditions - some of which were specific to *Theileria*-infected cells and others to uninfected cells. These phosphoproteins were characterised for ionic requirements, autophosphorylation activity and
cellular location. An assay for detecting many protein kinase enzyme activities in several samples simultaneously was developed, and revealed protein kinases both unique to, and altered in activity in, Theileria-infected leukocytes.

(4) A method was developed for the isolation of the intra-leukocytic parasite, in order to distinguish events which were occurring in the parasite itself, from those occurring in the host cell. The method was fully characterised for purity of the preparations, recovery and damage to the parasites by microscopic, protein content and DNA sequence criteria.

The relationship between those genes found to be stimulated in expression as a result of T.annulata-infection, and components of growth regulatory pathways is discussed, as is the potential importance of the various phosphoproteins and protein kinases which were unique to, or altered in, Theileria-transformed cells. The implications of these findings with respect to hypothesised molecular models of Theileria annulata-induced leukocyte transformation are explored.
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CHAPTER 1

INTRODUCTION
1.1. **THEILERIA ANNULATA (A REVIEW)**

1.1.1. **Tropical Theileriosis**

The genus *Theileria* contains protozoan parasites and is classified in the phylum *Apicomplexa* (*Sporozoa*), class *Sporozoa* and order *Piroplasmida* (Levine et al., 1980). The species *Theileria annulata* is the causal agent of tropical theileriosis in cattle and domestic buffalo, which is endemic in a geographical belt extending from North Africa, through the Middle East, Southern U.S.S.R. to India and China (Purnell, 1978) (Figure 1.1). *T. annulata* is transmitted by ticks of the genus *Hyalomma* in most of the areas where the tick vector is established (review by Robinson, 1982).

An estimated 250 million cattle are at risk to infection, and although tropical theileriosis is a major constraint on livestock production and improvement (Robinson, 1985), no accurate data is available as to the resultant loss of productivity from mortality and debilitation in many of the endemic areas. However, in one survey of Central Anatolia, Turkey, blood smear examination showed that 18% of a random sample of 996 cows were infected with *T. annulata*. In addition, tropical theileriosis was diagnosed in 53% of 306 sick cows brought to the clinics at Ankara Veterinary Faculty in the summer seasons of 1982 to 1985. The data showed that the mortality of tropical theileriosis in infected cattle varied from 43% to 95% according to cattle breed, (Sayin, 1986). A similar survey carried out at the Animal Disease Diagnostic Laboratory, NDDB, Anand in India, revealed that 64% of sick crossbred and exotic cattle were positive on blood smear examination for tropical theileriosis in 1985-1986 (Singh, 1986), thus confirming the widespread existence of infection. While mortality varies from 40% to 95% in susceptible exotic cattle (*Bos taurus*), indigenous cattle (*Bos indicus*) reared in endemic areas suffer mortality rates of only around 5% and this is confined mainly to calves (Neitz, 1957). Thus, the disease is most prevalent wherever crossbred and exotic cattle are maintained. Due to the advent of large scale crossbreeding programmes of low yielding indigenous cattle
Figure 1.1

The distribution of tropical theleriosis (adapted from Purnell, 1978)
with exotic breeds in order to enhance milk and meat production, a cattle population more susceptible to tropical theileriosis is rapidly increasing and losses have already assumed serious proportions.

1.1.2. Theileria: Life cycle (see also figure 1.2)

The Theileria parasite undergoes a complex life cycle in the tick vector and bovine host as depicted in figure (1.2). Ticks of the genus Hyalomma responsible for transmitting *T. annulata* may feed on cattle two or three times during their lives: three host ticks as larvae, as nymphs and as adults, or, if two-host ticks, as larva and nymph on the host and then as adults on a second host. Transmission is thought to occur most commonly when ticks feed on infected cattle as nymphs and then on disease-free animals as adults.

1.1.2.1. Tick Vector Stages

There are two stages comprising the life cycle of Theileria in the tick vector - gamogony and sporogony, as described by Cowdry and Ham (1932) and Mehlhorn and Schein (1984). Piroplasms within the erythrocytes of the bovine host are the parasite stage ingested by feeding ticks. These develop to gametes which undergo syngamy in the tick gut and the resultant zygotes enter the cell lining of the gut where they further differentiate into kinetes. These migrate via the haemolymph, appearing as sporoblasts in the salivary glands while the tick is moulting (Schein et. al., 1975; Schein et. al., 1977). The sporoblasts form an elaborate intracellular syncytium which undergoes segmental fission after the tick commences feeding at the next instar, thus giving rise to sporozoites (Samish and Pipano, 1978). The sporozoites are then secreted in the tick saliva (Purnell and Joyner, 1968).

1.1.2.2. Mammalian Host Stages

The life cycle of Theileria in the bovine host has been well characterised and has been reviewed by Wilde (1967). A bovine host is infected by the inoculation of sporozoites in the saliva of an
**Figure 1.2**

**LIFE-CYCLE**

- **Type III Acinus**
  - Sporozoites
  - Lymphocyte
  - Macrophage
  - Erythrocyte

- **Tick Salivary Gland**
  - Sporoblast syncytiun
  - Sporoblast
  - Kinetes

- **Tick Gut**
  - Zygote

- **Bovine Lymphoid Tissue and Blood**
  - Micromerozoite
  - Gamonts
infected tick while it feeds. These invade leucocytes (primarily of the type expressing class II MHC antigens – B cells and macrophages. Spooner et. al., in press) very rapidly. Some sporozoites of Theileria annulata were observed to complete penetration within 5 minutes, in an in vitro invasion experiment (Jura et. al., 1981) and most by 30-60 minutes. Thus rapid entry of theilerial sporozoites into host cells is thought to occur by passive endocytosis as it involves little or no motor activity on the part of the parasite or target cell and can occur at 4°C (Fawcett et. al., 1982). Jura et. al., (1983), however, disagree, since they showed the interiorisation to be an active, temperature-dependant process. It is thought to depend largely upon progressive circumferential binding of ligands on the parasite to receptors on the host cell membrane and invagination of the target cell plasmalemma, followed by shedding of the sporozoite surface coat, as it enters the cell. (Webster et. al., 1985). Once interiorised, the sporozoites round up and enlarge to form trophozoites (Jura, 1981; Stagg et. al., 1981) and by the third day post infection, the parasites undergo nuclear division to form macroschizonts by which time the host cells have undergone transformation to become large lymphoblastoid cells (Brown et. al., 1973). There is a synchronous division of the host cell as the macroschizont divides, with at least one parasite being distributed to each daughter cell, such that a huge population of parasitised lymphocytes results (Hulliger et. al., 1964; Morrison et. al., 1981). In the infected animal, the macroschizonts develop into forms containing many more nuclei, termed microschizonts and result in the host cell rupturing to release uninucleate merozoites. The merozoites invade erythrocytes and develop into piroplasms, the stage infective for the tick vector, thus completing the parasite life cycle (Sergent et. al., 1945).

1.1.3. Pathogenesis

The pathogenesis of tropical theileriosis is associated with both parasite induced lymphoproliferation and the progressive anaemia due to the phagocytosis and destruction of infected erythrocytes (Barnett, 1977). Parasitaemias as high as 80% can occur in exotic cattle infected with T. annulata and in contrast to the related T. parva, it is this stage of the parasite which probably results in the major
pathogenic changes, (Neitz, 1957).

The disease is first manifest as a swelling of the lymph nodes draining the sites where the infected ticks fed. As the cells of the lymphoid system and parasitised lymphoblasts are stimulated to a massive proliferation, fever develops and the animal loses condition. The appearance of killer cells in the peripheral blood (see 1.1.4) coincides with the onset of extensive destruction of parasitised and non-parasitised cells in the lymphoid tissues during the terminal stages of the disease (Preston and Brown, 1983). Death, in susceptible animals, if it occurs, is usually 3 to 4 weeks after infection. A record of 700 cases in the Hissar region of India, recorded clinical signs including lymphadenopathy, fever, anaemia and weakness as well as continuous feeding and ruminating, urticarial skin eruptions, and haemoglobinuria, (Sharma, 1986). Relapses are common and recovered animals act as a source of infection for the vector, since they maintain a low level of parasitised erythrocytes (Neitz, 1957).

1.1.4. Immunity

As well as resulting from natural, or deliberate infection, immunity to tropical theileriosis can also be induced by vaccination with attenuated macroschizont-infected cell lines (Pipano, 1981) although immunised cattle are not completely protected against challenge by heterologous parasites (Gautam, 1981). This method is widely used in many countries as a moderately effective vaccine which itself produces no clinical symptoms when used to inoculate susceptible cattle (Hall, 1988; Hashemi-Fesharki, 1988). Successful immunisation relies on establishing active infection in the bovine host. Since the immunoising cells will almost certainly be allogenic and hence rejected, it is thought that macroschizonts transfer into the hosts' cells, in order to establish a transformed population. Similarly, strong immunity is seen in cattle which have recovered from a primary infection of *T. annulata*, to challenge with the same stock of parasite and a variable degree of immunity is exhibited to challenge with heterologous stocks (Gill et al., 1981). A carrier status exists upon recovery, with persistence of piroplasms, and immunity wanes significantly within one year (Neitz, 1957).
The existing evidence indicates that antibody-independent, cell-mediated mechanisms comprise the most significant protective immune response to infection with *Theileria annulata* and that the primary target of this response is the macroschizont-infected lymphocyte (see Hall, 1988 for review). In particular, Preston et al., (1983) reported appearance of cytotoxic cells in the blood and lymph nodes, in calves recovering from tropical theileriosis. One population of these cytotoxic cells appeared BoLA (bovine lymphocyte antigen - Spooner, et al, 1979) (MHC - Major Histocompatibility Complex)-restricted, and to be analogous to cytotoxic T cells while another population comprised non-BoLA restricted natural killer-like cells. The results suggested "a role for cytotoxic cells in recovery from primary infection in inhibition of proliferation of macroschizonts which evade mechanisms of acquired resistance and in the lysis of macroschizont-infected cells deriving from challenge sporozoites which have evaded serum-mediated inhibition" (Preston, et al., 1983).

However, because those cytotoxic responses were observed to be transient, Preston and Brown, (1988) searched for other important effectors of the cellular immune response. In an *in vitro* assay, they demonstrated that adherent cells (presumed to be macrophages) isolated from the peripheral blood of immunised calves over a period of several weeks post-inoculation, exhibited strong cytostatic effects on macroschizont-infected lymphocytes. These cells were active against both autologous and allogenic cells infected with either homologous or heterologous parasite stocks to those used in the primary infection.

Spooner et al., (1986), demonstrated that animals could be efficiently protected against *T. annulata*, using MHC mismatched cell lines as a vaccine in doses as low as $10^2$ cells. In this case, the 'graft rejection' phenomenon may be beneficial by interfering with the development of the parasite within the animal allowing it time to develop protective immunity.

Monoclonal antibodies have now been raised and isolated, which recognise antigens specifically on the surface of macroschizont-infected lymphocytes; potential initiators of the cellular immune responses. (Shiels, et. al., 1986 -- see 1.3). Some of the
antigens which they recognise have been well characterised (Taracha, 1985; Preston, et. al., 1986; Hall, 1988) and the genes encoding them cloned for study. Such molecules may eventually provide the basis for a molecular vaccine.

Although at present there is no evidence of cell-mediated immunity operating against the sporozoite stage of Theileria, the sporozoite appears to represent an ideal target for the immune system, being an extra cellular stage, however transient. Preston and Brown (1985) have shown that sporozoite neutralising activity was present in sera from animals immunised after a primary challenge. Williamson, S., Centre for Tropical Veterinary Medicine, University of Edinburgh (personal communication), has since raised and characterised two monoclonal antibodies which neutralise sporozoites in vitro. The antigens recognised by these antibodies may be used alone or included as a component in a molecular vaccine along with macroschizont antigens to increase overall protection.

Another potentially effective component of such a molecular vaccine would be antigens from the merozoite and piroplasm stages of the parasite which elicit an immune response. Although there is at present no evidence for immunity to these stages, immunity, if induced, would aid in blocking transmission and reducing severity of the clinical symptoms by controlling the level of parasitaemia, and hence perhaps the anaemia.

1.1.5. Control Measures

The major methods used in the control of tropical theileriosis in the field are vector control and vaccination with attenuated macroschizont infected lymphocytes.

1.1.5.1. Vector Control

Vector control is achieved primarily by dipping, or spraying in acaricides. However, since a few ticks on animals ensures continued protection (through maintaining immune animals which recover from infection with a low inoculation) of the herd from epidemics of
tropical theileriosis, tick control at high levels can result in herds of susceptible animals which are at high risk in the event of breakdown of tick control. There is also a real risk of ticks acquiring resistance to acaricides. Moreover, anything interrupting a stringent acaricide programme provides a threat to the livestock at risk. This was recognised in Zimbabwe, where there have been rapid increases in tick numbers and tick-borne diseases where acaricide dipping programmes failed as a result of interruption by civil war; there had been total reliance on dipping as a means of control. (Lawrence, 1981; Suthest and Tahori, 1981). There are, therefore, certain benefits in maintaining tick levels high enough to ensure early infection of calves while not causing too heavy production losses from fatal infection or tick worry. Emphasis is now placed on minimal dipping, as part of an integrated approach to control (Sutherst and Tahori, 1981). Such an approach will enable host resistance to ticks and immunity to tropical theileriosis to be expressed rather than suppressed by intensive dipping.

Since well-adapted local breeds of cattle exhibit a significant degree of resistance to ticks, (Sutherst and Tahori, 1981) whereas many types of imported exotic cattle do not, this variation is sufficient to promote selection for tick resistance. Preliminary results (Sutherst and Tahori, 1981) suggest that host resistance can usefully contribute to the control of Rhipicephalus appendiculatus and thus limit the rate of transmission of Theileria parva, the related parasite which is responsible for East Coast fever - a similarly debilitating cattle disease. The same authors suggest that data from Uganda and South Africa show it will be even more effective against other important tick species including Hyaloma anatolicum, the vector for T. annulata.

The technique of tick control by sterile male release has also been considered (Srivastava, 1981; Cunningham, 1981). Srivastava and Sharma, (1976 a and b) demonstrated induction of sterility of H.anatolicum anatolicum males by exposure to gamma radiation. However, experiments must be performed to see if these sterile male ticks, released in large numbers, could compete with normal males in mating and survival ability in order to reduce the tick populations. Many physiological and ecological factors must be considered before this becomes a component of the control programme against tropical
Chemotherapy

In recent years, some progress has been made in chemotherapy of tropical theileriosis, due to the discovery of effective theilericidal drugs. However, as yet there has been no extensive use of these drugs in the field as there is none which has been tested and shown to fit all the requirements of effectiveness, safety, reliability and cost. The two existing classes of drugs in most common use against theileriosis are the febrifugines and the naphthoquinones.

Naphthoquinone. The first naphthoquinone to show anti-theilerial activity was menoctone, whose in vitro activity and in vivo activity against *T. annulata* was reported by McHardy (1978) and McHardy et al., (1980) respectively. This drug, which destroyed intralymphocytic macroschizonts was further developed, and an analogue, parvaquone (993C) (Clexon; Coopers Animal Health) has since proven to be the most promising of this class of compounds acting in vivo against *T. parva* (McHardy et al., 1983). 993C was not quite so effective against infection with *T. annulata*. In fact, another analogue, 720C (Butalex, Coopers Animal Health) has proven more promising in trials involving cattle infected with *T. annulata*. Singh (1986) reported that a single injection of this drug at 2.5 to 5.0mg/kg body weight was effective against tropical theileriosis.

Febrifugines The febrifugine, halofuginone hydrobromide has been shown to be active against *Theileria annulata* in clinical cases. However, when this salt came into contact with mucous membranes of the treated animal, it proved to be a severe irritant. This created a problem since this drug could only be used when administered orally, as injections caused violent local reactions. (see Schein and Voigt, 1981, for a review). Availability of halofuginone lactate circumvented this problem, since this compound was not a serious irritant. In vitro screening of this drug against *T. annulata* macroschizont-infected lymphocytes showed that 0.025ppm of halofuginone lactate reduced the schizont index from 82% (control cells) to 42%. (Schein, 1986). In a number of trials described by Schein and Voigt, (1981) using the lactate form of the drug on cattle
infected with *T. annulata*, all treated cattle responded. Within 24 hours, the number of macroschizonts found in lymph node smears was reduced and were showing increasing signs of degeneration, and after 4 days schizonts were no longer detected. Halofuginone did not, however show any effect on the piroplasm stage of the life cycle, and so cattle remained latent carriers of parasites after treatment. All cattle which recovered from acute tropical theileriosis were immune to a further homologous challenge.

So, although the febrifugines and naphthoquinones were not, at first, considered to be as effective against *Theileria annulata* as against *Theileria parva*, these developments give sufficient optimism to warrant further research into improving the therapeutic safety margin and to reduce costs.

1.1.5.3. **Immunisation**

As described fully in (1.1.4), the only effective method of immunising cattle against *T. annulata* infection which is of practical use in the field, is to establish an active infection with attenuated macroschizont-infected lymphocytes (Pipano, 1981). Attenuation is achieved by passaging an *in vitro* culture of lymphocytes infected with an isolate of *T. annulata*, until the parasites have lost their virulence and will not cause clinical symptoms when inoculated into susceptible cattle. $10^6$ to $5 \times 10^6$ cells comprise one dose of the vaccine, but since cells survive freezing down and resuscitating, they can be used to seed fresh cultures indefinately. Thus the vaccine is relatively simple to prepare (Hashemi-Fesharki, 1988 for review). It has been used in many countries such as Israel, Iran and Russia, and is being developed in many others at present (see Hall, 1988) It may be that different vaccines are best developed for each area, according to the strain(s) of the parasite present, (Sergent, et. al., 1945) although protection against heterologous challenge is often very good (Gill, 1981).

Another technique in control is now showing promise - that of "infection and treatment", whereby cattle are actively infected with the parasite and simultaneously treated chemoprophylactically with tetracyclines so that mild or inapparent reactions result, and an
immune status is produced (Pipano, 1981; Gill, et. al., 1976). Radley (1981) reported success in immunising against T.annulata infection by infecting with sporozoites and treatment with one or two doses of long-acting oxytetracycline. However, the degree of protection conferred to subsequent challenge with heterologous strains has not been fully investigated.

The cost of implementing these control measures must be carefully evaluated, as must the effectiveness of each, and the risks involved in developing a widespread carrier status in immune animals. Clearly much has to be considered before effective control can be attained - even with the effective methods available.
1.2. **CELLULAR GROWTH CONTROL and TRANSFORMATION**

The aim of this project (fully outlined in section 1.3.7) was to investigate the mechanism by which *T. annulata* induces host leukocytes to undergo transformation into lymphoblastoid cells (1.1.2.2). Before reviewing the current knowledge on Theileria-induced transformation, it is necessary to explain the present understanding of the mechanisms involved in growth regulation of vertebrate cells and how this can be interrupted to produce the transformed phenotype.

1.2.1. **Introduction**

In recent years, our understanding of the mechanisms which regulate vertebrate cell proliferation, has expanded at such a rapid rate that it has become increasingly difficult to keep abreast of the latest developments. For this reason, this review presents an overview of the current concepts, avoiding lengthy descriptions of primary data. In attempting to cover such a subject which has a relatively wide scope, it is difficult to provide a comprehensive account, and so emphasis has been placed on well-characterised systems and examples. This expansion of knowledge applies both to normal cells, whose growth is tightly controlled, and also to transformed or cancerous cells, which divide in an uncontrolled manner. A number of genes have been identified through molecular and biochemical studies, whose products are involved in regulating normal cell growth. In contrast, many genes which are capable of inducing a transformed phenotype have been identified. The remarkable progress of the past few years in dissecting mechanisms of growth control, both normal and abnormal, has been stimulated by the demonstration that these two groups of genes are in fact largely the same - a concept which had long been widely believed, but had remained speculative. Although unequivocal evidence for this notion was obtained only recently, the concept itself was formed over a period of time from the analysis of the genes which are responsible for malignant transformation by certain tumour inducing viruses.
1.2.2. Retroviruses

Retroviruses have a diploid genome, composed of two identical subunits of single-stranded RNA (3.5 to 9.0 kilobases in length). The subunits each have features of eukaryotic messenger RNAs (mRNAs) as they are capped and polyadenylated, and furthermore, their replication - competent genomes contain at least three genes for structural proteins, gag, pol and env. During the initial phase of infection of mammalian cells, the viral RNA is used as a template of synthesis of linear duplex DNA bearing Long Terminal Repeats (LTRs): the LTR units are composed of sequences present uniquely near the 3' and 5' ends of viral RNA (U3 and U5) plus a short sequence (R) present at both ends of viral RNA. The structure of the complete linear DNA may be represented as U3RU5 - (genes) - U3RU5. In addition to the linear species, there are two major forms of circular DNA in acutely infected cells - covalently closed molecules one bearing one copy of the LTR, and the other bearing two copies. Insertion of the retroviral genome into the mammalian genome can occur at many sites, perhaps at random, resulting in the proviral form with the simultaneous generation of a short duplication of 4-6 base pairs (bp) of host DNA which then flank the provirus. Viral genes are then transcribed from the 5' LTR, utilising host RNA polymerase II for synthesis of full length transcripts which terminate at the end of the 3' LTR. The transcriptional products may then be used directly (for gag proteins), packaged as virion RNA, or processed by splicing to generate mRNA's for pol and env (Varmus and Swanstrom, 1982). Most retroviral proteins are synthesised as polyproteins that must be cleaved (and sometimes glycosylated or phosphorylated) to form mature structural gene products (see Varmus and Swanstrom, 1982). The LTR sequences, as well as containing signals for initiation of transcription and polyadenylation, are also involved in transcriptional regulation (e.g. by steroid hormones - see Ucker, et al., 1983), and, in some cases, can enhance the efficiency of linked, heterologous promoters in a non-polar fashion (eg. Ucker, et al., 1983). They also act as sites for homologous recombination eliminating proviral DNA from the chromosome at a very low frequency.

Several strains of these retroviruses are capable of inducing transformation of their mammalian host cells.
1.2.3. **V-onc** Viruses

Several strains of retroviruses rapidly induce fatal tumours following infection of experimental animals of the appropriate host species. The same strains also induce the transformation of cultured cells from one or more lineages to a neoplastic phenotype (for review, see Bishop and Varmus, 1982). Analysis of these retroviral genomes has led to the identification of some two dozen different transforming genes, known as viral oncogenes (v-onc), which are distinct from the genes required for viral replication (see table in Fig. 1.3 for a list of oncogenes). Viruses lacking a v-onc gene are incapable of transforming cultured cells, and, with only a few exceptions (see 1.2.4), induce tumours only after a prolonged latency or are not oncogenic at all.

1.2.4. **Oncogenic Activity of v-onc Viruses**

v-onc- retroviruses, unlike v-onc+ retroviruses (1.2.3) contain no onc gene, and have a reduced capacity to transform their host cells. Two general hypotheses have received the most attention as explanations for the oncogenic activity of v-onc- viruses: (1) that some component of the viral genome itself may serve as an oncogene (or transforming gene); and (2) that proviruses may act as mutational agents, thereby affecting the control of normal host genes. These two hypothesised mechanisms are explained below:

(1) Since unassigned open reading frames of substantial length are rare among the viral genomes which have been sequenced, candidates for viral genes that could stimulate tumorigenesis have been sought mainly among the structural genes. For example, evidence implicates env genes of certain murine leukaemia viruses in leukaemogenesis (Oliff et. al., 1980), although the mechanism by which this occurs is not understood.

(2) Retroviruses could produce mutations in infected cells in several ways. As agents that introduce their genomes more or less randomly into host chromosomes, they are capable of inactivating host genes by interrupting them. This capacity is exemplified by the insertional inactivating mutations produced by murine leukaemia virus (MLV) proviruses that have been integrated within a Rous sarcoma virus
### Figure 1.3

#### Retinoviral oncogenes

<table>
<thead>
<tr>
<th>Virus</th>
<th>Origin</th>
<th>Gene</th>
<th>Protein</th>
<th>Virus Disease</th>
<th>Viral Product</th>
<th>Human Chromosome</th>
</tr>
</thead>
<tbody>
<tr>
<td>RSV</td>
<td>chicken</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>inner side of plasma membrane</td>
<td>19q13-13q21</td>
</tr>
<tr>
<td>MLV</td>
<td>chicken</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>inner side of plasma membrane</td>
<td>19q13-13q21</td>
</tr>
<tr>
<td>FeSV-ASV</td>
<td>FeSV-ASV</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>plasma membrane</td>
<td>15q24-q23</td>
</tr>
<tr>
<td>PK-FeSV-ASV</td>
<td>PK-FeSV-ASV</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>plasma membrane</td>
<td>15q24-q23</td>
</tr>
<tr>
<td>PKR-FeSV-ASV</td>
<td>PKR-FeSV-ASV</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>plasma membrane</td>
<td>15q24-q23</td>
</tr>
<tr>
<td>GFP-FeSV-ASV</td>
<td>GFP-FeSV-ASV</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>plasma membrane</td>
<td>15q24-q23</td>
</tr>
<tr>
<td>VSV-FeSV-ASV</td>
<td>VSV-FeSV-ASV</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>plasma membrane</td>
<td>15q24-q23</td>
</tr>
<tr>
<td>ALV-FeSV-ASV</td>
<td>ALV-FeSV-ASV</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>plasma membrane</td>
<td>15q24-q23</td>
</tr>
<tr>
<td>VSV-FeSV-ASV</td>
<td>VSV-FeSV-ASV</td>
<td>Ppa50</td>
<td>sarcoma</td>
<td>PK(p7)</td>
<td>plasma membrane</td>
<td>15q24-q23</td>
</tr>
</tbody>
</table>

**Note:**
- RSV = Rous sarcoma virus; ASV = avian sarcoma virus; FeSV = fowl sarcoma virus; PK = pre-B cell leukemia virus; MLV = murine leukemia virus; ALV = avian lymphoma virus; REV = retrovirus; MLV = murine sarcoma virus.
- The different letters indicate specific viral strains.
- The numbers indicate specific viral strains.

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*(adapted from Franks and Teich, 1986)*
(RSV) provirus responsible for the transformed phenotype of the host, thus reversing the transformed phenotype (Varmus, et al., 1981). Although not resulting in transformation, an insertional mutation by a retrovirus is also apparently responsible for the dilute - coat-colour allele in certain inbred mouse strains (Jenkins et. al., 1981). However, mutations that inactivate genes are likely to be recessive lesions, and the target gene for an oncogene mutation of this type would have to be either sex-linked, or inactivated on both autosomal chromosomes to produce the tumourous phenotype. A more promising possibility, is that a provirus would activate the expression of host genes, particularly genes adjacent to the integration site. Such lesions would be dominant in most cases, permitting a single insertion event to induce a tumour. Evidence for this mechanism in B-cell lymphomas will be provided (1.2.8.4.1).

A consideration of mutational mechanisms in which v-onc-viruses might induce tumours, focuses attention upon the host genes that might be implicated in the proposed mechanisms of transformation.

1.2.5. **Cellular Oncogenes (c-onc) Homologous to Retroviral Oncogenes**  
  (Proto-oncogenes)

The first transforming oncogene identified belonged to Rous sarcoma virus (RSV) which causes cancer in chickens; the gene was termed src for sarcoma. (Other oncogenes have since been designated by analogous three-letter abbreviations). The protein which is encoded by this gene was then isolated and termed p60src, because its molecular weight was found to be 60 kilodaltons. In 1975, J.M.Bishop and H.E.Varmus found that DNA sequences nearly identical to that of the src oncogene, were present in all chicken cells. Shortly after, Stehelin et. al., (1976) demonstrated, by hybridisation, that DNA sequences homologous to transforming gene(s) of avian sarcoma viruses were present in normal avian DNA. By now, all of the oncogenes which have been isolated from retroviruses (about two dozen - see fig.1.3) that cause carcinoma, sarcoma, leukaemia or lymphoma in chickens, other birds, rats, mice, cats or monkeys, have been found to be closely related to a normal gene in the host animal and to encode an oncogenic protein similar to a normal protein. All of these cellular oncogene homologues (c-oncs) have been highly conserved in the course
of evolution. For example, the \texttt{c-src} gene is found not only in all vertebrate animals, but also in the fruit-fly \textit{Drosophila}, while the \texttt{c-ras} gene is present in species ranging from yeast to man (Hunter, 1984).

Because of the identification of \texttt{c-onc} genes in the more primitive phyla, it seemed unlikely that the \texttt{c-oncs} represented genes of endogenous retroviruses, since their host cells are restricted to vertebrates. The application of restriction endonucleases and molecular cloning has provided evidence for this deduction, by revealing the vertebrate homologues of retroviral oncogenes to be normal cellular genes: most are unique loci, situated at constant chromosomal positions within any given species (with the exception of the \texttt{ras} genes which constitute a multigene family, Ellis et. al., 1981); some display minor structural polymorphisms of the sort found in recognised cellular genes; several have intervening sequences that interrupt the coding sequence of the gene; and none has been found either within, or even linked to the proviruses of endogenous retroviruses (Bishop, 1981).

The acute transforming retroviruses are believed to represent recombinants in which a transforming gene, derived from a homologous gene of normal cells, has been inserted into a retrovirus genome. That the \texttt{c-onc} genes are the progenitors of the viral \texttt{onc} genes is now generally assumed, from evidence provided by phylogenetic patterns: the \texttt{c-onc} genes are found in many genera, spanning long periods of evolutionary time, whereas the \texttt{v-onc} genes are generally found only in single strains of retroviruses which were isolated from particular species. Also, the homology (as defined initially by hybridisation studies and later by comparing DNA sequences) between \texttt{v-onc} and cellular DNA is greatest for the species from which the viral oncogene allegedly originated (Bishop, J.M., 1981).

Although it is also generally accepted that retroviruses assimilate \texttt{c-oncs} (proto-oncogenes) by recombination, the precise mechanism by which this occurs remains speculative, even though deliberate efforts to recover new oncogenic sequences from cells by viral transduction have proven successful (Rapp and Torado, 1980).
1.2.6. **Tumourigenicity of v-onc genes**

Two general mechanisms have been proposed for the activation of proto-oncogenes to transforming genes upon transduction by a retrovirus, both of which are implicated in different systems. Firstly, the resultant transforming genes (v-oncs) of these viruses are expressed at high levels in virus-infected cells as a result of being under the control of viral transcriptional regulatory sequences. For example, the amount of viral pp60src in chicken cells transformed by Rous sarcoma virus was reported to be around 100-fold higher than the amount of cellular pp60 src in normal chicken cells (Collet et. al., 1978; Karess et. al., 1979). It is therefore possible that transformation by these viruses is a consequence of abnormal expression of genes which are functionally identical to the cellular proto-oncogene. Secondly, transformation might result from structural differences between the viral and cellular proteins. A well-studied v-onc which comes under this categorization is v-erb B (see 1.2.8.2.2).

The fact that retroviruses which have captured cellular oncogenes and are able to induce grossly abnormal cell proliferation raised speculation that proto-oncogenes participate in regulating the growth of normal cells.

1.2.7. **Tumourigenicity of c-onc genes**

**DNA Transfection**

Evidence linking proto-oncogenes to the induction of neoplasias by non-viral mechanisms was obtained using the technique of DNA transfection. In this assay, detection of oncogenic activity of cellular genes depends on the biological activity of purified cellular DNA. Hill and Hillova (1971) achieved the first successful transfer of biologically active total cellular DNA, with the demonstration that DNA of cells transformed by Rous sarcoma virus, induced transformation of recipient cultures of chicken embryo fibroblasts as a consequence of transfer of the viral genome. Transfer of biologically active eukaryotic DNA's, termed transfection, is usually performed by exposure of recipient cell cultures to donor DNA in the form of a
calcium phosphate precipitate, and in a typical assay $10^6$ recipient cells might be exposed to 20 micrograms of donor DNA. The most commonly used recipient cells are NIH 3T3 (non-neoplastic) mouse cell lines, since transformation of these cells occurs by stable integration of donor DNA's with high efficiencies of approximately 0.1 to 1.0 transformants per microgram of suitably 'active' donor DNA (eg. Murray et al., 1981). These cells have been used as recipients for most transfection assays of either viral or cellular transforming genes and have revealed two mechanisms of activation of c-onc genes.

(a) C-onc Activation by Altered Expression: This technique has also been used to support the hypothesis that normal cell genes could induce oncogenic transformation. This has been demonstrated both by transfection of molecular clones containing DNA homologous to retroviral transforming genes, and by transfection of total genomic DNA of normal vertebrate cells. The cellular mos (v-mos being isolated from the Moloney sarcoma virus) and ras proto-oncogenes lacked transforming activity when associated with their normal cellular flanking sequences (De Feo et al., 1981). However, when these proto-oncogenes were ligated to viral transcriptional regulatory sequences, their transformation potential was activated (De Feo et al., 1981) to an efficiency comparable to that of the homologous sarcoma virus DNA's. These experiments demonstrated that in some cases, abnormal expression of the cellular homologues of retroviral transforming genes is sufficient for induction of oncogenic transformation.

In accordance with this, Cooper et al., (1980) found that transfection of 0.5 to 5 kilobase DNA fragments from normal cells induced transformation at low efficiencies (about 0.003 transformants per microgram of DNA). Subsequent use of DNA isolated from these transformed cells in the same assay, induced transformants at high efficiencies (0.1 to 1.0 transformants per microgram DNA), indicating that these transformed cells contained activated transforming genes that could be efficiently transmitted by transfection. It appeared that the DNA rearrangements which occurred during integration of donor DNA in transfection assays, resulted in the activation of proto-oncogenes. These potential transforming genes may, for instance, dissociate from their normal regulatory sequences and when placed under the control of efficient transcription promoters, result in
abnormal gene expression and transformation. Activated transforming genes would then be transmitted at high efficiencies as single transforming units in the secondary transfection assays of transformed cell DNA's.

(b) C-onc Activation by Mutation: Murray et. al., (1981) and Krontiris and Cooper (1981) found that DNA from a human bladder tumour cell line contained a gene which induced a tumourigenic phenotype at high efficiency (0.1 to 1.0 transformants per microgram of DNA) following transfer into non-malignant cultured cells. Subsequent molecular cloning and analysis of this gene revealed it to be the cellular homologue of the the viral oncogene v-ras which was previously identified in the Harvey strain of murine sarcoma virus. In addition, the critical difference between the normal ras proto-oncogene and its "activated" counterpart from the tumour cells resided in a single nucleotide change, which led to one amino-acid substitution in the ras gene product (Taparowsky et. al., 1982).

Since these initial experiments, analysis of DNA's from different types of neoplastic cells, derived from several different vertebrate species including man, induce transformation of NIH 3T3 cells with high efficiencies. These neoplasms have included spontaneously occurring tumours, chemically induced tumours, virus-induced tumours, and cell lines derived from them (eg. Shih et. al., 1981; Lane et. al., 1982). Carcinogenesis in various neoplasms thus appears to involve dominant genetic alterations, either by mutations or gene rearrangements, which result in the activation of cellular transforming genes that are then detectable by transfection. It should also be noted that approximately 50 per cent of chemically induced and spontaneous tumours do not efficiently transform NIH3T3 cells on transfection (eg. Shih et. al., 1981; Lane et. al., 1982). In these cases, carcinogenesis may have involved either epigenetic changes or recessive genetic alterations which would not be detectable by transfection of tumour DNA's. Alternatively, they may have been caused by particular dominant activated transforming genes which are not capable of inducing transformation of the NIH 3T3 cells used as recipients in the transfection assays.

Subsequent analysis of the isolated active c-onc genes which do transform cells in these assays, has demonstrated that some contain mutated, amplified and/or translocated proto-oncogenes, further
supporting the notion that alterations in proto-oncogenes play a role in malignant transformation.

1.2.8. **Function of Oncogenes**

Parallel with these studies on transforming abilities of oncogenes, progress was also being made in elucidating the biochemical function of certain viral oncogenes and, by implication, of the homologous cellular proto-oncogenes.

1.2.8.1.1. **Tyrosine Kinase Class of Oncogenes**

Levinson et. al., (1978) demonstrated that the oncogene present in Rous sarcoma virus (RSV), known as the v-src oncogene, encodes a product which catalyses the transfer of a phosphate group from ATP onto various protein substrates. Protein phosphorylation had long been known to occur in normal cells and was thought to play a role in regulating protein function (see 3.4.1). However, Hunter and Sefton (1980) found that the v-src protein kinase (pp60 v-src) differs from all other protein kinases previously described: the viral enzyme invariably transfers the phosphate group onto a tyrosine residue and not onto serine or threonine. The novel kinase activity was subsequently found to be shared by the products of several other retroviral oncogenes including v-abl, v-fes/fps, v-yes, v-fgr, v-ros, v-mos, v-erb B and v-fms as well as the c-src protein (for review, see Hunter and Cooper, 1985). Figure (1.4a) shows, in tabulated form, some features of these tyrosine kinases, while figure (1.5) shows their structures. These findings raised much interest, since phosphorylation of tyrosine residues in proteins appears to be a rare modification of cellular proteins: in normal cells, only 0.02% of phosphorylated residues are tyrosine; 90% are serine and 10% are threonine. However, in transformed cells, the percentage of phosphotyrosine may be increased to around 0.2% of the total phosphorylated residues, suggesting that tyrosine-specific protein kinases are in some way involved in transformation.
### (a) Viral

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Virus strain</th>
<th>Subcellular location</th>
<th>Protein product</th>
<th>Phosphate donor</th>
<th>Cation</th>
</tr>
</thead>
<tbody>
<tr>
<td>v-erb</td>
<td>Rous sarcoma virus (various strains)</td>
<td>Plasma membrane</td>
<td>p90**</td>
<td>ATP, GTP</td>
<td>Mg**</td>
</tr>
<tr>
<td>v-fps</td>
<td>Fujinami sarcoma virus</td>
<td>Plasma membrane</td>
<td>p150**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td></td>
<td>PRC II sarcoma virus</td>
<td></td>
<td>p105**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td></td>
<td>PRC IV sarcoma virus</td>
<td></td>
<td>p150**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td></td>
<td>Rochester sarcoma virus</td>
<td>(URI)</td>
<td>p90**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td>w-yes</td>
<td>Y73 avian sarcoma virus</td>
<td>Plasma membrane</td>
<td>p90**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td></td>
<td>Esh sarcoma virus (ESV)</td>
<td></td>
<td>p85**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td>w-ras</td>
<td>UR-3 virus</td>
<td>Plasma membrane</td>
<td>p55**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td>v-abl</td>
<td>Anemia murine leukemia virus</td>
<td>Plasma membrane</td>
<td>p110**</td>
<td>ATP, GTP</td>
<td>Mg**, C**, Mn**</td>
</tr>
<tr>
<td>v-fes</td>
<td>Feline sarcoma virus</td>
<td>(Sonder-Holt strain) Plasma membrane</td>
<td>p90**</td>
<td>ATP, GTP</td>
<td>Mg**</td>
</tr>
<tr>
<td>c-erl</td>
<td>Gardner-Arnstein strain</td>
<td>Plasma membrane</td>
<td>p115**</td>
<td>ATP, GTP</td>
<td>Mg**, Mn**</td>
</tr>
<tr>
<td>c-ons</td>
<td>Plasma membrane</td>
<td>p90**</td>
<td>ATP, GTP</td>
<td>Mg**</td>
<td></td>
</tr>
</tbody>
</table>

(Adapted from Swarup et al., 1983)

### (b) + (c) Cellular

<table>
<thead>
<tr>
<th>Proto-oncogene</th>
<th>Gene product</th>
<th>Special properties</th>
<th>Tissue with highest mRNA expression (species)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-arc</td>
<td>80 PM</td>
<td>Tyrosine kinase</td>
<td>Neuronal tissues (ck, m, D), smooth muscle cells of gut (D)</td>
</tr>
<tr>
<td>c-neo</td>
<td>98/92 PM</td>
<td>Tyrosine kinase</td>
<td>Kidney (ck)</td>
</tr>
<tr>
<td>c-yes</td>
<td>60 PM</td>
<td>Tyrosine kinase</td>
<td>Myeloid cells (ck, m, h)</td>
</tr>
<tr>
<td>c-erb-B</td>
<td>170 PM</td>
<td>Tyrosine kinase, transmembrane glycoprotein, homologous to EGF-receptor</td>
<td>Kidney (ck)</td>
</tr>
<tr>
<td>c-fos</td>
<td>140 PM</td>
<td>Tyrosine kinase</td>
<td>Extra-embryonic tissues, macrophages (ck, h)</td>
</tr>
<tr>
<td>c-erb</td>
<td>150 PM</td>
<td>Tyrosine kinase, tissue-specific splicing of transcript</td>
<td>Thymus, testes (m)</td>
</tr>
<tr>
<td>c-flt/c-kit</td>
<td>185 PM</td>
<td>Tyrosine kinase, transmembrane glycoprotein, partial homology with c-erl/B</td>
<td></td>
</tr>
<tr>
<td>c-src/c-met</td>
<td>75 C</td>
<td>Threonine kinase</td>
<td>Gonads (m)</td>
</tr>
<tr>
<td>c-mos</td>
<td>C</td>
<td>Extremely low level of expression</td>
<td></td>
</tr>
</tbody>
</table>

(Adapted from Wagner and Muller, 1988)

### (c) Proteins associated with tyrosine protein kinase activity

<table>
<thead>
<tr>
<th>Protein*</th>
<th>Gene found in</th>
<th>Gene name</th>
<th>Cellular protein</th>
<th>Size</th>
<th>Tyrosine protein kinase?</th>
</tr>
</thead>
<tbody>
<tr>
<td>EGF receptor</td>
<td>(cellular)</td>
<td>?</td>
<td></td>
<td>170 K</td>
<td>EGF-stimulated</td>
</tr>
<tr>
<td>PDGF receptor</td>
<td>(cellular)</td>
<td>?</td>
<td></td>
<td>175 K</td>
<td>PDGF-stimulated</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>(cellular)</td>
<td>?</td>
<td></td>
<td>130 K, 95 K</td>
<td>Insulin-stimulated</td>
</tr>
<tr>
<td>LSTRA cells (cellular or viral)†</td>
<td>?</td>
<td>?</td>
<td>?</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Viral transforming proteins are designated by their size, in kilodaltons, and genetic origin (most are encoded by fused gag-one genes in the viral genome); cellular proteins are designated by their presumed function.

(Adapted from Cooper and Hunter, 1983)

---

**Figure 1.4**

Tyrosine-specific Protein Kinases

- **(a)** Viral
  - Table of viral oncogenes and their associated protein kinases.
  - **w-arc**: Rous sarcoma virus (various strains).
  - **v-fps**: Fujinami sarcoma virus.
  - **w-yes**: Y73 avian sarcoma virus.
  - **w-ras**: UR-3 virus.
  - **v-ABL**: Anemia murine leukemia virus.
  - **v-fes**: Feline sarcoma virus.
  - **c-erl**: Gardner-Arnstein strain.
  - **c-ons**: Plasma membrane.

- **(b) + (c)** Cellular
  - Table of proto-oncogenes and their properties.
  - **c-arc**: 80 PM, Tyrosine kinase.
  - **c-neo**: 98/92 PM, Tyrosine kinase.
  - **c-yes**: 60 PM, Tyrosine kinase.
  - **c-erb-B**: 170 PM, Tyrosine kinase, transmembrane glycoprotein.
  - **c-fos**: 140 PM, Tyrosine kinase, homologous to EGF-receptor.
  - **c-erb**: 150 PM, Tyrosine kinase, tissue-specific splicing of transcript.
  - **c-flt/c-kit**: 185 PM, Tyrosine kinase, transmembrane glycoprotein.
  - **c-src/c-met**: 75 C, Threonine kinase.
  - **c-mos**: C, Extremely low level of expression.

- **(c)** Proteins associated with tyrosine protein kinase activity
  - Table of tyrosine and insulin receptor kinases.
  - **EGF receptor**: 170 K, EGF-stimulated.
  - **PDGF receptor**: 175 K, PDGF-stimulated.
  - **Insulin receptor**: 130 K, 95 K, Insulin-stimulated.

---

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STRUCTURES of two groups of viral oncogene products and of a normal serine-specific protein kinase (bottom) are given by the bars, whose length is proportionate to the number of amino acids in each protein; the NH$_2$ end of each chain is at the left. The Class 1 products display clear tyrosine protein-kinase activity; the Class 1-related products do not. Analysis of the amino acid sequence of the proteins (predicted from the nucleotide sequence of their DNA) shows they have in common a 250-amino-acid region related to the protein-kinase domain of p60src (color). In most cases part of the protein encoded by the viral gene gag (and in one case part of actin) is synthesized with the oncogene protein as single product. Two proteins extending outside cell have identifiable transmembrane domain (black).
1.2.8.1.1.2. **Src Protein**

Historically, pp60v-**src** was the first protein shown to contain phosphotyrosine in vivo and has since been the most intensely studied: about 30% of pp60 v-**src** molecules are themselves phosphorylated on tyrosine, at a single, major site (Sefton et. al., 1982), as well as being phosphorylated at serine, as are most of the other tyrosine protein kinases. All of the other viral transforming protein kinases in figure (1.4a) have since been shown to contain one or more sites of tyrosine phosphorylation when isolated from transformed cells (Cooper and Hunter, 1983). Thus all tyrosine-specific protein kinases have autophosphorylation capacity, and have activity independent of cyclic nucleotides or calcium divalent cations (Swarup et. al., 1983).

Hanafusa, (1986), despite previous findings that high levels of pp60 v-**src** were found in Rous sarcoma-virus-transformed cells, reported the inability of overproduced p60 c-**src** to induce cell transformation. Hanafusa (1986) reviews evidence indicating that activation of the c-**src** gene can be achieved by a single mutation at various sites in the coding sequence - a finding which is consistent with the relatively high frequency at which a recombinant c-**src**-containing virus mutated to generate a transforming virus (Iba et. al., 1984). Since all of the transforming **src** proteins have elevated protein kinase activity, these mutations must contribute to conformational alterations which activate the kinase activity. Correlating with the increased kinase activity of the activated **src** protein, is the fact that it also has a higher level of autophosphorylation (Iba et. al., 1985), suggesting that phosphorylation of the kinase itself may govern its activity. Iba et. al., (1985) also showed that tyrosine (amino acid 416) appears only to be available for phosphorylation in the active form of p60src, indicating its conformation to be altered, although in vitro studies had previously indicated this phosphorylation event to be unnecessary for transformation (see Hanafusa, 1986 for review). Cooper et. al., (1986) showed that p60 c-**src** is phosphorylated at Tyr (amino acid 527) and proposed that this phosphorylation event negatively regulates the kinase activity of p60 c-**src**.
With the established link between altered tyrosine kinase activity and cellular transformation (1.2.8.1.1.), this hypothesis that p60 c-src regulates its kinase activity by its own tyrosine phosphorylation (not necessarily autophosphorylation) is interesting in that this may be the initiation event of the mechanism by which c-src exerts its control on cell growth. However, there is, as yet, no evidence directly implicating this phosphorylation/dephosphorylation of the src protein in cellular transformation.

1.2.8.1.2. **Substrate Proteins of Tyrosine Kinases**

The above studies on src (1.2.8.1.1.2), and similar studies on the other tyrosine kinase-encoding oncoproteins such as v-abl (see review by Gebhardt and Foulkes, 1986), strongly suggest that the ability of the v-onc products to phosphorylate proteins on tyrosine residues is critical for their transformation function. As a result, a great deal of research has gone into identifying the physiologically important substrates for these known protein kinases (for a review, see Cooper and Hunter, 1983). As yet, none have been found, but this may simply be due to technical difficulties, namely: (1) The scarcity of phosphotyrosine in proteins in vivo (1.2.8.1.1) means that it is difficult to detect proteins modified in this way. (2) The promiscuous nature of retroviral protein-tyrosine kinases in vivo results in the phosphorylation of many major cytoplasmic proteins which are unrelated to the transformation event. Gebhardt and Foulkes (1986) estimate that more than 90% of the phosphotyrosine-containing proteins in certain transformed cell lines have no role in the transformation process. (3) Several reports have claimed that tyrosine kinases phosphorylate phosphatidylinositol (reviewed in Macara, 1985). If true, this might explain the apparent absence of important protein targets - perhaps the substrates are lipids.

Figure (1.6) tabulates the proteins (of both viral and vertebrate origin) which have been found to be phosphorylated at tyrosine in vivo, although others exist which are known only by their electrophoretic characteristics (Cooper and Hunter, 1983). It appears that in many transformed cells, the major phosphotyrosine-containing proteins are the tyrosine-specific kinases themselves: all tyrosine protein kinases characterised to date are themselves
Phosphotyrosine - Containing Proteins

**Figure 1.6**

**Phosphotyrosine - Containing Proteins**

<table>
<thead>
<tr>
<th>Source of kinases</th>
<th>Major endogenous phosphorylated protein</th>
<th>Subcellular location of protein kinase</th>
<th>Cation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>A. Growth Factor Stimulated</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. EGF stimulated</td>
<td>170 K</td>
<td>membrane</td>
<td>(\text{Mg}^{2+}, \text{Mn}^{2+})</td>
</tr>
<tr>
<td>2. Insulin stimulated</td>
<td>95 K</td>
<td>membrane</td>
<td>(\text{Mn}^{2+}, \text{Mg}^{2+})</td>
</tr>
<tr>
<td>3. PDGF stimulated</td>
<td>170 K</td>
<td>membrane</td>
<td>(\text{Mn}^{2+} &gt; \text{Mg}^{2+})</td>
</tr>
<tr>
<td><strong>B. Others</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. LSTRA kinase*</td>
<td>55, 58 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+}, \text{Mn}^{2+})</td>
</tr>
<tr>
<td>2. Liver</td>
<td>75 K</td>
<td>particulate</td>
<td>(\text{Mn}^{2+})</td>
</tr>
<tr>
<td>3. Spleen</td>
<td>56–53 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+} &gt; \text{Mn}^{2+})</td>
</tr>
<tr>
<td>4. Brain</td>
<td>58, 56–53 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+} &gt; \text{Mn}^{2+})</td>
</tr>
<tr>
<td>5. Lung</td>
<td>56, 53 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+})</td>
</tr>
<tr>
<td>6. Testis</td>
<td>58, 56, 53 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+})</td>
</tr>
<tr>
<td>7. Kidney</td>
<td>56, 53 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+})</td>
</tr>
<tr>
<td>8. T-Lymphocytes</td>
<td>58–56 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+} &gt; \text{Mn})</td>
</tr>
<tr>
<td>9. Sea urchin</td>
<td>45, 57, 74, 84, 126 K</td>
<td>particulate</td>
<td>(\text{Mg}^{2+} &gt; \text{Mn})</td>
</tr>
<tr>
<td>10. Band 3 (erythrocytes)</td>
<td>93 K</td>
<td>membrane</td>
<td>(\text{Mg}^{2+})</td>
</tr>
<tr>
<td>11. Leydig cells</td>
<td></td>
<td>particulate</td>
<td>(\text{Mg}^{2+})</td>
</tr>
</tbody>
</table>

(adapted from Swarup et al., 19...)
substrates for tyrosine protein kinase(s). In addition, cells transformed by either the v-abl or v-src kinases show an increase in protein-bound phosphoserine. Clearly, a major problem in studying the detailed biochemical mechanisms of cell transformation is to distinguish between primary effects which are a direct consequence of an oncogene product, and secondary effects. This was, in fact, to prove of major importance in considering the relevance of the results obtained in this project (4.2).

1.2.8.2. Growth Factors/Growth Factor Receptors: Relationship to Oncogenes

Shortly after the initial discovery that several retroviral oncogenes (and their cellular progenitors) encoded tyrosine protein kinases, the link between an oncogene and a cellular gene with a known role in growth control was suggested. Previously, Cohen (1962) had described the isolation of a molecule present in mouse submaxillary glands which had the effect of accelerating incisor eruption and eyelid opening in new born animals. This molecule which he designated as the epidermal growth factor (EGF), later proved to have potent mitogenic activity on fibroblasts in culture, and to deliver its mitogenic signal via specific transmembrane receptors present in responsive cells. Ushiro and Cohen (1980) analysed the EGF receptor which led them to discover its relationship with oncogenes: the receptor was found to possess protein kinase activity specific for tyrosine residues. Furthermore, they found that the receptor kinase is stimulated several fold by the binding of EGF (Ushiro and Cohen, 1980). These findings raised the possibility that tyrosine kinases could be responsible for transmitting growth factor induced mitogenic signals to the interior of the cell, and that oncogenes (notably the tyrosine kinase class of oncogenes, 1.2.8.1.1) might stimulate uncontrolled proliferation via some perturbation of this pathway.
1.2.8.2.1. **Growth Factors**

1.2.8.2.1.1. **Functions of Growth Factors**

Since the discovery of EGF, more than a dozen other polypeptide growth factors which stimulate the proliferation of fibroblasts or other cell types in culture have been described; indeed, specific growth factors are probably required for growth and differentiation of most vertebrate cells. Like EGF, these factors transmit a mitogenic signal to their target cells via specific cell surface receptors, several of which have been shown to possess tyrosine kinase activity. However, the physiological roles of growth factors have been more difficult to establish, although it is generally considered likely that they participate in regulating proliferation and/or differentiation during embryogenesis, differentiation, growth and wound healing. Indeed, Sporn and Roberts (1988) in reviewing the action of peptide growth factors, point out that many are multifunctional and may include both stimulatory and inhibitory properties on cell proliferation as well as effects unrelated to the control of cell growth (see fig. 1.7). It is now realised that the actions of an individual growth factor may be diverse on a single cell-type depending on the context of the other signal molecules present and also that the range of cells which respond to each factor may be wider than was first determined. For example, as shown in figure (1.7), the interleukins, first defined as peptide signalling molecules which controlled the activities of cells in the immune system, have now been shown to be more diverse in activity than first realised. Thus, interleukin-2 (IL-2) which was originally named T-cell growth factor is now known also to be an important growth factor for B-cells and to increase the cytotoxic activity of monocytes. IL-2 may also control the growth of glial cells of the nervous system. Conversely, interleukin-4 which was first demonstrated to be a B-cell growth factor, has since been shown to have the capacity to stimulate T-lymphocytes and to control the maturation of other haematopoietic cells such as granulocytes and macrophages (for review, see Sporn and Roberts, 1988).

The different growth factors, which are tissue-specific to varying degrees, have little in common structurally, but can be
Peptide growth factors are multifunctional

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Proliferative effects</th>
<th>Anti-proliferative effects</th>
<th>Effects unrelated to proliferation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal growth factor</td>
<td>Keratinocytes, fibroblasts</td>
<td>Hair follicle cells, squamous carcinoma cells</td>
<td>Suppression of gastric acid secretion</td>
</tr>
<tr>
<td>Fibroblast growth factor</td>
<td>Many mesenchymal cells, especially endothelial cells</td>
<td>Ewing's sarcoma cells, other tumour cells</td>
<td>Regulation of pituitary and ovarian cell function</td>
</tr>
<tr>
<td>(basic)</td>
<td>Fibroblasts, osteoblasts</td>
<td>Fibroblasts, epithelial cells, T-lymphocytes, osteoblasts</td>
<td>Suppression of immunoglobulin secretion and adrenal steroidogenesis</td>
</tr>
<tr>
<td>Transforming growth factor-beta</td>
<td>Fibroblasts</td>
<td>T-lymphocytes</td>
<td>Endogenous pyrogen, bone resorption</td>
</tr>
<tr>
<td>Interleukin-1</td>
<td>Lymphocytes, keratinocytes, fibroblasts</td>
<td>Breast cancer cells</td>
<td>Increased cytotoxic activity of monocytes</td>
</tr>
<tr>
<td>Interleukin-2</td>
<td>T- and B-lymphocytes, oligodendrocytes</td>
<td>T-lymphocytes, oligodendrocytes</td>
<td>Increased expression of cell surface antigens and secretion of immunoglobulins</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>Hybridoma, plasmacytoma cell lines</td>
<td>Breast cancer cells</td>
<td>Inhibition of lipoprotein lipase, stimulation of collagenase</td>
</tr>
<tr>
<td>Tumour necrosis factor-alpha</td>
<td>Diploid fibroblasts, epithelial cells</td>
<td>Many tumour cells</td>
<td></td>
</tr>
</tbody>
</table>

The table is not intended to be comprehensive.
(adapted from Sporn and Roberts, 1985).
grouped together functionally because they all stimulate DNA synthesis in cultures of the appropriate target cells, and for some tissues are not only required for stimulating the proliferation of cells, but also for their survival (see Burgess, 1985 for review).

1.2.8.2.1.2. Autocrine Secretion

Additional evidence relating growth factors to oncogenesis came from an entirely different line of investigation. It had long been observed that transformed cells in culture are generally able to grow in much lower concentrations of serum (the usual source of growth factors for cultured cells), than are non-transformed cells. In an attempt to determine whether transformed cells themselves produce growth factor-like activities, De Larco and Torado (1978) discovered that fibroblasts transformed in culture with certain oncogene-containing retroviruses secrete factors which could transiently induce normal cells to express a transformed phenotype. The discovery of these so-called transforming growth factors led Sporn and Torado (1980) to suggest that the ability of a cell to produce a factor(s) which can support its own growth might be a general mechanism contributing to the unlimited growth capacity of tumour cells. There is now much evidence to support this concept of autocrine secretion. For example, many types of tumour cells release polypeptide growth factors into the medium when grown in cell culture, and these same tumour cells often possess functional receptors for the secreted peptide. Such growth factors include type A transforming growth factor (TGF-A), peptides related to platelet-derived growth factor (PDGF), bombesin and type B transforming growth factor (TGF-B — see Sporn and Roberts, 1985 for review). The action of each of these four peptides is mediated by a distinct membrane receptor, which in turn activates a post-receptor signalling mechanism and eventually results in a mitogenic response. Indeed, Weissman and Aaronson (1983) demonstrated that it was possible to overcome the absolute dependance of some cell lines on their appropriate growth factor by infecting the cells with an acutely transforming v-onc retrovirus. This further strengthened the connection between oncogenes and the pathways by which growth factors induce cell proliferation.
v-sis is Related to PDGF (Platelet-Derived Growth Factor)

A further finding led the gradually converging paths of growth factors and oncogenes to finally intersect:- the simian sarcoma virus (SSV) carries an oncogene known as v-sis, which encodes the capacity of the virus to induce a variety of neoplasms in monkeys and to transform fibroblasts in culture. DNA sequence analysis of the v-sis oncogene revealed that its predicted amino acid sequence (residues 67-175) is virtually identical to the N-terminal 109 amino acids of the B-chain of human PDGF (Waterfield et. al., 1983; Doolittle et. al., 1983). The PDGF protein was isolated as a potent mitogen for fibroblasts and over a stretch of 109 amino acids, only three substitutions were found between the v-sis gene product and human PDGF, which may simply represent the species difference between monkey and man. The A chain of PDGF exhibits a 60% homology to the B chain (see Heldin and Westermark, 1988 for a review of PDGF). The finding of a structural homology between the v-sis product and PDGF strongly suggested that SSV has acquired the gene for one of the two polypeptide chains of PDGF. Cloning of human c-sis, the cellular counterpart of v-sis, later verified this assumption (Johnston et. al., 1984). The human c-sis transcript contains an open reading frame for a 28kDa precursor of the B-chain of PDGF, but does not encode the A-chain. This structural link between PDGF and a viral transforming protein implicated a functional similarity (i.e., that the transforming capacity of SSV is due to a PDGF-like growth factor activity). This interpretation is consistent with the following:-

(1) SSV induces sarcomas and gliomas in vivo (Deinhardt, 1980), i.e., transforms only cell types that contain PDGF receptors.

(2) SSV-transformed cells secrete a growth factor which binds to, and activates the PDGF receptor (Chin et. al., 1984). The receptor is phosphorylated at tyrosine, and DNA synthesis is induced in quiescent fibroblasts on addition of PDGF (Heldin and Westermark, 1988). Each of these activities were shown to be inhibited by antibodies to different regions of the v-sis gene product (Leal et. al., 1985).

SSV-induced transformation thus appears to be due to an autocrine stimulation (that is, the capacity of cells to synthesise their own
growth factor) brought about by a viral-encoded growth factor that mimics PDGF in its functions. This finding established unambiguously that a growth factor gene can function as an oncogene.

1.2.8.2.2. Growth Factor Receptors

1.2.8.2.2.1. V-erb B is Related to the EGF Receptor

Soon after this discovery that the v-sis oncogene product is virtually identical to part of a known growth factor, DNA sequence analysis revealed the origin of another oncogene. V-erb B, a tyrosine kinase-encoding oncogene present in an avian retrovirus which induces erythroleukaemias and sarcomas, was found to be a mutated version of the EGF receptor (Downward et. al., 1984). The v-erb B protein appears to function as an activated growth factor receptor: by virtue of its unregulated kinase activity, it is believed to deliver a constitutive mitogenic signal rather than one which is regulated by the growth factor.

As well as the fact that the v-erb B oncogene encodes a truncated version of the EGF receptor, several other lines of evidence suggest that this receptor can play a role in the uncontrolled proliferation characteristic of neoplastic cells and that the v-erb B oncogene can function as an activated EGF receptor:-

1. Several tumour cells of both human and other vertebrate origins, produce a growth factor called transforming growth factor-A (TGF-A, Todaro et. al., 1980). This peptide is highly related to EGF; both factors bind to the EGF receptor with similar affinities and induce the proliferation of cells bearing the EGF receptor. Todaro et. al., (1980) proposed that TGF-A plays a role in oncogenesis by inducing autocrine growth through activation of the EGF receptor.

2. The EGF receptor is found to be amplified and rearranged in a significant proportion of human brain tumours of glial origin (Libermann et. al., 1985). The resultant overexpression of EGF receptor may then play a role in the development or progression of these tumours.

3. Sequence analysis also reveals that v-erb B and the EGF receptor are both related to the family of oncogenes encoding tyrosine
kinases (Yamamoto et. al., 1983; figures 1.4 and 1.5), which have been implicated in growth regulatory mechanisms (1.2.8.1.1).

4. In addition, \textit{v-erb B}, when introduced into chick heart mesenchymal cells, abolishes the strict requirement of these cells for EGF and insulin (Balk et. al., 1984). This suggests that \textit{v-erb B} can substitute for an activated EGF receptor.

1.2.8.2.2.2. EGF Receptor Protein (see Beug et. al., 1988 for a detailed review)

The mature protein consists of 1186 amino acids which are preceeded at the N-terminal end by a signal sequence of 24 hydrophobic amino acids. The signal peptide is cleaved following insertion of the nascent receptor into the membrane of the endoplasmic reticulum. The receptor is cotranslationally glycosylated and transported through the Golgi apparatus to the plasma membrane. There are three major domains comprising the receptor: (1) A 622 amino acid extracellular ligand-binding domain. (2) An anchoring transmembrane region composed of 23 hydrophobic amino acids and (3) A cytoplasmic domain composed of 542 amino acids and containing the protein kinase domain and phosphorylation sites (see fig. 1.8 for structure).

Threonine (amino acid residue 654) is phosphorylated by protein kinase C, and there are three known autophosphorylation sites - all tyrosine residues - Tyr (1068), Tyr (1148) and Tyr (1173). In intact cells, autophosphorylation occurs mainly on Tyr (1173), a residue which is deleted in the \textit{v-erb B} protein (Yamamoto et. al., 1983). Although it is known that the binding of EGF to its receptor leads to the activation of the receptor tyrosine kinase which phosphorylates various cellular proteins as well as itself, the role of the autophosphorylation activity is not yet fully understood.

1.2.8.2.2.3. Signal Transduction via the EGF Receptor

Addition of the tumour promoter, phorbol ester (TPA) to fibroblasts and other cells was found to abolish the "high affinity" state of the EGF receptor and also to reduce its protein tyrosine kinase activity (Brown et. al., 1979). TPA activates the Ca$^{2+}$-
Figure 1.8

(a) Comparison of the EGF receptor with the erbB proteins from AEV-H and AEV-ES4 viruses. The diagram shows the membrane inserted forms including the potential glycosylation sites (small branches in the extracellular domain), the threonine phosphorylation site in position 654 and the three tyrosine phosphorylation sites at the C-terminal end (P1, P2, P3). Only that portion of AEV-ES4 erbB protein which differs from AEV-H is shown. Triangle indicates an internal deletion.

(Adapted from Beug et al., 1988).

(b) Signal transmission from cell-surface receptor to adenylate cyclase through G-protein. The activated (effecter) GTP-bound form of G-protein, $G_E$:GTP, which activates adenyl cyclase, is converted to the inactive form, $G_R$:GDP, by GTPase. ras protein is hypothesized to act like G-protein.

(Adapted from Newbold, 1984).
sensitive protein kinase C, which in turn phosphorylates the EGF-receptor on several sites. One of these sites is Thr (654), a residue located ten amino acids within the cytoplasmic domain of the EGF-receptor (Davis and Czech, 1985). Hunter, (1988) therefore suggested that the phosphorylation of EGF receptor on threonine residue (Thr 654) regulates the affinity of the extracellular domain towards the ligand and the enzymatic activity of the protein tyrosine kinase domain.

Two theories, so far, have been put forward to explain the mechanism by which the binding of EGF to the extracellular domain of its receptor stimulates the kinase activity in the cytoplasmic domain of the receptor, without the involvement of any other molecules:–

1. When the receptor binds EGF, the extracellular domain undergoes a conformational change which is transmitted through the membrane-spanning region to the kinase domain. This, in turn is structurally altered, resulting in its activation.

2. EGF-induced aggregation of EGF receptor molecules activates the kinase activity, thus bypassing the requirement of the previous hypothesis for a conformational change to be transferred through the transmembrane region. Since EGF does, in fact, induce receptor aggregation in intact cells, there is some evidence in favour of this mechanism (see Schlessinger et. al., 1988 for review).

1.2.8.2.2.4. **V-erb B Oncogene Product**

The transmembrane glycoprotein encoded by v-erb B, is synthesised on membrane-bound polyribosomes and is modified by the addition of one or two carbohydrate chains that are further processed into complex carbohydrate forms. During this processing, the v-erb B protein is translocated from the rough endoplasmic reticulum (E.R) to the plasma membrane in a manner similar to the EGF receptor (see fig. (1.8.a); Beug et. al., 1988). The v-erb B proteins of two strains of AEV (avian erythroblastosis virus), AEV-ES4 and AEV-H, both have an extracellular N-terminal domain of only 61 aminoacids, compared to 622 aminoacids in the EGF-receptor. The structure of all three is shown in fig. (1.8.a). This deletion abolishes the ability to bind EGF, and is also thought to be responsible for the fact that 85%-90% of the v-erb B protein synthesised in the rough E.R. is not correctly
processed and so persists in the intracellular membranes, where it seems to be degraded (Privalsky and Bishop, 1984). Obviously, receptor activation is not, therefore further stimulated by addition of EGF (Kris et. al., 1985). Despite the fact that the v-erb B proteins from both strains of the virus also lack part of the C-terminal domain, including one or two tyrosine phosphorylation sites found in the EGF-receptor (see fig. 1.8.a), they both were found to be autophosphorylated on tyrosine to some extent and to phosphorylate tyrosine residues on a variety of substrates in vitro. The AEV-ES4 v-erb B protein was found to differ from chicken EGF receptor in that it exhibited only residual autophosphorylation on tyrosine in virus-transformed fibroblasts metabolically labelled with $^{32}$P-orthophosphate (Decker, 1985). V-erb B was observed, however, to induce an enhanced tyrosine phosphorylation of cellular proteins such as p36 (which is also phosphorylated in v-src-transformed cells) and p42 (as in quiescent fibroblasts stimulated with serum, EGF, or phorbolesters) (Gilmore et. al., 1985).

The v-erb B protein thus represents a highly mutated and deregulated form of the EGF receptor which is unable to respond to growth factor signals and appears to be constitutively activated, resulting in the transformed phenotype. However, it is not known whether, as suggested for the EGF receptor (1.2.8.2.2.4), clustering of v-erb B proteins is required for mitogenic signalling.

1.2.8.2.3. Other Receptor-like Oncogenes

Shortly after the relationship between v-erb B and the EGF receptor was discovered, the v-fms oncogene was shown to be a modified version of the receptor for the macrophage colony-stimulating factor (CSF-1 or M-CSF) (Sherr et. al., 1985). Many investigators now suspect that other retroviral genes of the tyrosine kinase gene family (v-src, v-abl, v-fes/fps, v-yes, v-fgr and v-ros) as well as functionally related oncogenes derived from tumour cells (neu, trk, met), could also have arisen from receptor genes. However, this may not be the case, since the products of these remaining oncogenes which
encode tyrosine kinase activities generally reside at the cytoplasmic face of the plasma membrane, while growth factors are transmembrane proteins. Of course it remains a possibility that the viral oncogene proteins have become mutated in a manner similar to that shown for v-erbB (1.2.8.2.2.4), but to such an extent as to no longer have the sequences necessary for transmembrane location.

1.2.8.3. Non-Tyrosine Kinase Classes of Oncogenes

Even less is known about the functions of the oncogenes that do not encode tyrosine kinases, which include some whose products are localised in the cytoplasm and others which encode nuclear proteins. Our relative ignorance about these oncogenes is paralleled by the scarcity of information regarding how growth factor-induced mitogenic signals are transmitted beyond the growth factor receptors through the interior of the cell and ultimately to the nucleus, which must somehow be stimulated to undergo a round of DNA synthesis. However, the following sections on the ras and the nuclear-located oncogenes lend further support to the notion that oncogenes and growth factors utilise related, if not the same signalling pathways.

1.2.8.3.1.1. The ras Oncogenes

The ras gene family in mammalian cells comprises five closely related members: the Harvey and Kirsten ras genes (c-Ha-ras and c-Ki-ras), an inactive pseudogene of each (c-Ha-ras and c-Ki-ras) and the N-ras gene, as well as several distinctly related genes designated rho (ras-homologous). The genes of the ras family are related both by DNA sequence homology and by the fact that they encode protein products of approximately 21kDa (p21ras), which share the properties of GTP/GDP binding, weak GTP-ase activity and the presence of cysteine residues (potential sites for membrane localisation) (see Marshall, 1988 for review). The ras genes have been implicated in a variety of human cancers - approximately 10-20% of most types of human malignancies contain an "activated" ras oncogene ("activated" = its ability to induce transformed foci when transfected into recipient cells such as mouse NIH 3T3).
Ras proto-oncogenes can be activated in either of two ways: by mutations in the coding sequence, leading to an altered protein product, and by overexpression of the normal gene product. The former is much more common in human malignancies. Approximately 40 of these mutant ras genes have been analysed, and in all cases, the mutations responsible for transforming activity have been located to codons 12, 13 or 61, each being single base changes (Marshall, 1988).

The viral ras proteins, which are also able to bind the guanine nucleotides GDP and GTP and are localised at the inner surface of the plasma membrane, do differ from the normal and mutant c-ras proteins in that they are phosphorylated at position 59 where an ala --- thr substitution has occurred (Shih et al., 1980). However, transforming ras proteins with certain mutations at amino acids 12, 59 or 61, show at least a ten-fold reduction in GTP-ase activity (Temeles et al., 1985). This suggests that mutations at these sites generate a transforming protein because they lead to reduced GTP-ase activity. This idea is consistent with the observation that virtually any amino acid substitution or deletion at codon 12 or 61 activates p21 ras transforming function. However, the fact that overproduction of normal protein can also lead to transformation is not readily explainable in terms of alterations in GTP-ase activity.

1.2.8.3.1.2. Function of Normal and Mutant ras Genes

The sequence and functional homologies between p21 ras proteins and a family of membrane-associated proteins called G proteins has been well documented (see Masters and Bourne, 1988 for review) and provides an insight into the possible function and mechanism of transformation conferred by ras oncogenes. In short, the G-proteins transduce extracellular stimuli into a set of cellular responses by stimulation and inhibition of adenylate cyclase following the attachment of polypeptide hormones to specific cell surface receptors (fig. 1.8.b) (Gilman, 1984). As well as possessing a guanine nucleotide-binding site, the G-proteins have GTP-ase activity which regulates their function. In the case of stimulation of the adenylate cyclase system, binding of the relevant hormone to its receptor promotes conversion of the inactive GDP form of the G-protein into the GTP state which in turn activates the cyclase. Thereafter,
increased production of cyclic AMP (the second messenger) stimulates a cyclic AMP-dependant protein kinase which carries out specific regulatory phosphorylations that determine the response of the cell. The G-protein signal is cancelled by the slow hydrolysis of GTP to GDP, mediated by the GTP-ase activity of the G-protein.

Thus, homologies, both in function and at the sequence level between the G-protein and p21 ras proteins, argues that p21ras proteins mediate signals involved in the control of cell proliferation. One model envisages that the binding of a particular ligand (such as a growth factor) to its receptor stimulates p21ras to bind GTP and interact with an effector protein. This interaction, as with the G-protein system would be terminated by hydrolysis of GTP to GDP. The transforming activity of mutants defective in GTP-ase activity could therefore be due to a prolonged interaction with effector proteins.

It is also possible that transforming p21ras proteins bind GTP even in the absence of receptor binding and are therefore constitutively activated: An X-ray crystallographic study (McCormack et al., 1985) predicted that binding of GTP to p21ras would create a conformational change compared with the GDP form. It is therefore possible that the transforming mutations mimic the conformational change induced by GTP binding and thereby place the protein in a constitutively activated form. Computer modelling (Pincus et al., 1983) proposed that these mutant forms of p21ras would be more conformationally restricted than the wild type protein, and so could theoretically transmit a permanent signal, even in the absence of the ligand. p21ras also appears to interact with the EGF receptor since Kamata and Feramisco (1984) observed increased guanine nucleotide binding by ras proteins, on stimulation with EGF and insulin. This provides some evidence for a functional link between p21ras and receptor systems. Clearly, our knowledge of the functional role(s) of the ras proteins (both normal and transforming) in cellular growth control is at a preliminary stage. Perhaps further work will reveal the pathways in which the ras proteins operate, and if different ras proteins interact with different receptor or effector systems.
1.2.8.4.1. **Nuclear Oncogenes**

One further discovery also illustrates how research on oncogenes and growth factors has merged. Experiments designed to reveal the nature of the mitogenic response revealed that the stimulation of cultured cells with certain mitogens and growth factors induced the expression in the nucleus of two proto-oncogenes, myc and fos. Although the precise functions of these proto-oncogene products are not known, their early activation following growth factor stimulation suggests that they participate in the induction of cell division. This lends further support to the notion that oncogenes and growth factors utilise the same, or related signalling pathways.

1.2.8.4.2. **Myc Oncogene: Protein Functions and Role in Tumorigenesis**

The *myc* oncogene was originally discovered as a transforming gene in certain avian retrovirus strains and has also been implicated in the genesis of human hematopoietic neoplasms. Four types of abnormality involving *c-myc* indicate its role in tumour formation:

1. Provirus insertion adjacent to *c-myc* in chicken B-cell lymphomas (O'Donnell et al., 1985).
2. *c-myc* gene amplification in some human tumours (Kinzler et al., 1986).
3. Chromosomal translocations involving the complete *c-myc* gene in various Burkitt's lymphomas or mouse plasmacytomas (Robertson, 1984, for review).
4. Chromosomal translocations and rearrangements of the *c-myc* gene, leading to a truncation of exon 1 (Robertson, 1984, for review), also in some Burkitt's lymphomas.

One factor common to all these various rearrangements is the constitutive expression of relatively high levels of *c-myc* mRNA and protein throughout all stages of the cell cycle, a situation that might induce a constant proliferation stimulus. The regulation of transcription of *c-myc* involves a complex series of control elements both within the gene and in its 5' flanking region (see Fahrlander and Marcu, 1988 for review). It is thought that this regulation involves sequence-specific interactions with protein factors - one such site
for a DNA binding factor has been identified 5' of c-myc (see Fahrlander and Marcu, 1988). Although many issues concerning c-myc control remain unresolved, the fact that this gene is under complex regulation reflects the likely importance of this gene in normal growth and its contribution to malignant transformation.

The myc gene products were the first transforming proteins to be localised in the nucleus (v-myc: Donner et al., 1982; c-myc: Hann and Eisenman, 1984) and are therefore thought to be likely candidates for involvement in the regulation of gene expression. Properties of the v-myc proteins closely resemble those of the c-myc proteins. The myc proteins are synthesised in the cytoplasm around 7 minutes after addition of cellular growth stimuli, are rapidly transported to the nucleus in less than 1 hour, and exhibit a half-life of only about 15 minutes (Hann and Eisenmann, 1984; Beimling et al., 1985). According to an analysis carried out by Evan and Hancock, (1985), the c-myc protein is not linked to either the chromatin or to the nuclear matrix, whereas the v-myc protein has been found to be associated with chromatin (Bunte et al., 1982).

Donner et al., (1982) first demonstrated that purified v-myc protein binds to double stranded DNA in vitro and this has since been repeated with the c-myc protein (Persson and Leder, 1984). Moelling et al., (1986) reported preferential binding of both the viral and cellular myc proteins to cellular sequences upstream of c-myc. In addition, the myc proteins from three non-transforming MC29 deletion mutants exhibit a reduced ability to bind to DNA (Donner et al., 1983), indicating a correlation between DNA-binding of the myc protein with transformation. It is unknown whether the myc protein requires other cellular proteins to exert its specific function(s). In comparison, the SV40 T-antigen recognises specific DNA sequences without any such factors, whereas the E1A protein of adenovirus does not bind to DNA at all, except when supplemented by a host factor (for reviews, see Philipson, 1988; Schlokat and Gruss, 1988).

Particularly important in linking the myc proto-oncogene with physiological function(s) in metabolic pathways involved in the regulation of cellular proliferation, was the report that expression of c-myc increased, rapidly following treatment of different cell types with growth stimulating components including PDGF (Kelly et.
al., 1983). (This discovery was followed by the finding that c-myc mRNA accumulation is even preceded by the transcriptional activation of another proto-oncogene, the c-fos gene, which also encodes a nuclear protein - see Bravo and Muller, 1988 for review).

Coughlin et al., (1985), recognised that activation of the PDGF receptor is associated with induction of c-myc and c-fos expression and that occupancy of the PDGF receptor, as well as activating its own kinase activity, also leads to phosphoinositide turnover and the consequent production of inositol triphosphate and diacylglycerol. Diacylglycerols stimulate protein kinase C, the enzyme which is activated by the tumour promoting and mitogenic phorbol esters (for review, see 1.2.9.2). Furthermore, the observation that phorbol 12-myristate 13-acetate stimulates the expression of c-myc in Balb/c -3T3 cells (Kelley et al., 1983) suggests a relationship between the phospho-inositide/protein kinase C pathways and the expression of the c-myc gene.

Coughlin's studies were therefore based on the idea that a growth factor (eg PDGF), via receptor-mediated phosphoinositide turnover, leads to activation of protein kinase C which is an intermediate step in c-myc expression (Coughlin et al., 1985). Results indicated that this phosphoinositide turnover/protein kinase C pathway is one of several pathways which transduces receptor-mediated enhancement of c-myc expression, and that neither increased expression of c-myc, nor activation of kinase C alone were sufficient to account for the mitogenic effect of PDGF. These findings indicate, therefore, that other pathways are also essential, that activation of c-myc seems to be a direct response to the growth factor/receptor interaction, and that activation of c-myc alone does not necessarily lead to an increase in growth rate (see also 1.2.10).

1.2.9. Signal Transduction

Once a growth factor has been activated by the binding of ligand, the mitogenic signal must travel through the cytoplasm to the nucleus, ultimately leading to a round of DNA synthesis and cell division. It is apparent that some of the changes which occur in response to growth factor stimulation are also seen in cells transformed with
activated, or retroviral oncogenes. Fragments of some of these mechanisms by which this intracellular signalling is likely to occur have been briefly reviewed in previous sections:

Section (1.2.8.1) discussed the role of phosphorylation in signal transduction: the fact that many growth factor receptors and oncogene products possess protein-tyrosine kinase activity strongly implicates tyrosine phosphorylation as being important in the mitogenic response. These kinases lead to the phosphorylation of a large number of cellular proteins, but despite intensive efforts, it is still unclear which substrates are critical for the transformation process. However, a serine/threonine kinase which is stimulated by EGF, PDGF, TPA (a tumor-inducer), insulin and the oncogenes ras and src, results in phosphorylation of the 40S ribosomal protein S6. Multiple phosphorylations of this protein are thought to be associated with the increased rate of protein synthesis observed during the mitogenic response, caused by altered affinity of the 40s ribosomal unit for mRNA. (Thomas, 1988). Other substrate proteins of kinase enzymes which may be involved in transformation are discussed in section (4.2.3.2).

The G-protein family, which appear to have a signal transducing function, were discussed in section (1.2.8.3.1). These membrane-associated proteins transduce signals from hormone (or sensory) receptors, to various effector systems, including adenylate cyclase. The interaction of G-proteins with effector enzymes was seen to be regulated not only by the receptor molecules, but also by guanine nucleotides. DNA sequence analysis revealed significant homology between one of the G-protein subunits and the ras proto-oncogene family whose protein products have similar properties to G-proteins. Like the G-proteins, normal c-ras proteins are associated with the plasma membrane, bind GTP and have GTP-ase activity, raising the possibility that ras proteins participate in the transduction of mitogenic signals between unidentified growth factors and effector systems. In contrast, the transforming ras proteins bind GTP but hydrolise it inefficiently, suggesting that they may be constitutively active (see 1.2.8.3.1).

Nuclear - located oncogene products were reviewed in section
(1.2.8.4), which were stimulated in expression as a direct result of growth factor/receptor interactions, and are hypothesised to contribute to cellular proliferation through regulation of gene expression.

1.2.9.2. **The Inositol Lipid Signal Pathway** (see figure 1.9)

Some growth factors and oncogenes stimulate the metabolism of inositol lipids in the membrane (Mitchell, 1982), yielding two products which can each act as a second messenger in signal transduction: inositol (1, 4, 5) triphosphate (Ins 1, 4, 5P₃) and diacylglycerol (DG) (Berridge and Irvine, 1984; Nishizuka, 1984). These two second messengers then regulate two separate ionic events, both of which are crucial for the onset of DNA synthesis. Inositol (1, 4, 5) triphosphate acts to mobilise calcium from intracellular stores in the endoplasmic reticulum (Berridge and Irvine, 1984), whereas diacylglycerol stimulates protein kinase C (Nishizuka, 1984; Parker and Ullrick, 1988), which then activates a Na⁺/H⁺ exchanger to raise intracellular pH (Moolenaar, 1988). Nishizuka (1988) also reviews the circumstantial evidence for the ubiquitous nature of protein kinase C such as involvement in endocrine and exocrine secretion, neurotransmitter release, platelet granule release, neutrophil activation, Na⁺/H⁺ exchange, cell-cell interaction, cell surface expression of receptors, and gene expression which may contribute to the transformed phenotype. These two branches of the pathway which result in increases in intracellular calcium and pH are responsible, somehow, for initiating the sequence of events which culminates in DNA synthesis and cell division. This may be via activation of *myc* and *fos* transcription, which can be stimulated by phorbol-esters or calcium ionophores. The importance of these two branches of the pathway for the initiation of cell growth is apparent from experiments on lymphocytes which demonstrate that DNA synthesis can be triggered by combining the action of a calcium ionophore (which mimics the effect of Ins 1, 4, 5 P₃) with that of phorbol-ester (which mimics the stimulatory effect of DG on protein kinase) (Guy et. al., 1985). Stimulation of both these branches of the pathway is necessary to induce DNA synthesis.
Summary of the main components of the inositol lipid pathway. The pathway is activated by growth stimuli acting on their specific receptors (R) in many different cell types. Substrate for the pathway is formed by the conversion of PtdIns into PtdIns4,5P2. The transducing mechanism acts via a G-protein (Np) which couples the receptor to the phosphodiesterase (PDE) that cleaves PtdIns4,5P2 to the two second messengers DG and Ins1,4,5P3. The latter is converted into additional inositol phosphates (Ins1,3,4,5P4 and Ins1,3,4P3) which may also have messenger functions. DG acts through protein kinase C to activate the Na+/H+ exchanger, which reduces the intracellular hydrogen ion concentration, while Ins1,4,5P3 mobilizes intracellular calcium. The increase in pH and calcium contribute to the onset of gene transcription and protein synthesis which culminate in DNA synthesis.

(adapted from Berridge, 1988).
(a) Major components of mitogenic signal pathways. The position where different oncogenes might participate in signaling is included below (adapted from Berridge, 1988).

(b) Classes of cooperating oncogenes

<table>
<thead>
<tr>
<th>Class 1</th>
<th>Class 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ha-ras</td>
<td>myc</td>
</tr>
<tr>
<td>Ki-ras</td>
<td>N-myc</td>
</tr>
<tr>
<td>N-ras</td>
<td>myb</td>
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<tr>
<td>src</td>
<td>p53</td>
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<tr>
<td>Polyoma middle T</td>
<td>Adeno E1A</td>
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<tr>
<td></td>
<td>Polyoma large T</td>
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<td></td>
<td>SV40 large T</td>
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(adapted from Land, 1988).
One of the growth factors which appears to act through this inositol lipid pathway is platelet-derived growth factor (PDGF). As discussed in (1.2.8.2.1.3), the v-sis oncogene encodes a protein which is almost identical to PDGF, and so is thought to transform via an autocrine mechanism utilising the inositol lipid pathway.

One principle which is emerging, is that inter-connected pathways exist, some of which may eventually converge, while others remain separate. Figure (1.10.a) shows a generalised mitogenic signalling pathway. The positions where different oncogenes are thought to participate in signalling are also shown. It is now apparent that mechanisms for oncogenesis by retroviruses and by non-viral carcinogens are likely to have the same molecular basis, and also that in principle, any gene encoding a growth factor, a receptor, an intracellular signalling molecule or a protein which regulates transcription may be considered as a proto-oncogene.

1.2.10. Multi-step Process of Cell Transformation

The discussions of transformation in preceding sections have focussed on the modes of action of individual oncogenes and their effects on cellular growth pathways. The initial interpretation of transformation of NIH 3T3 fibroblasts in vitro and fatal tumour induction by retroviruses, was that malignant transformation was induced in a single step. The NIH 3T3 cells used in these transformation assays had been grown indefinitely in monolayer culture and then passaged extensively in vitro and so may have acquired several alterations usually developed by a cell during its tumorigenic progression. These alterations may then pre-dispose the NIH 3T3 to tumorigenic conversion by a single-hit event. More recent evidence suggests that a single oncogene is generally not sufficient to induce full neoplastic transformation, as this usually results from the accumulation of multiple oncogenic events:

1) Co-operation of oncogenes has been demonstrated in the transformation of cultured cells by a DNA tumour virus, the polyoma virus. The transforming capacity of this virus lies in its "early region", which encodes the proteins known as large T, middle T and small T. While established cell lines of rodent fibroblasts can be
transformed by middle T alone, the transformation of primary fibroblasts requires the combined action of large T which confers upon the cells an indefinite lifespan ("establishment" or "immortalisation") and middle T, which induces a transformed phenotype (see Mougneau and Cuzin, 1988 for review). Transformation by this virus also involves co-operativity between a viral oncogene and a proto-oncogene: middle T protein forms a complex with the c-src gene product. This leads to an increase in the tyrosine kinase activity of c-src protein - a change which is necessary, but not sufficient by itself, for cell transformation (Mougneau and Cuzin, 1988).

2) **Ha-ras** and **myc** were the first pair of cellular oncogenes found to co-operate in giving rise to fibrosarcomas in primary rat embryo fibroblasts (Land et al., 1985). Subsequently, replacement of ras or myc with other oncogenes has permitted the establishment of two functionally different classes of oncogene, listed in figure (1.10.b) - those which can substitute for ras and those which can substitute for myc in bringing about transformation of non-immortalised cells (Weinberg, 1985 for review). The gene products of the first class are localised at the plasma membrane, while those of the second class are localised in the cell nucleus.

3) Avian lymphoid leukosis virus (LLV)-induced B-cell lymphomas were shown by Cooper and Neiman (1981), to contain at least two transforming genes: a cellular myc gene activated by integration of viral DNA sequences, and a distinct cellular gene that is not linked to viral DNA, but which efficiently induces transformation of NIH 3T3 cells on its own.

4) There is mounting evidence for the involvement of so-called "suppressor" genes and "anti-oncogenes" in the development or onset of neoplasia.

Suppressor genes in cellular growth control are genes which are capable of modulating the activity or effect of the genes responsible for the neoplastic phenotype. Although none have yet been isolated, their existence has been demonstrated in a number of experimental systems. Firstly, in vitro studies of somatic cell hybrids between normal and tumour cells have repeatedly demonstrated extinction of the neoplastic phenotype (see Wyke and Green, 1988 for review). For
example, Noda et al. (1983) reported that revertants (in vitro) of Kirsten-murine sarcoma virus-transformed fibroblasts were found to suppress ras, fos and src-transformed partner cells, but not cells transformed by mos, fms and sis, after somatic hybridisation. Similarly, Newmark, (1987) reports a finding by Dotto, (Yale University), that keratinocytes containing the viral ras gene which result in rapid development of carcinomas when grafted onto mouse skin, are not carcinogenic if the keratinocytes are mixed with fibroblasts from normal skin dermis. In the same report, Newmark describes an experiment by Kirsten, (Weizmann Institute, Rehovot) whereby addition of an interferon antibody to the medium in which (untransformed) ras-containing rat kidney cells were growing, resulted in transformation of the cells, as if a co-operating oncogene had been placed inside the cell. This strengthens the argument that autocrine inhibition is a major mechanism in the normal control of cell growth, and that its disruption can be a factor in tumour development.

Genes in which recessive mutations are associated with a predisposition to cancer have been termed "anti-oncogenes". These genes may have to be deleted or functionally inactivated before a tumour can arise. In retinoblastoma, both alleles of the gene rb-1 are lost, suggesting that the rb-1 may have a function in negatively regulating cellular growth (Klein and Klein, 1985). Other neoplasms, such as Wilms tumour, familial renal cell carcinoma, neuroblastoma and small cell carcinoma of the lung also seem to require the loss of both alleles of a single gene before tumorigenesis occurs.

The suppressor genes and anti-oncogenes may turn out to be as diverse as the oncogenes. Such negative regulatory genes may act by themselves, or in concert with oncogenes to bring about the neoplastic phenotype.

1.2.11. Conclusions, and Relevance to this Project

This review (1.2) has outlined the current state of knowledge of the mechanisms of normal cellular growth control, the involvement of oncogenes, and how these processes may be interrupted to bring about cellular transformation. Thus the ultimate aim of this project, in determining the mechanism by which Theileria annulata brings about the
transformation of bovine leukocytes, is to elucidate how the parasite interacts with the host cell growth regulatory pathways. This knowledge of some of the molecular processes involved in growth regulation allowed methods of looking at alterations in various components of these pathways in Theileria-transformed cells to be devised (See 1.3.7. for design of experimental approach).
1.3. THEILERIA-INDUCED TRANSFORMATION OF BOVINE LEUKOCYTES

1.3.1. Introduction

As mentioned in section (1.1.2.2.), one of the outcomes of Theileria infection is the uncontrolled proliferation of bovine leukocytes. Once infected, the leukocytes can be grown indefinitely as a cell line in the laboratory (Brown, 1979 and 1983). Such cell lines provide a unique and interesting model system for study of the mechanisms involved in induction of leukocyte transformation. To date, little as been reported in the literature on the growth regulation of these cells, either at a molecular level or at a phenotypic level. What information exists, is often contradicted by other experiments, and so it is difficult to conclude much about the way in which Theileria transforms its host cells. An additional complication in interpreting data from separate experiments is that two species of Theileria have been used in the study of host-leukocyte transformation; Theileria annulata (as described in this thesis) and Theileria parva (the causative agent of East Coast Fever). It is not known whether the mechanisms by which these two Theileria species transform leukocytes are the same, particularly since the host cell targets for infection seem to differ; T. parva seems preferentially to invade T-lymphocytes (I.L.R.A.D., 1983) whereas T. annulata appears to preferentially invade MHC class II positive cells (B-lymphocytes and macrophages) (Spooner et. al., in press). As a result, in the following resume of knowledge about Theileria-induced transformation, it will be made clear in each case whether the information was obtained from a T. annulata or a T. parva system.

Reichenow (1940), first postulated that division of the Theileria parasites within the host leukocytes occurred by binary fission, and not by schizogony (the schizonts breaking up into merozoites, which then infected other leukocytes) as was previously assumed. With the developments which permitted the prolonged cultivation of T. parva and T.annulata in vitro (see Brown 1979, 1983), it was made much easier to study the mode of multiplication of Theileria in host leukocytes. Hulliger et. al., (1964) confirmed, by observation, that the parasites divided by binary fission as hypothesised by Reichenow, but demonstrated that further infection of leukocytes did not (as
Reichenow believed) occur by re-infection with macroschizonts, but occurred by the parasites dividing in synchrony with the host cells, with one parasite being distributed to each daughter cell. This was further substantiated by observations of dividing Theileria-infected leukocytes in an infected cow lymph node (De Martini and Moutton, 1973). Since then, many fine-structural analyses have been carried out by electron microscopy in order to study the host-cell/parasite relationship. Jura and Brown (1983) thus demonstrated that in the interphase cell, there appeared to be some connection between the host cell nucleus and the macroschizont via annulate lamellae, although no conclusions could be made as to the precise nature and function of this connection.

The transformed leukocytes resemble normal lymphocyte blasts, except for the presence of the parasite which is visible in the cytoplasm. "Blast" is the name given to large proliferating cells which appear after stimulation of normal uninfected leukocytes or lymphocytes by antigens or by mitogenic lectins. These cells are neither immortal nor tumorigenic. (See Ling and Kay, 1975 for review). However, Theileria-infected leukocytes are transformed by the following criteria:-

(1) **Growth in Culture.**

Both *T. annulata* and *T. parva*-infected leukocytes are capable of unlimited growth in liquid culture. This capacity for unlimited growth ("immortalisation") is characteristic of transformed cells (see 1.2.10).

(2) **Tumour formation in Nude Mice.**

In an assay for tumour-forming ability, tumour-like growth of bovine lymphoid cells infected with *Theileria parva* has been observed in whole-body irradiated athymic (nude) mice inoculated with cells direct from fatal cases of East Coast Fever, and also with lymphoid cells infected with *T. parva* maintained as cell lines in culture (Irvine et al., 1975). Irvine et al. (1975), compared the growth, in irradiated athymic mice, of *T. parva* infected cell lines with that of similar bovine transformed lymphoid cells of known neoplastic origin. The results showed that cells infected with *T.*
parva grew malignantly in mice in tumourous masses, whereas bovine lymphosarcoma cells formed localised, non-invasive tumours. These findings contrasted with those recorded in the natural conditions in cattle, - lymphosarcoma being a malignant neoplasia - and East Coast Fever, a condition with no evidence of tumour formation. In the mice inoculated with T. parva-infected cells, there was no rejection, cellular degeneration or evidence that mouse cells became parasitised, although parasitised bovine cells became widely disseminated in the host's tissues and organs, in some cases causing death. In addition, tumours grew consistently only in irradiated athymic mice, and not in non-irradiated athymic mice, suggesting that non-irradiated mice retained some immune activity which was abolished by irradiation. Similar experiments were recently carried out by Fell, (Department of Zoology, University of Edinburgh, personal communication) who compared rates of growth of tumours which formed in irradiated mice injected sub-cutaneously with T. parva and T. annulata macroschizont infected cell lines. The rate of tumour growth was significantly higher in the mice injected with T. annulata-infected leukocytes. In all cases, no metastatic spread of parasitised cells to other organs was observed and eventually tumours became infiltrated with host cells and regressed within 3 to 4 weeks.

1.3.2. Reversibility of Theileria Transformation

One type of experiment with T. parva-infected leukocyte cell lines suggests that these cells are different from those transformed by viruses or carcinogens in that the transformation may be reversed. Treatment of Theileria parva-infected T-lymphoblastoid cells with the (non-mitogenic) naphthoquinone derivative 993C gradually eliminates intracellular parasites (see 1.1.5.2), but cell proliferation is not inhibited until several (approximately 11) days after parasite elimination (Pinder et. al., 1981). The proliferating cells no longer contain macroschizont particles and appear to be medium-sized lymphocytes and small blast cells whose surface phenotype (as defined by lectins and monoclonal antibodies) is unaltered after parasite elimination. In addition, elimination of the parasites was not observed to reduce the ability of these cells to stimulate DNA synthesis in autologous lymphocytes (see 1.3.4). It had been previously believed that these treated lymphoblastoid cells reverted
to small resting lymphocytes (McHardy, 1978), but the study of Pinder et. al., (1981) showed that there were morphological and functional differences between these treated cells and resting small lymphocytes. The authors considered a number of explanations for the continued division of treated cells for a limited period of time. The most plausible was that the transformation process, once initiated, no longer required the presence of active macroschizonts, but remained mediated by parasite product(s). The decline in proliferation could then be explained by a gradual elimination of these parasite product(s) (see 1.1.3).

A very similar experiment was carried out using the drug cytochalasin B which at low doses inhibits macroschizont multiplication without inhibiting the multiplication of the host cell. Thus, when Theileria parva-infected lymphocyte cells divide after treatment with this drug, the Theileria macroschizont does not divide, so only one daughter of the host cell receives a parasite. In these cultures, daughter cells which receive macroschizonts were shown to continue to proliferate, whereas non-parasitised daughter cells did not (I.L.R.A.D., 1983), thus indicating the cell transformation and proliferation to be a parasite-dependant, reversible process. However, this account did not report whether there was a delay between loss of parasite and cessation of proliferation, or whether the two events occurred simultaneously.

These two experiments appear to indicate that the continued presence of the parasite is necessary for maintaining the transformed phenotype, but that host lymphocytes may continue to proliferate and have transformed characteristics for a period of time after loss of the macroschizont - possibly due to persistence of some parasite product(s) responsible for sustaining neoplastic characteristics.

The difficulties in interpreting this type of experiment, involving the effect of drugs, however, should not be understressed. For example, it is also possible that the transformation event is non-reversible, but the effects of drug-treatment cause the cells to lose their transformed phenotype. These two hypotheses cannot be distinguished on the basis of the published results, although the authors have not considered the latter of the two explanations. This type of experiment has not been carried out with T. annulata-infected
lymphocytes, and so even though consensus of opinion seems to favour the transformation being parasite-dependant and reversible, this cannot be extrapolated to apply to *T. annulata* transformation.

1.3.3. **Involvement of Autocrine Mechanisms**

Williams et al., (1988) have presented evidence for stimulation of an autocrine mechanism in *Theileria parva*-infected T-lymphocyte cell lines. T-lymphocytes, on infection with *T. parva* macroschizonts, were observed to become independent of Interleukin (IL-2) (a T-cell growth factor) for their growth in culture. Hydroxynaphthoquinone-treated cells, which had lost their parasites, on the other hand (see 1.3.3), regained their dependence on addition of IL-2 to the growth medium and appeared to express fewer IL-2 receptors on the cell surface. *T. parva* macroschizonts thus appear to induce the expression of IL-2 and IL-2 receptors. However, the same phenomenon has been observed in uninfected lymphocytes stimulated to proliferate in culture with the T-cell mitogen concanavalin A (Con A) (Iyer, et al., 1984). So, although this IL-2 autocrine mechanism may be responsible for stimulating proliferation of infected cells, the point(s) at which *T. parva* interferes with the pathway(s) is unknown.

1.3.4. **Mixed Lymphocyte Reactions**

Pearson et al., (1979) reported that incubation of irradiated *Theileria parva*-infected lymphoblast cells with normal peripheral blood lymphocytes (PBL) from either the same, or unrelated cattle induced DNA synthesis in the PBL. The same stimulatory capacity has also been demonstrated in un-irradiated *T. annulata*-infected leukocyte cell lines (Ahmed J. S., 1986). This was unlike the classical mixed lymphocyte reaction (MLR), first clearly shown by Bain et al., (1964), since: (1) The stimulator cells in this case were T-lymphocytes, whereas strong MLR reactions are a property of B-lymphocytes (Lonai and McDevitt, 1977); (2) The response was several orders of magnitude higher in this case, than in the classical proliferative response which is induced by antigens of the major histocompatibility complex (BoLA).
It was postulated, by Pinder et. al., (1981) that the nature of the stimulus for lymphocyte proliferation by Theileria-infected cells could be 'parasite associated' antigens expressed on the surface of infected cells. However, there is no further evidence to support this, with the exception that such antigens have been shown to exist (see 1.3.5).

1.3.5. Transformation Antigens

Sheils et. al., (1986) identified a series of monoclonal antibodies which reacted strongly with epitopes present in Theileria-annulata-infected cell lines and less strongly, or not at all in some cases, against a panel of uninfected cells [including an uninfected bovine (non-viral) lymphosarcoma cell line, normal peripheral blood lymphocytes (PBL) and proliferating blasts derived from mitogen-stimulated PBL]. Several antigens were detected both on the surface and in the cytoplasm of only the Theileria-infected cells. Those antigens which were not expressed by the uninfected 'blast' cells were considered to be directly associated with the infection of bovine leukocytes by T.annulata and not more generalised alterations associated with actively proliferating leukocytes. Another group of antigens was found to be present in both Theileria-infected and uninfected leukocytes, but which appeared to be increased in expression in the infected cells. These probably represent host-encoded antigens which are stimulated by T. annulata infection.

These antigens represent alterations, both internal to and on the surface of T.annulata-infected leukocytes.

1.3.6. Models for Transformation by Theileria

In order to bring about the rapid proliferation of bovine leukocytes that results from invasion by Theileria, the mechanism(s) which maintain the normal growth characteristics of the host cell, are somehow disrupted. The general mechanisms by which this may occur as a consequence of Theileria-infection are summarised as follows:-

(1) The Theileria macroshizont may produce molecule(s) which mimic component(s) of the host cell mitogenic pathways in a manner similar
to a retrovirally transduced oncogene (see 1.2.6), thus stimulating cell division. This could occur at any level of the pathways, from growth factors and their receptors through secondary messenger molecules to nuclear transcription factors (see 1.2.9).

(2) The parasite may produce a molecule which is unlike any component of the leukocyte mitogenic pathways, but interferes with them, resulting in loss of normal growth control. This may be achieved through stimulating a positive regulatory component of the pathways, or by inactivating a negative regulatory component. This could also occur at any level(s) of the mitogenic pathways. A positive regulatory component of the leukocyte mitogenic pathways may be stimulated, for example, by activation of a growth factor or secondary messenger via phosphorylation of particular residues (see 1.2.9). Either a parasite-encoded kinase or a host-encoded kinase which has itself been activated (directly or indirectly) by a parasite molecule, may be responsible for the stimulation of the growth factor or secondary messenger. Alternatively, parasite molecules may directly or indirectly increase transcription of leukocyte growth factors or secondary messengers, thus bringing about loss of normal growth constraints. Alternatively, a possible target could be inactivation of a negative regulatory component of leukocyte growth control pathways, such as an 'anti-oncogene' or 'suppressor gene' (see 1.2.10.4) - either at the transcription, or gene product level.

(3) Theileria DNA sequences inserted into the leukocyte genome could transform the host cell in a manner similar to transformation by a v-onc retrovirus (see 1.2.7). Insertion into or near a host gene encoding a component of the mitogenic pathway may result in its inactivation, altered structure, or de-regulation, either of which could lead to altered growth characteristics.

(4) Alternatively, the Theileria macroschizont may be utilising a transforming virus to bring about leukocyte transformation. This may occur in a number of ways. Firstly, the parasite may carry a virus into the host cell, and so the virus might then either integrate with the leukocyte genome, or actively express transforming products from within the macroschizont. Alternatively, infection of the host cell by the parasite may activate expression of viral transforming products from viruses already present in the host cell, in a latent state.
None of the work carried out on the transformation of leukocytes by Theileria annulata or Theileria parva (see 1.3.2. to 1.3.5) has provided data which allows one to positively discriminate between any of these models. The primary reason for this is that all possible mechanisms of transformation have the same end result - stimulation and/or repression of various components of the host cell normal growth regulatory pathways. Thus the one or two causative events induced by Theileria macroschizonts which initiate the transformation, are obscured by the number of secondary events which produce and maintain the transformed phenotype. There are only two findings relevant to the causative events of Theileria-induced transformation, neither of which result in solid conclusions. Firstly, Pinder et. al., (1981) examined several Theileria parva-infected leukocyte cell suspensions by electron microscopy, and failed to observe any morphologically-recognisable viral particles. This by no means rules out the possibility of viral involvement in transformation since the virus, if present, may be stably integrated in either the parasite, or host cell genome (see model 4). The second finding, that the transformation of leukocytes by Theileria parva is a reversible process dependant upon continued presence of the macroschizont, if true (see 1.3.2), would imply that the transformation does not involve stable integration of viral DNA sequences (model 4a) or parasite DNA sequences (model 3) into the host genome. However, as discussed in section (1.3.2), the results from this type of experiment were not entirely conclusive, due to the use of drugs for macroschizont elimination which may themselves have interfered with the transformed state of the Theileria-infected cells.

1.3.7. Aims of This Investigation and Experimental Approach

The ultimate long-term aim of this project is to determine the mechanism by which the protozoan parasite Theileria annulata brings about the transformed phenotype of bovine leukocytes. However, bearing in mind the difficulty in distinguishing the causative events of Theileria-Induced leukocyte transformation from the secondary responses throughout the mitogenic pathways, (see 1.3.6) it was decided to first obtain a more general picture of the response of leukocyte growth regulatory pathways to Theileria annulata infection.
An approach involving the techniques of protein chemistry and molecular biology was considered most appropriate in determining the molecular changes which a host leukocyte undergoes, upon infection with *T. annulata*.

In order to detect molecular events occurring as a result of *T. annulata* infection, a collection of cell lines, both *T. annulata*-infected, and uninfected were used for direct comparison. The origins of the cell lines are described in section (2.1.1). Peripheral blood mononuclear cells (PBL) were isolated from cow 110 (PBL 110) and compared with the same cells, infected (in vitro) with *T. annulata* (Hissar) (Brown, 1983; cell line TaH-PBL 110) in order to detect events which were a direct result of both *T. annulata* infection and also those which were more generalised alterations associated with actively proliferating leukocytes. To distinguish which of the alterations were specific to *Theileria*-infection, and which were a result of proliferating leukocytes, comparison was also made with proliferating blasts derived from treating PBL 110 with the T-lymphocyte mitogen, concanavalin A (ConA-PBL 110) (see 2.1). These cells would reveal the alterations in T-lymphocytes which were a result only of mitogenic stimulation. Those events directly associated with parasite infection could then be deduced by inference. In order to strengthen the evidence for any such events detected by these comparisons, two further cell lines were used: direct comparison of the bovine lymphosarcoma cell line (BL-20) with the same cell line infected with *T. annulata* (Hissar) (TaH-BL-20) were used to reveal events directly related to *T. annulata* infection - the events associated with actively proliferating leukocytes in general, would not be apparent as differences since BL-20 is itself stimulated to divide by an unknown agent.

Thus, using these cell lines, molecular events could be detected which are (a) specific to *T. annulata* infection of bovine leukocytes and (b) more generally associated with leukocyte proliferation.

Caution was exercised in interpreting data obtained from comparison of PBL 110, TaH-PBL 110 and ConA-PBL 110, since the proportions of different sub-populations of leukocytes are likely to vary between the cell lines: while PBL 110 comprise a general preparation of mononuclear leukocytes, ConA-PBL 110 will be enriched.
for T-lymphocytes since concanavalin A is a T-lymphocyte-specific mitogen (Ling and Kay, 1975). TaH-PBL₁₁₀, on the other hand, is likely to be enriched for B-lymphocytes and macrophages, since these appear to be the preferred host cells for T. annulata infection (Spooner et. al., in press). Successive passaging in long-term cultures will ensure that the infected cell-type which proliferates fastest will predominate in the cell-population. In this respect, comparison between BL-20 and TaH-BL-20 gives a more controlled representation of events specific to Theileria annulata infection, since the cell populations are both the same type.

Consideration of the hypothesised molecular models by which T. annulata induces proliferation of host leukocytes (1.3.6) raises the possibility that the gene(s) by which the parasite (or passenger-virus) transforms its host cell may be (or be similar to) a host proto-oncogene. (See section 1.2.8 and Dyer and Tait, 1987). Since the rate at which novel oncogenes are being discovered is now levelling off, it appears that there exist a limited number, many of which have been cloned into plasmid, or viral vectors. Thus, it was decided to utilise a collection of oncogenes (see table 2.1) as molecular probes to investigate the possibility that parasite-encoded oncogenes may be involved in the transformation process. Use of these oncogene probes also allowed a preliminary study to be carried out of stimulation of expression of various host-encoded proto-oncogenes as a result of Theileria-infection, thus giving information as to the stimulation of host cell mitogenic pathways.

Another strategy to define molecular events relevant to the transformation process was adopted, as a result of the emphasis placed by the literature on the importance of protein kinases and phosphorylation of substrate proteins, in cell growth and transformation (see 1.2.8 for review). Thus, it was decided to investigate the effect of T. annulata infection on both the species of phosphoproteins, and the activity and species of protein kinases present in the leukocyte host cells. Using these experimental approaches, it was hoped to characterise the phenotype of T. annulata-infected leukocytes at a molecular level, and in doing so, to provide a better understanding of the mechanism by which the parasite interferes with the leukocyte growth regulatory pathways to induce proliferation.
CHAPTER 2

MATERIALS AND METHODS
2.1 THEILERIA-INJECTED CELL LINES AND CONTROL CELLS

2.1.1 Source

A number of Theileria annulata infected and uninfected cell lines and cells were used for the work described in this thesis:

(1) \text{PBL}_{110} \quad \text{Normal white blood cells, freshly isolated from cow 110.}

(2) \text{TaH-PBL}_{46} \text{ and TaH-PBL}_{110} \quad \text{Lymphoblastoid cell lines derived from cows designated 46 and 110 respectively, infected in vitro with macroschizonts of } P. \text{ annulata from a parasite isolate from Hissar (India)} \ (Gill, \ et.al., \ 1976).

(3) \text{BL20} \quad \text{An uninfected bovine (non-viral) lymphosarcoma cell line. } (\text{Morzaria, S.P. et al., 1984}).

(4) \text{TaH-BL20} \quad \text{The BL20 cell line, infected with TaH macroschizonts.}

(5) \text{Con A-PBL}_{110} \quad \text{Proliferating blasts derived from treating PBL}_{110} \text{ cells with the mitogen Concanavalin A, and left 2-3 days before harvesting.}

Livestock were maintained and blood made available for leukocyte isolation by C.G.D. Brown, Centre for Tropical Veterinary Medicine, Edinburgh. The infected cell lines were produced by Mr C.D.G. Brown, Centre for Tropical Veterinary Medicine, Edinburgh, using the method of Brown, (C.D.G., 1983). Parasites of the Hissar isolate used in infection were obtained from India (Gill, et.al., 1976).

2.1.2 Maintenance of Cultures

Lymphoblast cell lines were maintained at $10^6-10^7$ cells/ml in Roswell Park Memorial Institute medium 1640 (RPMI) pH 7.4,
supplemented with 15% heat inactivated (55°C, 30mins) foetal calf serum, 0.1% gentamycin sulphate, 0.2% NaHCO₃, 20mM L-glutamine and 0.75μg/ml fungizone (amphotericin B). The cells were incubated in a 37°C humidified incubator at 5% CO₂, and diluted with fresh medium every 2-3 days to maintain the correct density. Due to the different growth rates of the various cell lines, the dilutions were determined to be (approximately) as follows, but growth was checked visually with an inverted microscope.

BL 20 1 + 2.5 fresh medium every 2 days
TaH-BL20 1 + 4  fresh medium every 2 days
TaH-PHL46/110 1 + 9  fresh medium every 2 days

2.1.3 Isolation of Bovine Peripheral Blood Leukocytes

50ml of lithium heparin (14.3 U.S.P. Units/ml) treated blood was centrifuged at 2800g, 5°C for 20min and the resultant buffy coat removed in a volume of 3ml or less. The leukocytes were then mixed with 9ml of PBS (PBS = Phosphate buffered saline = 0.01M Phosphate 0.15M NaCl, pH 7.3) to give a suspension which was carefully layered onto 8ml of Ficoll-paque (Pharmacia-Fine Chemicals AB, Uppsala, Sweden) and centrifuged at 800g, 15°C for 35 minutes. Mononuclear cells at, and below the interface were recovered with a 5ml pipette and washed by mixing with 20ml PBS, followed by recentrifugation at 300g, 15°C for 10 minutes.

The supernatant was poured off and the cells rewashed as before, but centrifuging for only 5 minutes. The final leukocyte pellet was resuspended in 10ml cold, complete culture medium (see 2.1.2). The cells were counted using a haemocytometer (or Coulter counter), to estimate erythrocyte contamination, and their viability was determined by visualising uptake of Trypan Blue (Mishell and Shiigi 1980). The cell concentration was adjusted to (10⁶-10⁷/ml) in complete medium (see 2.1.2) and smears were made for Giemsa staining.

2.1.4 Giemsa Staining

Drops of cell suspension were air-dried on a microscope slide and
methanol-fixed (1 minute). Slides were then immersed in 0.045% giemsa staining solution in 3% methanol for 20 min. The slides were then rinsed in water, air-dried and observed using light microscopy.

2.1.5 Preparation of 'Con A Blasts'

Bovine leukocytes were stimulated to divide by addition of the T-cell mitogen Concanavalin A. (Bradley, 1980) as follows:-

Bovine peripheral blood leukocytes were prepared (2.1.3) and suspended in complete medium (2.1.2) at a concentration of $10^6$-$10^7$ cells/ml. The mitogen concanavalin A Type IV-S (supplied by Sigma) and B-2-Mercaptoethanol were added to final concentrations of 2.5μg/ml and $5 \times 10^{-5}$ M respectively. The culture was incubated for approximately 3 days in a 37°C humidified incubator with a 5% CO$_2$ atmosphere until cells were seen to be blasting. This stage is recognised by the large increase in size of the cells, as well as an increase in cell number.

2.1.6 Harvesting Cultures

Cell viability, was checked by the Trypan Blue exclusion method (Mishell and Shiigi 1980) and cells were counted using a haemocytometer.

The required amount of culture was centrifuged at 300g for 5 mins at 4°C. If a high percentage of dead cells and/or debris were present in the culture, the cells were centrifuged through a cushion of foetal calf serum at 300g for 10 mins at 4°C to effect removal of the former. The resultant cell pellet was resuspended by tapping, and the tube filled with PBS. These cells were washed 3 times as described above, before resuspending the final cell pellet in an appropriate buffer for the various experimental procedures described.
2.2.1 Direct Isolation of mRNA onto Nitrocellulose

This protocol is designed to selectively immobilise mRNA directly from a cell lysate onto nitrocellulose (Bresser et al., 1983). Many of the adaptations are from the Schleicher and Schuell Quick-Blot Kit manual.

Cells were harvested as described previously (2.1.6) but washed three times in PBS which had been made RNAse-free by stirring in 0.1% Diethylpyrocarbonate (DEP) and then autoclaving. (For four replicate filters, each with four dilutions) 5 x 10^6 cells were resuspended in 90μl PBS and proteinase K in RNAse-free water was added to a final concentration of 200μg/ml and incubated with the cells at 37°C for 30 mins. The cells were subsequently lysed on ice with 0.5% Brij 35 detergent and 0.5% Deoxycholate. Nucleic acids were solubilised by addition of an equal volume (100μl) of supersaturated NaI (heated to 75°C) followed by 200μl of saturated NaI [NaI to a final concentration of 12.2M]. Dilutions were prepared in saturated NaI and subsequently filtered through nitrocellulose which had been presoaked in RNAse-free water, then 6 x SSPE (1 litre of 20 x SSPE = 174g NaCl, 27.6g, NaH₂PO₄, H₂, 7.4g EDTA, pH 7.4), for 5 minutes. The filtering was carried out on a minifold apparatus constructed from a culture flask with a grid of 6mm diameter holes drilled in the upper surface and a vacuum pump connected to the flask neck.

After filtration, the nitrocellulose membrane (Schleicher and Schuell, pore size 0.45μm) was washed 3 times in RNAse-free water and then in 3 changes of 70% ethanol for 5 minutes each rinse, in order to remove excess NaI. Any residual basic proteins were then acetylated by soaking the membrane in freshly prepared acetic anhydride solution (0.25% v/v acetic anhydride in 0.1M triethanolomine). Since baking of these filters is unnecessary, they were simply air dried and either stored in a heat sealed bag at 4°C or prehybridised immediately. (see 2.2.4).
2.2.2 **Poly-A+RNA Isolation**

Total RNA was first extracted from cells according to Chirgwin et al., 1979, in conjunction with centrifugation through caesium chloride, according to Glisen et al., 1974. In all the work involving RNA, the following precautions were taken to minimise RNAse contamination:

1. Glassware was baked at 200°C for 4 hours.
2. Disposable laboratory equipment was treated with 0.1% (v/v) DEP for 30 minutes at room temperature and then autoclaved at 16 psi for 30 minutes.
3. Gloves were worn throughout (as with experiments involving DNA).
4. All solutions were filtered through nitrocellulose (0.45μm pore) and autoclaved where possible. DEP treatment and autoclaving was to be avoided with Tris-buffers. However, water used in Tris-buffers was DEP treated.

A 1ml pellet of washed cells was thoroughly homogenised in 10ml of millipore-filtered (pore size 0.45μm) guanidine thiocyanate buffer (5M guanidine thiocyanate, 50mM Tris-HCl, pH 7.5, 10mM EDTA, 5% 2-mercaptoethanol, 4% w/v N-lauroyl sarcosine and 0.15g/ml caesium chloride). The resulting viscous solution was left at room temperature for 30 minutes before centrifuging in Corex glass tubes at 30 000g for 20 minutes at 20°C to pellet cell debris. The resulting supernatant was layered onto 5.7M caesium chloride, 100mM EDTA, and centrifuged for 20 hours at 160 000g at 20°C. The upper layer, containing denatured protein was discarded, the top of the caesium chloride cushion removed, and the sides of the tubes were washed with water to remove traces of the proteins. The DNA and caesium chloride were removed, leaving the RNA pellets which were resuspended in ice-cold 10mM Tris-HCl pH 7.4. RNA was precipitated by addition of 2.5 volumes of cold ethanol and incubation for 30 minutes at -70°C, followed by incubation for 60 minutes at -20°C. RNA was collected by centrifugation at 30 000g for 30 minutes at 4°C. The RNA pellet was resuspended in 10mM Tris-HCl pH 7.4 and re-precipitated by addition of sodium chloride to 300mM followed by 2.5 volumes of ethanol and overnight storage at -20°C.

Poly A⁺ RNA was then isolated using essentially the procedure of
Avid and Leder (1972), utilising affinity chromatography on oligo-dT cellulose at 27°C. An oligodeoxythymidylic acid (oligo-dt) cellulose column was prepared by packing 0.5g of the latter (suspended in 10mM Tris-HCl 7.5, 0.5M NaCl, 1mM EDTA) into a 2ml syringe with a glass wool plug. The column was washed through 10 x with this buffer, and then equilibrated with high salt buffer (0.2% SDS, 0.5M NaCl, 10mM Tris-HCl, 1mM EDTA).

The total RNA samples were centrifuged as before, redissolved in high salt buffer, and heated at 65°C for 10 minutes to disrupt aggregates. Once cooled, the RNA was loaded onto the column and washed through with 10 column volumes of high salt buffer until the absorbance at 260nm of the eluate was negligible. Low salt buffer (0.2% SDS, 10mM Tris, 1mM EDTA) was used to elute the poly A+ RNA. 0.5ml fractions were collected and those with the highest concentrations of poly A+ RNA (as assessed by their absorbance at 260nm - see Fig. 2.1) were pooled, adjusted to 300mM NaCl and ethanol precipitated. Typically, from a starting culture of 5 x 10^9 cells, 20-80ug of poly A+ RNA was isolated.
Figure 2.1

mRNA Recovery from Tah-BL-20 total RNA.

Pooled fractions 24 - 26
mRNA Recovery = 2 O.D.'s / 68.3 O.D.'s
= 2.9% (80 ug)
2.2.3 Slot Blot Transfer of RNA to Nitrocellulose

Nitrocellulose (Schleicher and Schuell 0.45um pore) was pre-soaked in 6 x SSC (1 litre of 20 x SSC = 175g NaCl, 88.2g sodium citrate, pH = 7.0) and placed on a Schleicher and Schuell Slot-blot mini-fold apparatus, with a vacuum pump connected. RNA was dissolved in saturated NaI and was slowly applied to the slots, being concentrated onto the membrane by the minifold. The filter was then treated as in the quick-blot protocol (2.2.1).

2.2.4 Hybridisation to RNA Blots (2.2.1 and 2.2.3)

Filters were prehybridised in a shaking water bath at 42°C for at least four hours in a solution containing 10% w/v Dextran sulphate (pre-dissolved in H₂O as a 50% solution), 50% v/v deionised formamide, 5 x Denhardt's solution (50 x Denhardt's = 5g Ficoll, 5g polyvinylpyrrolidone, 5g BSA Pentax fraction V, per 500ml) 5 x SSPE, 20 ug/ml boiled sonicated salmon sperm DNA. The filters were hybridised in a small volume of 10% w/v Dextran sulphate, 50% formamide, 5 x Denhardt's solution, 5 x SSPE, 5ug/ml boiled sonicated salmon-sperm DNA and >10⁶ counts per minute of ³²P labelled probe (2.2.15) which had been denatured by boiling for 5 minutes. Incubation was carried out for at least 12 hours at 42°C with shaking. The filters were then washed as follows:

(1) 50ml 5 x SSPE for 15 minutes at 42°C.
(2) 50ml 5 x SSPE for 15 minutes at 42°C.
(3) 50ml 2 x SSPE 0.1% SDS for 30 minutes at 42°C.
(4) 50ml 1 x SSPE, 0.1% SDS for 30 minutes at 42°C.

The filters were then air dried and exposed to Kodak X-Omat film for 12 hours to 1 week at -70°C.

2.2.5 Gel Electrophoresis of RNA

The gel apparatus used was a BRL model H4 250 x 200mm gel tray. For a 250ml 1.2% agarose gel, 3.0g of Sigma agarose medium EEO was added to 195ml H₂O and dissolved in a microwave oven. When the
solution cooled to hand temperature, 5mls of stock (37%) formaldehyde and 50mls of x 5 MOPS buffer (41.8g MOPS, 6.8g Na Acetate, 10mls 500mM EDTA, pH=7.0 per litre) were added and the gels cast in a fume cupboard. RNA samples (2-8ug polyA+ or 10-20ug total RNA) were diluted to 10.1ul in H2O, and then 4.5ul of x 5 MOPS, 7.9ul 37% formaldehyde and 22.5ul of recrystallised formamide were added. They were then denatured by heating to 55°C for 15 minutes and chilling on ice. 5ul of 20% Ficoll, 0.2% Bromophenol blue was added and if staining of any tracks was desired, ethidium bromide was added to 0.5ug/ml. Samples were loaded into the dry wells of the gel and run into the gel at 100 volts, using 1 x MOPS as an electrophoresis buffer. The gel was then submerged completely and run with buffer recirculation at 30-40 volts overnight.

2.2.6 Electroblotting (Using BioRad Apparatus)

After separation of the RNA by electrophoresis, the RNA was transferred to Pall Biodyne membrane as follows:- The transfer buffer used was 0.025M NaPO4 buffer pH 5.5 (made by dissolving 10.35g monobasic sodium phosphate to 3 litres and pH to 5.5 with 25mM stock dibasic sodium phosphate). Transfer pads were soaked in buffer, and the gel was inverted and placed on a pad so that a Pall Biodyne filter cut to the correct size could be placed directly on the underside of the gel. This sandwich was submerged in a tank of transfer buffer and the RNA transfer was carried out in a cold room at 20 volts overnight, then at 50 volts for 30 minutes. RNA was then fixed onto the filter by laying on an ultra-violet trans-illuminator, (RNA-side facing the lamps) for three minutes.

2.2.7 Northern Hybridisation

Prehybridisation and hybridisation were both carried out in hybridisation solution (50mM Pipes pH 6.8, 100mM NaCl, 50mM NaPO4=0.53g Na2, 0.414g Na per 100ml, 1mM EDTA, 5% SDS). Filters were prehybridised in two rinses of 10mls of hybridisation solution in a sealed bag for 10 minutes at 65°C for a homologous probe or 59°C for a non-homologous probe. Hybridisation was carried out for 12-24 hours at the same temperature in 10mls of the same solution containing >10^6
counts per minute of $^{32}\text{P}$ labelled probe (2.2.15) which had been
denatured by boiling for 5 minutes. Filters were then washed at the
hybridisation temperature in three changes of 1 x SSC, 5% SDS for 15
minutes. Filters were then wrapped in saran-wrap and exposed to
Kodak X-Omat film for 12 hours to 1 week at -70°C.

2.2.8 **Stripping Filters**

Nylon filters were stripped of probe by heating to 70-80°C for 2-
3 hours in 0.1% SDS. This allowed re-use of a filter for subsequent
hybridisation to a new probe.

2.2.9 **Isolation of Piroplasms from Infected Blood for Subsequent DNA
Preparation (2.2.10)**

250mls of heparinised blood was supplied to us on several
occasions by Mr C.G.D. Brown (C.T.V.M., Edinburgh). Blood was
obtained from cattle infected with the Hissar isolate of *Theileria
annulata* and parasitaemia varied from 30% to 90%, often with several
parasites in each erythrocyte visible by Giemsa stain (2.1.4) and
light microscopy. Piroplasms were isolated using the method of
Martin et. al., (1971), used for the isolation of schizonts of
*Plasmodium berghei*.

The majority of leukocytes were removed, by spinning the
heparinised blood twice at 2800g for 20 minutes and topping up with
PBS. Each time, the serum, and buffy coat were removed and
discarded. Eight 30ml plastic columns were set up by plugging with
glass wool, adding Whatman CF11 and washing with 2 column volumes of
PBS. Blood was passed through the columns to remove leukocytes,
followed by 1 column volume of PBS. The resultant erythrocyte
preparation was checked for leukocyte contamination by addition of
white blood cell counting fluid (1% acetic acid), which selectively
lyses erythrocytes. Leukocyte contamination was then estimated using
a haemocytometer. Red blood cells were subsequently lysed in 10
volumes of prewarmed (37°C) 0.83% NH$_4$ Cl buffered 9:1 with 0.17M Tris
pH 7.65 at 37°C until lysis was observed. The released piroplasms
were spun down at 2800g for 10 minutes, and washed twice in PBS and
once in 10mM Tris pH 7.65 (10 minute spin at 600g). Typically, a 0.2-
0.5ml piroplasm pellet was obtained from 250ml of infected blood.

2.2.10 Preparation of DNA from Piroplasms

DNA was extracted from piroplasms using a method derived from DNA
extraction procedures designed for trypanosomes (Fairlamb et.al., 1978
; Borst et.al., 1979; Frasch et.al., 1980; Borst et.al., 1980b). Pi
roplasms were lysed by resuspending in 9 volumes of 10mM Tris, 0.25M
EDTA, 100mM NaCl, and addition of Sarkosyl (to 1%), and digested by
proteinase K at 100ug/ml overnight at 50°C. The mixture was
deproteinised three times using an equal volume of phenol (saturated
in 0.1M Tris pH 8), then phenol –chloroform (1:1) and finally
chloroform. Back extraction of the interfaces with a small volume
of lysis buffer was carried out to maximise yield. The resulting
aqueous layers were pooled, diluted 1:1 with 10mM Tris pH-8, 100mM
NaCl and incubated for 3 hours at 37°C with 0.1mg/ml boiled
ribonuclease A. The solution was deproteinised by extraction with
phenol then phenol-chloroform and finally chloroform. The aqueous
phase was dialysed extensively against 10mM Tris pH 7.6, 1mM EDTA.
Typically, between 0.5mg and 1.5mg of DNA was obtained from 250ml of
infected blood.

2.2.11 Optical Density Measurements on DNA

All DNA samples were checked for their absorbance at 260nm and
280nm. The optical density at 260nm (O.D. 260) was used to estimate
the concentration of DNA (and RNA). An O.D. 260 of 1.0 is equivalent
to 0.05mg/ml of DNA. The ratio of the O.D. 260 to the O.D. 280 was
used to estimate purity. Ratios ranging from 1.7 to 1.8 were
considered protein-free and ratios lower than this were purified
further with phenol and chloroform to remove protein. Ratios higher
than 1.8 indicated the presence of RNA and/or nucleotides. RNA and
oligonucleotides were removed from these samples by passing through a
Sepharose CL 2B column (see 2.2.14.2).
2.2.12 **Restriction Enzyme Digestion**

Restriction enzyme digests were carried out as described previously (Maniatis et al., 1982) using the appropriate (10 x) buffer for the given enzyme. For Southern blots, typically 2-3 micrograms of DNA were loaded per track and for restriction mapping of clones, 1 microgram was loaded. For each microgram of DNA, 10 units of restriction enzyme were used, and the digestion carried out for 4 hours at 37°C or the temperature appropriate to the restriction enzyme (Maniatis et al., 1982). One tenth volume of 10 x sample buffer (Maniatis et al., 1982) was added to each sample and heated to 65°C for 5 minutes before loading on agarose gels.

2.2.13 **Agarose Gel Electrophoresis**

Agarose gel electrophoresis was carried out as described previously (Maniatis et al., 1982). The gels were run in Tris Borate (TBE) buffer (0.089M Boric Acid, 0.089M Tris, 2mM EDTA) and ethidium bromide (0.5 micrograms per ml final concentration). When separating fragments of 1-20kb (kilobases) in size, 0.6% agarose was used, while 1.5% agarose was used for smaller fragments (0.2-4kb) separation. Gels were run overnight at 40 volts (200mm x 250mm gels) or 60-120 volts for 0.5 to 4 hours for the smaller gels (50 x 75mm; 140 x 110mm). DNA was visualised under long wave ultraviolet illumination (366nm), and photographed where necessary, using a polaroid camera. A commercial 1Kb ladder (BRL) was used to estimate the fragment sizes. The $R_f$ value (relative mobility) of each standard fragment was plotted against $\log_{10}$ [molecular size (kb)] to create a standard curve. Sizes of unknown fragments were estimated from their $R_f$ values using this standard curve.

2.2.14 **Plasmids**

Several recombinant plasmids were used either wholly or in part, to search for homologous sequences in parasite DNA by Southern blot analysis (2.2.16) and by library screening (2.2.19) and to study gene expression by Northern dot blot analysis (2.2.3; 2.2.4).
pUC 18 (supplied by BRL) was also used in library construction (2.2.21). Details of the plasmids are given in table (2.1) and in chapter (3.2).

2.2.14.1 Transformation of E.Coli with Plasmid DNA

Some plasmids to be used as probes in Southern blotting and northern dot blotting, were obtained as a DNA preparation in very small quantities and so in order to produce our own large scale supply, it was necessary to transform the plasmids into their appropriate host cells.

A 5ml culture of the appropriate strain of Escherischa coli (eg. JM83 for pUC 18, HB101 for pBR322 - see Table 2.1) was incubated at 37⁰C with vigorous shaking overnight in L-Broth with the appropriate antibiotics (2.2.14.2). 1ml of the resultant culture was then used to inoculate 100mls of L-Broth (again with the appropriate antibiotics) in a 1 litre flask. The culture was then grown as before with vigorous shaking at 37⁰C until an O.D. 600 of 0.3 was reached. The culture was then chilled on ice for 10 minutes, followed by centrifugation for 10 minutes at 10000g at 4⁰C. The pellet was then resuspended in 50ml of ice-cold, sterile 50mM CaCl₂, 10mM Tris pH 8.0, left on ice for 15 minutes and then centrifuged as before. The pellet was then resuspended in 6.6mls of ice-cold CaCl₂/Tris, dispensed into 0.2ml aliquots in pre-chilled eppendorf tubes and stored at 4⁰C for 12-24 hours.
### TABLE 2.1

<table>
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<th>PLASMID</th>
<th>ANTIBIOTIC</th>
<th>HOST INSERT</th>
<th>REST. ENZYME</th>
<th>SIZE</th>
<th>FOR EXCISING INSERT</th>
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<td>p-src(EcoRI-B)</td>
<td>Tet&lt;sup&gt;R&lt;/sup&gt; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>p-yes(pyes1)</td>
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<td>HB101 4.0 kb Pst (3 fragments)</td>
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<tr>
<td>p-myb</td>
<td>Tet&lt;sup&gt;S&lt;/sup&gt; Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>DH1 7.3kb BamHI</td>
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<tr>
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<td>DH1 5.1kb EcoRI</td>
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<tr>
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<td>DH1 0.7kb EcoRI</td>
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<td>HB101 1.0kb Pst (4 fragments)</td>
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<td></td>
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<tr>
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<tr>
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<td>HB101 1.0kb EcoRI</td>
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<tr>
<td>pras(pBS9) Ha</td>
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<td>JM83</td>
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</table>

*Plasmid Obtained with Permission from:*

1. Dr J. Michael Bishop, Dept. of Microbiology, University of California.
2. Dr Mitsuaki Yashida, Cancer Research Institute, Tokyo.
3. Dr R. A. Weinberg, Massachusetts Institute of Technology, Cambridge.
4. Dr Howard Temin, University of Wisconsin.
6. Dr David Baltimore, Massachusetts Institute of Technology.
7. Dr D. R. Lowy, Nat. Inst. of Health, Bethesda, Maryland.
8. Dr Natalie Teich, I.C.R.F.
9. Dr Charles Sherr, St. Jude Childrens Research Hospital, Memphis, Tennessee.
10. Dr Flossie Wong-Staal, Nat. Inst. of Health, Bethesda, Maryland.
11. Dr G. M. Cooper, Sidney Farben Cancer Institute, Boston.
12. Gibco/BRL.
For each plasmid, 3 dilutions of plasmid DNA were prepared in ice-cold 10mM Tris (pH 7.4) 1mM EDTA and added to the aliquots of cells, (final plasmid concentrations of 100ng/ml, 1ng/ml, 0.01ng/ml). The mixture was then left for 30 minutes on ice before transferring to a 42°C water bath for 2 minutes. This serves to heat shock the bacteria, inducing them to take up the DNA. 1ml of L-Broth without any antibiotics was added to each tube, with subsequent incubation at 37°C for 30 minutes if Tetracycline is to be used or 1 hour if Ampicillin or Kanamycin is to be used. This incubation is carried out without shaking to allow recovery of the bacteria. 100ul aliquots of transformed cells were spread on L-broth plates which were then incubated at 37°C for 12-16 hours.

The L-broth plates with 1.2% agar were cast (Maniatis, et.al., 1982), containing the appropriate antibiotic(s). Ampicillin was used at 50ug/ml or Tetracycline at 25ug/ml (from a stock made up in 50% ethanol). For pUC 18, 60ul of 25mg/ml X-gal (5-bromo-4-chloro-3-indoly-B-galactopyranoside) in dimethylformamide was prespread on each plate, before inoculating with 100ul bacteria plus 5ul 1M IPTG (isopropyl B-D-thiogalactopyranoside).

2.2.14.2 Preparation of Plasmid DNA:

Before preparation of plasmid DNA was carried out, the bacterial strains were checked for growth on L-Broth plates containing the appropriate selective antibiotics for the plasmid concerned. (See Table 1). The antibiotics were added to working concentrations of 0.025mg/ml for tetracycline and 0.05mg/ml for Ampicillin and Chloramphenicol. A single bacterial colony was then taken from the appropriate test plate, and used to inoculate a 20ml culture of L-Broth containing the appropriate antibiotics. This culture was incubated at 37°C for 20 hours with vigorous shaking. The resultant culture was used to inoculate 200ml of L-Broth in a 1 litre flask. Appropriate antibiotics were also added to this flask. This was incubated with vigorous shaking at 37°C for exactly 2.5 hours. A blocking antibiotic (Chloramphenicol at 0.15mg/ml) which allows plasmid replication, but blocks bacterial replication, was then added and the culture again incubated at 37°C with vigorous shaking.
overnight. The cells were harvested by spinning at 10,000g for 20 minutes at 4°C. The cells were resuspended in 40mls of ice-cold 10mM Tris pH 7.4, 1mM EDTA and centrifuged again. The pellet was then resuspended in 4ml of ice-cold 25% sucrose, 50mM Tris pH 8.1, 40mM EDTA and egg white lysozyme added to 5mg/ml. This was left to stand on ice for 15 minutes, after which, 1.2ml of 0.5M EDTA was added. After a further 5 minutes, 10.8ml of 10mM Tris pH 8.1, 0.1% Triton X-100, 60mM EDTA was added, and the suspension incubated for 10 minutes before centrifugation at 20,000g for 30 minutes at 0°C. The supernatant was decanted into a sterile tube. To 7ml samples of the supernatant, 6.65g caesium chloride was added, followed by 0.7ml of 10mg/ml ethidium bromide. The mixture was then centrifuged at 35,000g at 25°C for 60 hours in an MSE 10 x 10ml angle rotor using an MSE Superspeed 65 ultracentrifuge. Following this caesium chloride gradient centrifugation step, the plasmid band was visualised under long wave ultra violet illumination and recovered by piercing the tube with an 18G hypodermic needle. The plasmid DNA band was extracted, with propan-2-ol (equilibrated with caesium chloride - saturated water), at least 3 times to remove the ethidium bromide. The aqueous phase was then removed, dialysed against 10mM Tris pH 7.8, 0.3M NaCl, 1mM EDTA and extracted 3 times with phenol, phenol/chloroform and chloroform. The DNA was then precipitated by the addition of two volumes of ice-cold ethanol followed by storage at -20°C overnight. The precipitate was centrifuged for 10 minutes in a microfuge and then resuspended in 0.1ml of 10mM Tris pH 7.6 1mM EDTA and checked for degradation by running a sample on an agarose gel. If any small fragments were present, they were removed from the DNA as follows: NaCl was added to the sample to a concentration of 0.3M and this was then run through a Sepharose CL 2B column (equilibrated with 10mM Tris pH 7.8, 0.3M NaCl, 1mM EDTA). Fractions were collected and analysed by agarose gel electrophoresis (2.2.13) to identify those containing the plasmid. These fractions were pooled and precipitated as before.

**2.2.14.3 Rapid Preparation of Small Quantities of Plasmid DNA**

If small quantities of plasmid DNA were required, a more rapid method of isolation was utilised. The method used was the alkaline lysis method (Maniatis, et.al., 1982), which was followed exactly.
2.2.15 **In Vitro Labelling of DNA with $^{32}$P dCTP.**

DNA fragments to be used as radio-labelled probes in the techniques of Southern blotting, northern blotting and library screening, were labelled by the random primer technique described in the P & S Biochemicals manual supplied with the Polymeraid RH Kit.

The specific fragment of DNA to be labelled was excised from the plasmid using the appropriate restriction enzymes as described (2.2.12). The fragment was separated by agarose gel electrophoresis, but using low-melting point (LMP) agarose, visualised under long wave ultraviolet light and cut out with a scalpel. The fragment was electrophoresed once again on LMP agarose and cut out in order to ensure purity. 1.5ml of water was added per gram of agarose. Before each labelling reaction, the DNA/agarose was placed in a boiling water bath to melt the agarose and denature the DNA, and then incubated at 37°C for at least 10 minutes prior to use. 25 nanograms of DNA were added to the reaction mixture along with $^{32}$P dCTP (P & S Biochemicals Instruction Manual) for a minimum of five hours at room temperature and the reaction then stopped using the reaction terminator supplied. The reaction mixture was then passed through a Sephadex G-50 column to remove unincorporated nucleotides (Maniatis et al., 1982). Typically, 1-5x10^8 cpm/microgram DNA were achieved by this method, as measured by scintillation counting of aliquots.

2.2.16 **Southern Transfer**

Hybridisation of DNA fragments, separated by gel electrophoresis, to specific labelled DNA probes was carried out by the method of Southern (1975), as described in section 2.2.16.1 and 2.2.16.2.

2.2.16.1 **Transfer of DNA from Gels to Nitrocellulose**

The agarose gels were first soaked in denaturing solution (1.5M NaCl, 0.5M NaOH) for 1.5 hours at room temperature with constant shaking. They were then neutralised by soaking in several volumes of neutralising solution (0.5M Tris pH=7.0, 3M NaCl) for 1.5 hours. The gel was then transferred to a strip of Whatman 3MM paper which had
been previously soaked in 20 X SSC and which was cut to the width of the gel. The ends of the 3MM paper were placed in 20 x SSC to act as wicks for the transfer buffer (20 x SSC). A piece of nitrocellulose (Schleicher and Schuell 0.45μm pore size), presoaked in 20 x SSC and cut to the exact size and shape of the gel was placed on top of the gel. Four layers of 3MM paper were then placed on top of the nitrocellulose and a pile of paper towels placed on top of this. The DNA was thus allowed to transfer overnight, using the capillary action of the transfer buffer being soaked up by the paper towels. The filter was removed and rinsed in 6 x SSC and baked in a vacuum oven at 80°C for 1.5 hours. The filter was then ready for hybridisation to the probe.

2.2.16.2 Hybridisation

The baked filters were rinsed for 15 minutes in 500ml of 4 x STE (1 x STE = 100mM NaCl, 10mM Tris pH=8, 1mM EDTA). They were then washed for 3 hours (at 59°C for a non-homologous probe and 65°C when using a homologous probe) in 300ml of the following solution per 20 x 20cm filter:-- 150ml of 20 x Denhardt's solution (Maniatis et.al.,1982), 60ml 20 x STE, 3mls of 10% sodium pyrophosphate, 3ml 10% SDS, 84ml of distilled water. The filter were then prehybridised in 50ml of the above solution containing 1ml of 5mg/ml sonicated boiled salmon sperm DNA. Prehybridisation was carried out at 59°C or 65°C as appropriate for 2 hours with vigorous shaking (200 rpm). The filters were then hybridised in 10ml final volume (per 20 x 20cm filter) of hybridisation solution. This solution is made as follows (for 25ml):-- 2.5g of dextran sulphate was dissolved in 1ml of water plus 5ml of 20 x STE. Then 5ml of 50 x Denhardt's solution, 12.5mls of deionised formamide, 0.25ml of 10% SDS and 0.25ml of 10% (w/v) sodium pyrophosphate were added. Labelled probe (>10^6 counts per minute of 32P) was added to 0.5ml of 5mg/ml salmon sperm DNA and 1ml of distilled water, boiled for 5 minutes to denature the probe and added to 10mls of hybridisation solution. The filters were incubated in this solution at 37°C for a non-homologous probe and 42°C for an exactly homologous probe for 12-24 hours with vigorous shaking (200 rpm). The filters were then washed as follows:

(1) 500ml 4 x STE, 0.1% SDS at room temperature for 30 minutes.
(2) 300ml 4 x STE, 0.1% SDS at 59°C/65°C as appropriate, for 1 hour with shaking.
(3) 300ml 2 x STE, 0.1% SDS at 59°C/65°C for 1 hour with shaking.
(4) 300ml 1 x STE, 0.1% SDS at 59°C/65°C for 1 hour with shaking.
(5) 300ml 0.5 x STE, 0.1% SDS at 59°C/65°C for 1 hour with shaking.

The filters were then air-dried and exposed to Amersham Hyperfilm for 12 hours to 1 week at -70°C.

2.2.17 **Host Vector Systems**

2.2.17.1 **Lambda gt-11/E.coli Y1090 System**

The initial isolation of clones was from a genomic piroplasm DNA library, constructed by Roger Hall, (Wellcome Unit of Molecular Parasitology, Glasgow) from Theileria annulata. The structure of the bacteriophage lambda (expression) vector _gt11_ (lac _5_ c1857 _nin_ 5 _S100_) is shown in figure (2.2). This phage is grown up on a host _E_. _coli_ strain, _Y1090_ (lac _11169_ _proA+_ _lon ara D139 strA sup F [trp C22::fn10_] (pMC9)). The vector has a single _EcoRI_ cleavage site within the _lac Z_ gene into which up to 7.2kb of foreign DNA may be inserted. Insertion of DNA into the _EcoRI_ site inactivates the structural gene for _B_-galactosidase. Thus recombinant phage are recognised by their property of producing colourless plaques on a _sup F lacT2_ - containing host (eg _Y1090_) in the presence of the _lac_ operon inducer IPTG and the chromogenic _B_-galactosidase substrate _X-gal_. Non-recombinant phage produce blue plaques as _B_-galactosidase turns _X-gal_ blue. (Young and Davis, (1983)). Host cells _Y1090_ carry the gene for Ampicillin resistance.

2.2.17.2 **Lambda EMBL 3/E.Coli Q359**

Lambda _EMBL 3_ is a replacement vector (see figure 2.2 for structure). Since the core of the _Bam H1_ recognition sequence is the recognition sequence of _Sau 3A_ and _Mbol_, _EMBL 3_ DNA can accept exogenous DNA fragments prepared by partial digestion with _Sau 3A_ or
MboI in the 9-22kb range, (Kaiser and Murray 1985).

Gam+ phages are unable to form plaques on E. coli lysogenic for phage P2. (Hendrix, et.al., 1983). Thus inclusion of a functional gam gene in the central replacement fragment of wild type EMBL 3, means that only recombinant and therefore gam- phages should form plaques on an E. coli (P2) strain. This is known as "sensitivity to P2 Interference", or "SPI selection."

Such a library, constructed by Dr Roger Hall, Wellcome Unit of Molecular Parasitology, Glasgow, was used for screening as described in section (2.2.18; 2.2.14). The library was made from a partial Sau 3A digest of genomic piroplasm DNA from Theileria annulata (Hissar). Host cells used for growth were Q359 (hsdRk-, hsdMk+, supF, 80, P2) which are lysogenic for P2 phage (Karn et al., 1980).

2.2.17.3 pUC 18/JM83

A small library from Theileria annulata (Hissar) DNA was constructed, from DNA restriction enzyme fragments of specific size (section 2.2.21). This was to aid the isolation of clones containing Theileria DNA fragments of a size class known to include sequences which hybridise to a particular probe on Southern blot analysis.

A map of the pUC 18 plasmid vector which was used, is shown in figure (2.2). Because the plasmid carried the ampicillin resistance gene (B-lactamase), when it is transformed into AmpS (Ampicillin-sensitive) host cells, (see 2.2.14.1), such as JM83 used here, only successfully transformed cells will grow in the presence of ampicillin. pUC 18 also contains a fragment containing the functional gene for B-galactosidase and a multiple cloning site. DNA fragments may be inserted into the unique restriction sites located in the multiple cloning region. Insertion is monitored by the loss of B-galactosidase activity upon transformation of host strain JM83 (2.2.17.3). Thus JM83 cells containing recombinant pUC 18 will produce white colonies, while those containing non-recombinant pUC 18 will produce blue colonies in the presence of the lac operon inducer IPTG and the chromogenic B-galactosidase substrate X-gal.
Figure 2.2

Restriction Maps of Cloning Vectors

(a) λgt 11

Features of λgt 11. Restriction endonuclease cleavage sites are designated in kb from the left end. +, A attachment site. The transcriptional orientation of λcd (CD) is given by the horizontal arrow. The sequence of the unique EcoRI site (boldface letters), the nucleotides that immediately surround it, and the amino acids encoded are shown below the phage map.

(b) λEMBL 3 (Kaiser and Murray, 1985)

(c) pUC 18

(from Gibco, BRL)
2.2.18 **Plating out Bacteriophage Lambda**

Cultures of the host bacteria were grown up at 37°C to log phase (optical density of 0.5-0.6 at 600nm) in L-Broth (10g Bactotryptone, 5g Yeast extract, 10g NaCl per litre, pH 7.5) plus 100ug/ml ampicillin for Y-1090 cells and in minimal medium plus 0.4% maltose (5g NaCl, 10g Bactotryptone pH 7.5) for Q359 cells. They were then spun down and resuspended in 1/10 volume 50mM Mg++. Then 0.1ml of the host bacteria and 0.1ml of the appropriate dilution of phage in SM buffer (Maniatis et.al., 1982) were incubated together for 15 minutes at 37°C. To this mixture, 2mls of L-Broth or minimal-Broth as appropriate, containing 0.7% Bacto-agar at 45°C were added. The top agar was then quickly mixed and poured onto a 9cm petri dish containing 25ml of pre-set and dried bottom agar. Bottom agar is L-Broth plus 100ug/ml ampicillin (or minimal broth) containing 1.5% Bacto-agar. Plates were then left for a few minutes at room temperature to set and then inverted and incubated overnight at 37°C. Plaques were examined and counted the next day. Bacteriophage were initially titred by plating out at different dilutions.

2.2.19 **Library Screening by Filter Hybridisation**

Recombinant phage clones containing sequences homologous to the desired probes were identified by hybridisation to plaques which had been transferred to nitrocellulose (Benton et. al., 1977).

2.2.19.1 **Plaque Transfer**

This was carried out according to Benton and Davis (1977). The library was plated on a suitable host bacteria (2.2.17.1; 2.2.17.2), at such a density that neighbouring plaques were distinct from each other (approximately 500 plaques per 9cm diameter petri dish or 3000 plaques per 25 x 25cm square plate). The plates were then chilled for 1 hour at 4°C to harden the top agar. Then a dry nitrocellulose filter was layed over the plate and marked to identify the orientation. The filter was left in place for 1 minute, transferred to denaturating solution (1.5M NaCl, 0.5M NaOH) for 1-2 minutes, and then transferred to neutralising solution (1.5M NaCl, 0.5M Tris pH= 7) for 2 x 5 minutes. The filter was rinsed in 2 x SSPE (20 x SSPE =
for 2 x 5 minutes. The filter was rinsed in 2 x SSPE (20 x SSPE = 3.6M NaCl, 200mM sodium dihydrogen orthophosphate pH 7.4 and 20mM EDTA pH= 7.4). Filters were air dried and then baked in a vacuum oven at 80°C for 90 minutes.

2.2.19.2 Hybridisation

Hybridisation with the required probe was carried out as described in (2.2.16.2).

2.2.19.3 Identifying and Purifying Recombinants

Recombinants were identified on the plates by matching up the signal detected by autoradiography with the plaques on the plate. Positive plaques were then picked using a Pasteur pipette, and then resuspended in 0.5ml S.M. phage buffer (Maniatis, et. al., 1982). Duplicate filters were used to ensure identification of positive plaques. Each positive plaque picked was replated and rescreened by the same procedure, and single positive plaques picked from the second screening were considered to be pure clones.

2.2.20 DNA Extraction from Phage Lambda

In order to analyse positive clones, DNA was extracted for restriction digest analysis, for subcloning and for construction of homologous probes.

2.2.20.1 Large Scale Isolation of DNA from Phage Lambda

The protocol used was one derived by Dr Hide, (Wellcome Unit of Molecular Parasitology, Glasgow), for Lambda NM149, based partly on methods described by Maniatis et.al., 1982).

Ten to twenty L-Broth plates were set up as described (2.2.18) except that the dilution of phage was adjusted to produce confluent lysis of the bacteria on the plate (approximately 50 000-100 000 phage per plate). The plates were then grown up overnight at 37°C and the
phage harvested by adding 4ml of SM medium (1 litre SM = 5.8g NaCl; 2g MgSO$_4$ 7H$_2$O; 6g Tris (pH 7.5); 10mg gelatin) and a drop of chloroform to each plate and rocking for 2 hours. The SM/phage suspension was then poured into centrifuge tubes and centrifuged at 800g for 10 minutes to remove bacterial debris and any agar. The supernatant was recovered, and approximately 10" phage were recovered from 10 plates. These phage were stored at 4°C with a few drops of chloroform, until required. A culture of host bacteria (Y 1090) was set up at a starting optical density of 0.01 at 600nm. The culture was grown with vigorous shaking to an O.D. 600 of 0.4. (2$^{10}$ bacteria per 100mls). At this point, the phage lysate, which had previously had the chloroform removed by pipetting and warming at 37°C for 10 minutes, was added to the culture. For 10 minutes, the culture was left without shaking to allow the phage to adsorb to the bacteria. Shaking was then commenced and the O.D. 600 monitored. Typically, the O.D. peaked at 0.8->1.2 and then fell to around 0.3-0.4. At this point, 2ml of chloroform was added and shaking continued for 30 minutes. The culture was then spun at 10000g for 10 minutes to remove the bacterial debris. The supernatant was then spun at 25000g for 3 hours to pellet the phage. Following this, the supernatant was removed, the pellet teased out with a glass rod and then resuspended in 4ml of SM buffer by rotating gently overnight. The phage were then gently homogenised and purified by caesium chloride step gradient centrifugation (Maniatis et al., 1982). The phage band was recovered and dialysed overnight against three changes of 1000-fold volumes of 10mM Tris pH= 8.1, 1mM EDTA. This dialysate was then aliquoted to 0.3ml per eppendorf tube and NaCl added to a final concentration of 0.1M. The phage solution was extracted three times with phenol, phenol/chloroform and chloroform. 0.06ml of 10M ammonium acetate was added to the final aqueous phase and an equal volume of isopropanol was used to precipitate the DNA from the mixture. The precipitation was carried out at -70°C for at least 2 hours. The DNA was pelleted and washed once in 70% ethanol and finally resuspended in a small volume of 10mM Tris pH 7.6, 1mM EDTA.

2.2.20.2 Small-Scale Isolation of DNA from Phage Lambda

In order to check recombinant bacteriophage for the presence of inserts by restriction enzyme digestion and hybridisation, the 'Aloha'
A protocol was used to prepare DNA from 10-20 clones simultaneously. It is based on the procedures of R. Taylor (NCI-FCRF), M. Graham and M. Olson (Washington University Medical School) and D. Ish-Horowiz (ICRF, London) which have been modified by Luiz Shozo Ozaki and Shobaona Sharma (New York University) (Dr P. Beck, personal communication).

The phage, usually from a single plaque, were plated to confluent lysis on one 7cm diameter petri dish as described (2.2.18). Phage were then harvested and bacterial debris and agarose removed (2.2.20.1). The following procedure was carried out on a 0.8ml aliquot of the supernatant - the rest was stored at 4°C for subsequent use if necessary, after addition of a few drops of chloroform. The supernatant (0.8ml) was treated with 0.5ml DE52 cellulose suspension (see footnotes) by inverting the mixture 30 to 50 times. The tube was then spun for 5 minutes in a microfuge and 0.8ml of the supernatant transferred to a new eppendorf tube along with 150ul of 2.5% SDS, 0.5mM Tris-Cl pH=8.0, 0.25M EDTA. The tube was heated to 67°C for 15 minutes to disrupt the bacteriophage and then cooled to room temperature. Phage proteins and RNA were then precipitated out by adding 200ul of 8M ammonium acetate, leaving on ice for 15 minutes and then spinning for 10 minutes in a microfuge. The DNA from the supernatant (0.9ml) was precipitated by adding 0.6ml isopropanol, mixing, leaving at room temperature for 10 minutes and pelleting for 10 minutes in a microfuge. The pellet was dissolved in 500ul 0.3M sodium acetate pH=5.0 and again precipitated by isopropanol as above. The pellet was then washed with 70% ethanol, dried for 5 minutes under vacuum and redissolved in 30-50ul TE buffer. 10ul aliquots were digested with relevant restriction enzyme(s) and run on agarose gels.

Footnotes.

DE52 cellulose (from Whatman), was prepared as follows:-

The DE52 cellulose was placed in several volumes of 0.05N HCl (pH <4.5). With gentle stirring, concentrated NaOH was added until the pH approached 7. The resin was then allowed to settle, the supernatant decanted off and several volumes of L-Broth added, mixed and the DE52 cellulose again allowed to settle. The L-Broth was
replaced and the rinsing process repeated until the pH of the supernatant was 7.4-7.5. The DE52 cellulose was finally resuspended in a slurry of 75% resin + 25% L-Broth, and ampicillin added to 50ug/ml. Aliquots were stored at -20°C.

2.2.21 Construction of a Genomic Library in pUC 18 Enriched for Specific Sequences

In order to increase the chances of detecting a clone which was homologous to a particular probe (v-yes), a library was constructed which was enriched for these sequences. This was achieved by accurately determining the size of a fragment of restricted genomic piroplasm DNA which hybridised to the v-yes probe on Southern blot analysis (2.2.16). The digest was then be repeated, run on an agarose gel, and the appropriate size fragments of DNA excised in an agarose block and extracted. This was then ligated into vector pUC 18, which was chosen for its high stability, easy growth and simple selection system for recombinants (2.2.17.3). A high efficiency was not necessary, since being an enriched library, relatively few recombinants were required (see 2.2.21.4). The library was then screened using the appropriate probe.

2.2.21.1 Purification of Theileria DNA Fractions from Agarose Gels

Genomic piroplasm DNA from Theileria annulata (Hissar) parasites was digested with a restriction enzyme (Hind III) which had been used previously in a Southern blot analysis with the same DNA and the probe of interest had hybridised to specific fragments of calculable size (3.35kb and 1.3kb - see section 3.3) using BRL lambda kilobase ladder markers (2.2.12, 2.2.15-2.2.16). Viewing the ethidium bromide-containing gel on an ultraviolet transilluminator, using long-wave uv light, the relevant fraction of digested genomic DNA was excised, (3-4kb). The agarose block was trimmed to remove excess agarose, sliced into roughly 2mm cubes and placed in a sterile eppendorf tube at 45°C-55°C for 5-7 minutes until the agarose was completely dissolved. Two to three volumes of a stock solution of sodium iodide (as supplied in a GeneClean Kit by Stratech Scientific Ltd.,) were added, mixed and then 5ul of Glassmilk was added per 5ug DNA, plus 1ul for every
additional 0.5ug. (Glassmilk was also supplied as part of the GeneClean Kit). The solution was then mixed well and placed on ice for 5 minutes to allow binding of the DNA to the silica matrix. The bound DNA was then centrifuged with the silica matrix in a microcentrifuge for 5 seconds. [The NaI supernatant was saved and after elution of DNA from the glass milk, (as described below *) this NaI supernatant was again bound to the same glass milk and the elution repeated]. The pellet was then resuspended in 200-400ul of NaI stock that was diluted 1/3 with water. The suspension was placed again at 45°C-55°C for 2-3 minutes, pelleted as before, and the supernatant discarded. The pellet was then washed three times with 200-700ul of NEW (Sodium Chloride - Ethanol - Water as supplied in the kit) by resuspension and pelleting in a microcentrifuge for 5 seconds. After the final wash, the last traces of NEW were removed using a capillary tube. * DNA was then eluted from the glass milk by resuspending the pellet in the same volume of 10mM Tris, 1mM EDTA pH=7.6 as the volume of glass milk used, incubated at 45°C-55°C for 2-3 minutes and centrifuged for 30 seconds to make a solid pellet. The supernatant containing the eluted DNA was then transferred to a new tube and the elution repeated with another volume of TE buffer.

The yield of DNA was estimated as follows: 0.1 to 1.0ul dilutions of each sample were dotted onto an ultraviolet-transparent petri-dish and a series of dilutions from 100ug/ml to 10ng/ml of a known DNA standard each in 1ul aliquots were dotted below. 1ul of 10ug/ml ethidium bromide solution was added to each dot and each made up to 5ul with water. The petri-dish with the DNA dilutions was then photographed under short wave U.V. light and concentrations of the unknown DNA preparations estimated by comparison of fluorescence intensity with the series of standard dilutions. Typically, around 80% of the excised DNA was recovered, and the final yield was 80ng from an initial input of 2ug of piroplasm DNA.

2.2.21.2 Hind III Digestion of Vector DNA

The pUC 18 vector DNA was cut with the same restriction endonuclease which was used in the preparation of insert DNA: Hind III (2.2.21.1). Since linear DNA molecules run further on agarose gels than circular or supercoiled molecules of the same size, the
cutting efficiency of the plasmid was checked roughly by comparing the migration of 100ng aliquots of the vector before and after cutting. The restriction enzyme was then inactivated by heating at 65°C for 5 minutes.

2.2.21.3 Ligation of Vector and Insert DNA

Ligation was carried out in the presence of polyethylene glycol 6000 (present in the buffer supplied by BRL - Bathesda Research Laboratories), which according to Pheiffer and Zimmermann, (1983) can stimulate blunt-end ligation by up to a factor of 10. The same stimulation is also seen with cohesive ends, at low DNA concentrations (Buckel and Kessler, 1987). Immediately after isolation of cut vector and insert DNA with identical 'sticky ends', the Theileria annulata (Hissar) and pUC 18 DNA were mixed in the desired proportions (see 3.3.3.4) and 5 x ligation buffer (BRL) was added to a final concentration of 1 x, followed by 40 units of T₄ ligase (BRL). The mixture was incubated overnight at 16°C then a further 10 units of T₄ ligase added and the incubation carried on at 37°C for 30 minutes. The ratio of plasmid molecules to inserts was critical, since if there are too few inserts per plasmid, then re-ligation of plasmids to themselves will occur, and if there are a large number of inserts per plasmid, then plasmids containing concatamerised insert will predominate (see 3.3.3.4 and figure 3.9a). In total the reaction was carried out using a total of 50ng-200ng DNA in a volume of 5-10ul. The resultant mixture was diluted five-fold with water and transformed into E. coli JM83. (2.2.14.1).

2.2.21.4 Transformation of E. coli JM 83 with Ligated Plasmid/Insert Mix

Competent JM 83 cells were prepared as described (see 2.2.14.1); and dilutions of 100ng/ml - 1ng/ml final concentration of ligation mix transformed into these cells. (see 2.2.14.1). The selection system for recombinant plasmids was as described in (2.2.17.3). A 100ng aliquot of uncut pUC 18 was always transformed into JM 83 at the same time in order to estimate the transformation efficiency. Typically,
efficiency of restriction enzyme digestion of the vector was estimated by transforming JM83 with 100μg of the cut plasmid - only uncut plasmid would transform cells. This background was found to be below 1%. The efficiency of ligation, calculated by comparing the total number of colonies grown on a plate after transforming with cut and ligated pUC 18 with those transformed by an equal amount of uncut pUC 18, was usually around 10%. The percentage of recombinant plasmids was observed by counting the blue and white colonies produced (see 2.2.17.3). The percentage varied according to the ratio of insert molecules (see 3.3.3.4). Since the library was enriched for the specific sequences, only 300 recombinant plasmids were considered necessary to give a high probability of including the particular fragment which was to be isolated. This calculation is based on a size estimation of the genome of *Theileria parva* (Allsopp, et.al., 1988) of $1.2 \times 10^4$ kb and on cutting out a size fraction from the agarose gel containing 1/20 of the restricted genome.

A genomic library enriched for sequences homologous to the v-yes oncogene was constructed as described above, containing 2500 recombinant colonies.

### 2.2.21.5 Library Screening: Checking the Library for Insertion of Foreign DNA and Screening for Specific Sequences

For ease of handling, the recombinant plasmids were first transferred to larger (18cm diameter) master plates. This also facilitated identification of clones after subsequent screening for (a) *Theileria annulata* DNA. (b) specific sequences. The screen for *Theileria* DNA insertion was necessary since some white colonies may result from two pUC 18 plasmids joining and circularising during the ligation process. In addition, other white background colonies appear, which may contain no plasmids conferring ampicillin resistance at all. Such colonies can usually be distinguished from recombinant-containing colonies by their smaller size.
Recombinant colonies were pooled onto a master agar plate and also onto a nitrocellulose filter laid on the surface of a second agar plate (Grunstein and Hogness, 1975). Nitrocellulose filters (Schleicher and Schuell BA45) were placed onto agar plates containing the appropriate antibiotic, ampicillin. Using sterile toothpicks, individual bacterial colonies were transferred first to the filter and then to a master agar plate that contained the selective antibiotic but no filter. Small streaks 2-3 mm in length were arranged in a grid pattern, with each colony in an identical position on both plates. Finally, a colony which contained a non-recombinant plasmid was transferred to the nitrocellulose filter. This was a useful negative control, necessary to discriminate between specific hybridisation of the radioactive probe to a recombinant plasmid and nonspecific background hybridisation. The master agar plate was stored in an inverted position at 4°C until the results of the screening procedure on the nitrocellulose filter were available.

The plates containing the filters were inverted and incubated at 37°C until the bacterial streaks had grown to 0.5 to 1.0 mm in width. At this stage, when the bacteria were still growing rapidly, the filter was transferred to an agar plate containing 10 µg/ml chloramphenicol and incubated for a further 12 hours at 37°C in order to amplify the recombinant plasmid. Nitrocellulose filters and the corresponding plates were marked in three or more asymmetric locations for future re-alignment and identification of positively hybridising clones.

Colony hybridisation was carried out in the same way as plaque hybridisation (2.2.19.1; 2.2.16.2).

The library was first checked for insertion of foreign DNA sequences by hybridisation with the same fraction of Theileria annulata (Hissar) DNA which was used in the construction of the library (2.2.21.1) 58% percent of the colonies screened produced a positive hybridisation signal. (See 3.3).

The library was then used for screening with the sequences of interest by hybridisation with a suitable radioactively labelled probe, (3.3) using the methods described above.
2.3 CELL FRACTIONATION AND PREPARATION OF LYSATES

2.3.1. Extraction of Crude Cell Lysate

Leukocytes were centrifuged in an MSE Chillspin at 600g for 15 minutes at 4°C and were then washed twice in phosphate-buffered saline (PBS). The cells were then resuspended in 0.5ml and the protein concentrations of each cell line estimated using the standard Bradford Assay (2.3.4). The cell concentration was then adjusted to 100ug per 60ul in 50mM Tris. 10% Triton X-100 was added to a final concentration of 1% and the cells vortexed well.

2.3.2. Crude Fractionation

An initial crude fractionation was carried out in order to enrich for nuclear and cytoplasmic proteins. This was carried out simply by homogenising the leukocytes in PBS and microcentrifuging for 5 minutes at 4°C. The supernatant was then removed, and frozen (-20°C) (cytosolic fraction). The pellets were resuspended in 2mM EDTA, homogenised and centrifuged as before. The supernatant was also frozen at -20°C for subsequent use (Particulate fraction).

2.3.3. Cellular Fractionation from Leukocytes

2.3.3.1. Plasma Membrane Preparation

Membranes were prepared from leukocytes, essentially according to the method of Standring and Williams, (1978). Around 100ml of cultured leukocytes (at 5 x 10^6/ml) were pelleted by centrifugation in an MSE Chillspin at 600g for 15 minutes at 4°C. Cells were then washed twice in PBS pH=7.2 and resuspended in 1ml of Tris-saline. An equal volume of 4% Tween 40 in Tris-saline (T.S. = 25mM Tris-HCl pH= 7.4, 150mM NaCl, 10mM Na Azide, 1mM MgSO_4, 2mM CaCl_2) was then added and the suspension stirred vigorously in a 5ml bijou for 1 hour at
(4°C). A small aliquot of cells was then taken for inspection by light microscopy to check that the cells were lysed. The cell suspensions were then made up to 5ml in T.S. and centrifuged at 2800g in an MSE Chillspin for 25 minutes. The pellet, enriched for nuclei, was saved for further use (2.3.9.2).

The supernatant was removed and centrifuged at 35 000g in an MSE 65 Superspeed Ultracentrifuge for 45 minutes at 4°C. The resultant pellet (crude membrane), was resuspended in 0.5ml 10mM Tris-HCl pH=7.4 using a homogeniser for further purification. Alternatively, the crude membrane pellet was resuspended in 200 ul of SDS sample buffer for running on SDS-PAGE, or Tris-saline for phospho-protein studies. To further purify the crude membrane fraction, the suspension in Tris-saline was centrifuged through a sucrose step gradient. A 15ml clear polycarbonate tube was very carefully filled with 4ml layers of (bottom to top) 40%, 28%, 10% sucrose in Tris-saline with no mixing of the layers. The sample was put on top of the step gradient and centrifuged at 28 000g for 15 hours at 4°C in a swing-out rotor. Two bands resulted, one between the 10% and 28% sucrose steps which was the purified membrane fraction and the other between the 28% and 40%, which consisted also of mitochondria (Standring, and Williams, 1978). The bands were taken off into 10ml polycarbonate tubes and diluted with 20mM Tris-HCl pH=8.0. They were then centrifuged for 1 hour at 40 000g in an MSE -65 superspeed ultracentrifuge at 4°C and the pellets resuspended in 100ul of the appropriate buffer. Samples were either boiled for 10 minutes, in SDS sample buffer, chilled and run on SDS-PAGE, or used (in Tris-saline) for phospho-protein or kinase studies.

2.3.3.2. Preparation of Nuclei

The pellet, enriched for nuclei, which was prepared during the process of making purified membranes (2.3.9.1), was not further purified. According to Standring and Williams, (1978), this fraction contained nuclei and mitochondria.
2.3.3. **Preparation of Macroschizonts from Theileria annulata (Hissar) - infected leukocytes**

As part of this project was aimed at developing a method for extracting a pure fraction of macroschizonts from *Theileria annulata (Hissar) - infected leukocytes*, a full description of methods attempted, yields and purity can be found in Chapter 3.6. The final method which was used for phospho-protein profiles and kinase experiments was the Tween 40 lysis method (3.6).

2.3.4. **The Bradford Assay of Protein Estimation**

The concentration of protein in solution, or cell suspension was measured by the Bradford assay (Bradford, 1976): 3ml of Bradford reagent was added to 0, 12.5, 25, 50, 100ug BSA in 200ul Tris-saline, and the optical absorbance at 595nm measured. A standard curve of absorbance against known protein concentration, was plotted in this manner for each batch of Bradford reagent. Thus, the protein concentration of an unknown solution could be measured by taking a measured aliquot of the solution, diluting to 200ul with Tris-saline, mixing with 3ml Bradford reagent, measuring the absorbance at 595nm and reading the protein concentration from the standard curve.

2.3.5. **Concentration of Proteins in Solution**

When the volume of protein solution limited the amount of protein which could be loaded onto a gel, the solution was concentrated by the following method.

The protein solution was sealed into a sterile dialysis bag, and the outside of the bag dried. Dry Sephadex G-150 (Pharmacia Fine Chemicals) was then sprinkled onto a petri dish and the dialysis bag rolled in the dish until it was covered with sephadex. The sephadex soaked up water through the dialysis membrane, and formed a damp 'skin' around it. When the volume in the dialysis tubing was sufficiently reduced (usually 10 to 15 minutes), the sephadex 'skin' was rolled off the membrane.
2.3.6. **Changing the Buffer of a Protein Solution**

In order to equilibrate protein solutions from one buffer to another more appropriate for the technique to be used, the following procedure was used. Biogel P-6DG was first swollen in the sample buffer in which the protein would finally be in. The Biogel suspension was then applied to suitable-sized columns (at least 1.5 times the volume of the samples to be applied). Columns were then spun in an MSE Chillspin at 600g for 30 seconds. The samples were then applied to the tops of the columns which were then spun as before. The solution eluted from the bottom of the columns was collected. The columns were then washed through with a half-volume (of the initial sample), spinning as before. Again the solution eluted was collected and pooled with the first. This was the protein solution. The system was first checked by adding bromophenol blue to the original solution and observing whether or not the eluted protein contained any of the dye. Contamination was not significant. In addition, protein loss in the column as measured by the Bradford assay (2.3.4) before and after applying samples to Biorad columns was not measurable.
2.4. PROTEIN ELECTROPHORESIS AND ISOELECTRIC FOCUSING

2.4.1. SDS-PAGE Separation of Proteins

Proteins were separated using the technique of SDS (Sodium dodecyl sulphate) - polyacrylamide gel electrophoresis (Laemmli, 1970). The vertical slab gel technique was used, as described previously by Studier, (1973). Samples were dissolved in a final concentration of 0.0625M Tris-Cl pH=6.8, 2% SDS, 10% Glycerol, 5% 2-Mercaptoethanol, 0.001% Bromophenol blue (SDS sample buffer) and boiled in order to denature the proteins.

The gels (13cm x 16cm x 1.5mm) were prepared from a stock solution of 30% acrylamide, 0.8% bis acrylamide, and were diluted to the desired concentration in 0.375M Tris. HCl pH = 8.8, 0.1% SDS (final concentration). Gels were either 10% acrylamide, or a 7-17% linear gradient. Polymerisation was initiated by addition of 100ul 10% Ammonium Persulphate and 10ul N,N,N',N'- Tetramethylethlenediamine (TEMED) per gel, before pouring and gels were overlaid with butanol during polymerisation. After rinsing off the butanol, the stacking gel (4% acrylamide - from the 30% stock, 0.125M Tris-Cl pH=6.8, 0.1% SDS, then 50ul ammonium persulphate, 5ul TEMED per 20ml stack) was poured and the gel comb inserted. The stacking gel was necessary to sharpen bands, by the phenomenon of stacking of protein bands at pH boundaries (Laemmli, 1970).

Samples were applied to the wells, and electrophoresis carried out using a Tris-glycine electrophoresis buffer (0.025M Tris pH=8.3, 0.192M Glycine, 0.1% SDS) at 10mA per gel overnight. Electrophoresis was terminated when the bromophenol blue dye front neared the end of the gel.

A set of marker proteins (Sigma High Molecular Weight Marker Kit for SDS-PAGE) were routinely run to calibrate the gels and so determine the molecular weight of unknown proteins. The molecular weights of unknown proteins were determined by running marker proteins alongside, staining with coomassie blue (2.4.2.1), and measuring the mobilities of the standard proteins. A standard curve of log$_{10}$ (distance migrated) against known molecular weight was then plotted, which was used to determine the molecular weights of unknown proteins.
A fresh standard curve was drawn for every gel run.

2.4.2. Staining Proteins in Polyacrylamide Gels

Where there was sufficient quantities of protein loaded onto gels, staining with Coomassie blue was carried out, but occasionally when material was in limited supply, the more sensitive technique of Silver Staining was used.

2.4.2.1. Staining with Coomassie Blue

Gels were submerged in 50% methanol, 7.5% acetic acid, 0.25% Brilliant Blue (filtered through Whatman 3MM paper) for at least one hour on a gently rocking platform. Destaining was then carried out for at least one hour in 50% methanol, 7.5% acetic acid and then in several changes of 5% methanol, 7.5% acetic acid until protein bands were visible and the background was clear.

2.4.2.2. Silver Staining

Silver staining was carried out according to Morrisey, et al., 1981). After electrophoresis, gels were fixed for 30 minutes in each of the following solutions sequentially : 50%methanol, 10% acetic acid, then 5% methanol, 7% acetic acid; and finally 10% gluteraldehyde. The gels were then left in a large volume of water overnight, before transferring to a solution of 5ug/ml dithiothreitol for 30 minutes. They were soaked in 0.1% silver nitrate for 30 minutes, rinsed for 1-2 minutes in distilled water and then agitated in a small amount of developer (50ul 37% formaldehyde in 100mls 3% Na₂CO₃). The reaction was stopped by addition of 5mls of 2.3M citric acid when protein bands were sufficiently intense but before the background darkened. The gels were finally rinsed in several changes of water.

2.4.3. Non-Denaturing Polyacrylamide Gel Electrophoresis.

SDS Polyacrylamide gel electrophoresis (2.4.1) is a sensitive technique for the characterisation of small amounts of protein and the
determination of the molecular weights of constituent proteins. SDS however causes the denaturing of proteins, typically dissociating oligomers to their subunits and thus leading to the loss of most enzymatic or biological properties. The method presented here allows the retention of the characteristics of the "native" protein. (Davies, 1964).

Glycerol and bromophenol blue were added to the samples (leukocyte crude cell extracts - see 2.5.6) at concentrations of 18% and 0.02% respectively. Each well was loaded with 5-10ug protein, as measured by the Bradford assay (2.3.4) and run on Laemmlli gels under non-denaturing conditions at 5°C. Gels were 2mm thick x 13cm x 16cm and were made up at various final concentrations from 5% to 10% acrylamide as desired (usually 4% except for molecular weight estimation). The 3% stacking gel contained 0.125M Tris. HCl pH = 6.8, and the running gel 0.375M Tris. HCl pH = 8.8. Both were polymerised by addition of ammonium persulphate to 0.1% and TEMED to 3.3mM. Samples were applied to the gels in volumes of 20 to 60ul, and were electrophoresed at 10mA per gel overnight using a Tris-glycine electrode buffer (0.025M Tris, 0.192M Glycine pH 8.4) (Laemmlli, 1970).

2.4.4. Molecular Weight Estimation of Proteins Separated in Native Gels

The procedures used for indirectly determining the molecular weight of particular proteins separated on native gels, were taken from Sigma Chemical Company Technical Bulletin No. MKR-137. They, in turn, were derived from the procedures of Bryan (1977) and Davis (1964).

Firstly, a standard curve was derived as follows: A set of proteins of known molecular weight: B-lactalbumin (from Bovine Milk); Carbonic Anhydrase from (Bovine erythrocytes), Chicken Egg Albumin, Bovine Serum Albumin and Jack Bean Urease were electrophoresed on a set of non-denaturing gels of various polyacrylamide concentrations (4% to 10%). For each protein, the electrophoretic mobility (Rf) of the protein in each gel relative to the tracking dye front was measured, and $100 \log (Rf \times 100)$ was plotted against the percent gel
concentration for each protein (Fig. 2.3.1). From these plots, individual slopes were calculated. These figures were the Retardation Coefficients ($K_R$), and the logarithm of the negative slope was plotted against the logarithm of the molecular weight of each protein. This produced a linear plot (Fig. 2.3.2) from which the molecular weight of unknown proteins could be determined, by first working out the Retardation Coefficient as with the standard proteins - by running gels of various polycrylamide concentrations.
Figure 2.3

Non-Denaturing Gel Molecular Weight Calibration Curves

![Graph showing molecular weight calibration curves for various proteins such as lactalbumin, chicken egg albumin, BSA monomer, carbonic anhydrase, BSA dimer, urease hexamer, and urease trimer. The graph plots molecular weight against percentage acrylamide.]
2.4.5. **Agarose Gel Iso-Electric Focusing (IEF)**.

The agarose gels (0.3g Pharmacia Agarose IEF, 3.6g Sorbitol, 27ml H₂O) contained 0.38ml of Ampholines pH 3-10 and 0.38ml pH 5-7 (supplied by L.K.B.), which were added after the agar had been dissolved. Gels (18.5 x 11.3cm) were poured directly onto the hydrophobic side of Gel Bond (L.K.B.) for ease of handling. Gels were poured at 37°C, left for half an hour to set, and then transferred to a 4°C cold room for a further 30 minutes.

Samples were prepared in a buffer containing 2% Triton X-100, 2.5mM Dithiothreitol, 10mM Tris. HCl pH=7.5, 1mM EDTA (IEF sample buffer). A crude cell extract was prepared by spinning down leukocytes and washing twice in PBS as already described (2.1.6). The pellets were resuspended to 5 x 10⁸ cells/ml in the above lysis sample buffer, left on ice for 30 minutes with occasional gentle vortexing and homogenised by hand (twenty strokes). Debris was then removed by centrifuging in a microfuge for 5 minutes. Samples were frozen at -70°C.

Other cellular fractions (eg. crude membranes, purified membranes and macroschizonts) prepared as described (2.3.3), were dissolved from pellets directly into the IEF sample buffer. If samples to be used were in inappropriate buffers or were too dilute, this was often corrected (see 2.3.5. and 2.3.6).

50µg of sample protein, in a volume of 20µl, was soaked into a piece of 3MM filter paper (approximately 3mm x 9mm) for 20 minutes before laying onto the gel, 3cm from the cathode. Up to 9 samples were applied side-by-side in this manner. The cathode buffer was 1M NaOH, while the anode buffer was 0.05M H₂SO₄. The electrode wicks were soaked in the appropriate buffer, cut to the required length and laid on top of the gel, in contact with the electrode wires, which ran the length of the gel. The gel was run at 1000 volts, 5 watts, with the maximum current set to 20mA. After 30 minutes, the filters which contained the samples were removed and the gel run until the current dropped to 4mA (approximately 40 minutes). At this point, protein bands were focused (had come to an equilibrium point on the pH gradient across the gel). Alternatively, when visible marker
2.4.6. **Calibration of IEF Gels**

In order to determine the Isoelectric point (IEP) of unknown proteins, a series of defined marker proteins with known I.E.P. values were run on one track of each gel. The kit was supplied by Bethesda Research Laboratories (BRL), and covered I.E.P. values from 4.7 to 10.6.

Thus, the pH gradients across the gels were calibrated and I.E.P. (P.I.) values of unknown proteins could be estimated.
2.5. **PHOSPHOPROTEIN/PROTEIN KINASE ASSAYS**

2.5.1. **Labelling of Cell Extracts with $^{32}$P ATP.**

In order to detect phosphoproteins, cell extracts were labelled in vitro with $^{32}$P ATP. To each 60ul aliquot of cell extract (see 2.3.1), 7.5ul of 0.1mM (ortho-) vanadate and 7ul of $^{32}$P ATP solution (8ul 2mM ATP, 20ul $^{32}$P ATP (10mCi/ml, 3000Ci/mMol), 132ul H$_2$O were added. Vanadate was included to inhibit the action of phosphatases (Foulkes, 1983). Addition of any other ions was included in 3.8ul 50mM Tris which was added regardless to keep the volumes constant. All reagents were kept at 0°C.

The reaction was initiated immediately after addition of $^{32}$P ATP, by incubation of the tubes at 24°C for 15 minutes. It was terminated by addition of 35ul of 3 x SDS sample buffer (2.4.1) per aliquot and these were then boiled for 10 minutes to denature the proteins. The samples were then either run on SDS PAGE systems immediately after cooling, or frozen at -20°C for future use.

2.5.2. **Analysis by Autoradiography**

Labelled phospho-proteins (and protein kinases) which had been separated on SDS-PAGE or IEF or native gels, were visualised by either wrapping the gels in Saran-wrap, or drying the gels down, and then exposing to Amersham Hyper-film for 12 hours to one week at -70°C. If gels were not being further treated, it was found that drying the gels down onto Whatman 3MM paper on a slab gel drier had the effect of sharpening the labelled protein bands and hence improving the resolution. [However, gels were often exposed initially to reveal all phosphorylation events and then treated to remove a subset of these (2.5.3) before drying down and re-exposing.]

2.5.3. **Specific Removal of Phosphate Groups from Serine and Threonine**

Having exposed gels to reveal all phospho-proteins resolved, they
were treated by heating to 56°C for 2 hours in 1M KOH to remove labelled phosphate from serine and threonine residues. Gels were then placed in 5% methanol, 7.5% acetic acid to reconstitute their original size, dried down on Whatman 3MM paper and exposed to autoradiograph film. Thus, proteins phosphorylated at tyrosine residues were enhanced (Cooper, et al., 1983). Although removal of phosphate was not 100% efficient at serine, threonine, comparison of bands with the original autoradiographs of all phosphorylated proteins, revealed if the proteins were phosphorylated at either serine/threonine or tyrosine. This was seen by whether or not relative intensities of bands were reduced after alkali treatment (for further discussion, see 3.4.4).

2.5.4. In Vivo Incorporation of Inorganic Radio-labelled Phosphate into Lymphoblasts

2 x 10^6 cultured cells (see 2.1) were taken, pelleted by centrifugation in an MSE Chillspin at 600g for 15 minutes and washed twice in phosphate-free medium (1 x Autopow, supplied by Flow Laboratories = 5.4g Autopow, 2.97g Hepes, 5mg gentamycin pH=7.4 per 500ml). Cells were then resuspended in 2ml Complete Autopow which had been pre-warmed to 37°C. (Complete Autopow = 1 x Autopow, 5% heat inactivated foetal calf serum, 0.2% NaHCO_3, 20mM L-Glutamine, 0.75ug/ml Fungizone). 0.5ml aliquots (5 x 10^5 cells) were then distributed into wells of a 24-well culture plate already containing 600uCi ^32P, 10mCi/ml (Amersham), in 0.5ml warm (equilibrated for 1-2 hours in 5% CO_2) Autopow. Cells were incubated in a 37°C humidified incubator with 5% CO_2 for a period determined by monitoring the incorporation of ^32P into cellular protein (2.5.5).

2.5.5. Trichloroacetic Acid Precipitation and Scintillation Counting

This was carried out by precipitating 10ul of the cell suspension in 2ml Trichloroacetic Acid (TCA) with 100ul 5 x PBS and passing through a Whatman Glass fibre filter. The filters were washed through twice with 2ml 10% TCA and then with ethanol and air-dried.
The filters were counted in a liquid scintillation counter in vials containing 3mls scintillation cocktail (Scintran Cocktail EX, BDH Chemicals).

2.5.6. Protein Kinase Assays

Proteins were assayed for protein kinase activity by separating the proteins under non-denaturing conditions (2.4.3), (2.4.5) and then assaying for activity by a method which permits visualisation of bands of protein kinase activity in situ in the separation gel.

2.5.6.1. In Situ Gel Assay of Protein Kinase Activity

Crude cell extracts were prepared from leukocytes for protein kinase assays, as follows. Cells were harvested as described previously (2.1.6) and washed three times in PBS. Cells were resuspended at 1.25 x 10^9 cells/ml in a buffer which was modified from that used by Grove and Mastro (1987). It contained 60mM KCl, 15mM NaCl, 15mM Tris-Cl (pH=7.5), 0.5mM spermidine, 0.15mM B-mercapto-ethanol, 1mM phenylmethylsulfonylfluoride (PMSF) and 5mM NaF. The leukocytes were then sonicated with four bursts (0.4mA) of 15 seconds each, with a 15 second rest between bursts. Triton X-100 was added to a final concentration of 0.25% and the protease inhibitor leupeptin to 25ug/ml. Samples were placed on ice for 30 minutes with occasional gentle vortexing before removing particulate material by centrifugation in a microfuge for 20 minutes at 4°C. The supernatants were collected, aliquoted and either used immediately or frozen at -70°C.

Protein kinase activity was assayed by adapting the procedure of Grove and Mastro (1987) which itself was a modification of the method of Iyer et al., (1984). Following electrophoresis on a non-denaturing gel (2.4.3), gels were soaked in 2 changes of 500ml of ice-cold 50mM Tris -HCl, pH=7.5 for 1 hour. Each gel was then incubated in a small plastic box at 37°C with agitation, containing 50ml of 30mM Tris-acetate pH=7.4, 4mM Mg-Acetate, 10mM NaF, 0.1mM sodium phosphate pH=7.4 and 2mg/ml of Histone type II AS (Calf Thymus mixture from Sigma). After the thirty minute incubation, the kinase assay was
initiated by the addition of 100uCi of $^{32}$P ATP made up to 1.25Ci/mmol (1.6uM) by dilution with 'cold' ATP and the incubation continued with gentle shaking for 30 minutes at 37°C. The reaction was terminated by removal of the incubation mixture followed by a rinse with 50ml of 10% trichloroacetic acid (TCA), 1% phosphoric acid. Gels were left on a rocking platform overnight at 4°C in a fresh change of the same acid solution. They were then given two further washes, each for 1.5 hours in the same solution and then incubated with 30% methanol, 10% acetic acid for several one hour washes and finally washed overnight in this solution. Gels were then rinsed in the same acid solution before drying down onto 3MM paper and exposing to Amersham Hyper-film in a cassette containing one intensifying screen for 3-72 hours.

2.5.6.2. **Modifications to In Situ Kinase Gel Assay for IEF Gels**

The in situ gel assay for kinase activity was carried out as described in (2.5.6.1), but with the following modifications. The incubation periods were cut down drastically, thus reducing any protein diffusion which may occur before fixation. Gels were also agitated more vigorously than was possible with the polyacrylamide due to being fixed on a membrane support (Gel Bond). The initial soaking of gels in 50mM Tris-HCl pH 7.5 was carried out twice (10 minutes each), with agitation. Pre-soaking in the incubation mixture (see 2.5.6.1) was cut down to 10 minutes, with a subsequent 15 minute reaction period. Fixing and washing of the gels was as described in (2.5.6.1). The incubation time before fixing, was cut from 120 minutes to 45 minutes.

2.5.7. **Quantitation of Kinase Activity**

Some autoradiographs were scanned on a laser densitometer (Shimazu Thin Layer Chromatogram Scanner, model CS-930). Although this did not help distinguish bands which were merging into one, it did give a quantitative comparison between various cell lines of kinase activity. The instrument was used as recommended by the manufacturer. The variable parameters were set as follows:-

Delt. "Y" = 0.10; Linearizer = OFF; Abscissa = XI; Drift Line
= ON; Background Correct = OFF; Detect Signal = Area; Min. Width = 0; Average Peak No. = 1; Photo Mode = Abs. Transmission; Ordinate = x 8; Output = Curve and Print; Accum. No. = 1; Peak Detect = 1; Detect Sensitivity = Medium; 1D Mede = Ext; Signal Average = 8; Drift Line = 0.1; Min. Area = 100.
2.6. ELECTRON MICROSCOPY

Electron microscopy was carried out in order to check the condition of macroschizonts isolated by the method described fully in Chapter 3.

2.6.1. Specimen Fixation

Washed pellets of material to be analysed by electron microscopy were fixed as follows, using a procedure recommended and refined by Dr A. Jurand (Department of Animal Genetics, University of Edinburgh).

The pellets to be sectioned were placed in 5-10mls of Fixation Buffer I for 15 minutes on ice and then moved to room temperature for 45 minutes (Fixation Buffer I = 0.0018M Sodium Barbitone, 0.003M Sodium acetate, 0.02N HCl, 2.5% gluteraldehyde (supplied by Taab Laboratories Equipment), 0.13M Sucrose, 0.003M Calcium Chloride) and was made up as follows: 7.357g Na Barbitone and 4.857g Na Acetate were dissolved in 250ml H2O to give buffer A. 25ml H2O was then mixed with 50ml 0.1N HCl to give buffer B. Buffers A and B were then mixed in proportions of 2:3. For Fixation Buffer I, an equal volume of 5% gluteraldehyde was added, followed by 0.045g/ml sucrose and 0.4mg/ml Calcium chloride.

The pellets were next rinsed twice (each for 15 minutes) in 5-10mls of Rinse II, which was Buffer (A + B) (as above), diluted 1:1 with water and sucrose and calcium added as before.

The pellets were then transferred to a solution of osmium tetroxide (from Taab Laboratories Equipment) for 30 minutes with occasional agitation. Osmium tetroxide was made up to 2% w/v in water and then diluted 1:1 with buffer (A and B) as above. The pellets slowly turned black.

Osmium tetroxide was removed as much as possible and the pellets given a quick 5 second rinse with Fixation Buffer I before carrying out the dehydration steps as follows: The pellets were taken through a series of alcohols of increasing concentration:
1. 70% v/v ethanol/water for 10 minutes.
2. 95% v/v ethanol/water for 10 minutes.
3. 100% v/v ethanol/water for 10 minutes. This step was carried out 3 times.

2.6.2. Embedding the Specimens

Specimens were embedded in araldite resin for sectioning purposes. Embedding kit 502 was obtained from Graticules Ltd., and the components mixed first in the recommended quantities. However, this led to poor sectioning and so the quantities were modified to those shown below.

Before embedding, the ethanol was removed from the pellets and replaced by epoxypropane and the pellets left for 10 minutes. The pellets were then placed in 1:1 mix of epoxypropane and araldite resin for 2-3 minutes. This soaking step was then repeated using a fresh solution for a further 2-3 minutes.

Pellets were made a suitable size for embedding (0.2-2.0mm diameter) by breaking with a needle and were then transferred with a glass rod to a watch glass containing 100% araldite mix [10g Epoxy resin, 10g D.D.S.A. (dodecyl succinic anhydride), 0.7g dibutyl phthalate, 0.45g D.M.P. -30 (2, 4, 6 -tri dimethylamino methyl phenol) - well mixed], and placed 3-5cm from a 60 watt bulb, for 1 hour on an orbital stirrer. (See Fig. 2.4). The pellets were then transferred using a thin glass rod into gelatine capsules which were then topped up with 100% araldite mix*. The araldite was then polymerised by incubating at 58°C overnight.

2.6.3. Preparation of Specimen Support Grids

Copper grids (mesh 200, 3.05mm diameter) were supplied by Taab Laboratories Equipment. The grids were coated with colloidion (from Graticules Ltd,) in order to support specimens. This was carried out as follows:-
Collodion was first prepared by decanting 20-50ml of supplied collodion into a watch-glass, covered with a loose-fitting lid and allowed to dry (3-5 days). The solid collodion was then weighed and dissolved in amyl acetate, to 1% w/v. An 18cm round dish was then half-filled with distilled water, and a paper ring (inside diameter 14cm approximately) floated on top. (See Fig. (2.4)). Using a pasteur pipette, a drop or two of the collodion solution was dropped onto the centre of the ring and the resulting skin of collodion used for coating the grids. Regions of the collodion skin suitable for coating grids were recognised by a non-wrinkled area showing a smooth silver-grey interference colour. The copper grids were then carefully placed close together, shiny side-down on the surface using fine-point tweezers. The grids were then lifted from the surface en masse by covering the grid-covered area with Bibulous paper, which was left to soak for a few seconds. The collodion skin surrounding the paper was then torn away using tweezers, and the Bibulous paper, now with the grids attached lifted clear. The paper was left overnight in a petri-dish, grid-side up, in order to dry.

2.6.4. Carbon-Coating E.M. Grids

The grids, still attached to the Bibulous paper, were next coated with a layer of carbon in order to enhance the contrast in the specimens and to strengthen the membrane support. The paper, with grids attached, was trimmed and taped onto a microscope slide. A drop of oil was placed on a piece of white plastic beside the slide, to serve as an indicator of when carbon-coating was complete - this was visualised as a sharp contrast between the white beneath the oil-drop and the surrounding grey of the carbon-coat. Carbon-coating was carried out under high vacuum in an evaporator chamber. The grids were placed 4cm below the carbon rods. Evacuation of the chamber and evaporation of carbon were carried out according to the manufacturer's instructions. Evaporation was usually carried for 10-30 seconds, until a satisfactory coating was visible. Grids were then removed from the chamber following the manufacturer's instruction manual and the chamber left under vacuum.
2.6.5. Sectioning Embedded Specimens.

2.5.5.1. Knife-Making

Glass knives were freshly made for sectioning the embedded specimens, and their quality was of prime importance in achieving good sections - avoiding knife-marks (scores) on the sections.

Knives were made from strips of glass 1/4 inch thick, 1 inch wide. The glass was cut using a glass-cutter especially designed for the task of making sectioning knife-edges (supplied by L.K.B.). Strips of glass were cut first into squares by holding firmly in the machines' clamps, drawing the diamond knife across the strip thus scoring it, and making the break with a lever system. The squares were then held in a second set of clamps such that the diamond knife would score diagonally, from corner to corner. Again the break was made as before. Thus two knife edges could be made from one glass surface (See Fig. (2.4)). The cutting edges of the knives were then inspected under a binocular microscope for irregular or ragged edges. These areas were completely destroyed using a file with a triangular cross-section to avoid accidental use. Thus, while a complete knife edge may not have been smooth, certain areas were often useable along its edge.

Onto knives with good cutting edges, foil collars were fitted (See Fig. (2.4)). These were sealed with a molten wax (1g embedding wax: 1g beeswax: 0.5g vaseline) and held for a few moments until the wax hardened.

2.6.5.2. Trimming Specimen Blocks

Before presenting to the ultra-microtome, specimen blocks had to be trimmed in order to reduce the section size to one which was suitable for mounting several sections on one microscope grid. This was carried out by shaving the block with a razor blade under a binocular microscope until the specimen pellet was revealed at the end of a 'pyramid' shape of resin (See Fig. 2.4).
2.6.5.3. **Sectioning Specimen Blocks**

Sections about 600 Å thick were cut from the resin blocks using an ultra-microtome (model MT1 from Sorvall), which was operated according to the user's manual. The block was held by vice jaws in the ultra-microtome, which moved in a circular motion when the cutting wheel was operated. The block was thus passed down over the cutting edge of the stationary knife, while the block moved a set distance forward onto the knife, with each stroke. The knife was set to lean 5-10° towards the block. Great care was taken to ensure that the square face of the block was held parallel in all planes, to the knife edge. This was carried out looking through a binocular microscope mounted on the ultra-microtome. The block was positioned correctly when both of the following conditions were fulfilled, the block being suitably close to the cutting edge:

1) The mirror image of the knife edge, visible in the block face, should be horizontal, and straight.

2) As the block is moved up and down slightly, the mirror image of the knife edge on its face should not appear to move closer or further away.

With the block correctly positioned, the foil collar on the knife was filled to the cutting edge with 10% acetone in order to float off the sections. The block was then advanced using the cutting motion, set to cut thick sections. This was continued until cutting commenced. The thick sections were cut until the block was being sectioned across its full face and these discarded. The machine was then set to mark 2-4, to cut thin sections, and the process continued. Suitable sections were silver in colour, from interference colour, and were therefore around 600 Å in thickness. They would have no visible knife-marks, and often trailed off the knife edge in 'ribbons'. Such sections were collected onto coated grids (2.6.3., 2.6.4), by touching the grids, coated surface down, onto the sections, and allowing them to float on the 10% acetone for 30 seconds - 1 minute. Grids were then picked up with fine-point tweezers and turned section-side up,
onto a piece of filter paper in a petri-dish to dry.

2.6.6. **Staining the Sections.**

A succession of two stains were used on the sections, both of which were heavy metals which bind to lipids in membrane structures, making them dense to an electron beam. The first stain was lead citrate - Reynold's Stain (Reynolds, 1963), and the second was uranyl acetate.

Reynold's stain was made by dissolving 1.33g lead nitrate in 15ml distilled water, and then adding 1.76g sodium citrate which had also been dissolved in 15ml distilled water. A heavy precipitate of lead citrate formed immediately, but was dissolved completely on addition of 8ml 1N NaOH (CO₃-free, supplied by B.D.H.) and the volume made up to 50ml with distilled water. The uranyl acetate stain was a 4% solution of uranyl acetate which was freshly-filtered before each use.

Staining of sections on grids with Reynold's stain was carried out with a fresh drop of stain for each grid, in a CO₂-free atmosphere. This was to prevent contamination of the sections with lead carbonate. Removal of CO₂ from the atmosphere was achieved by staining in a wax-bottomed petri-dish containing 2 wells filled with potassium hydroxide (KOH) pellets (See Fig. 2.4).

Firstly, a few drops of Reynold's Stain were placed on the wax base of the petri-dish, and a grid placed on each drop, the sections facing downwards. The petri-dish lid was immediately replaced, and after 40 seconds to 1 minute, the grids were removed and rinsed first with 0.02N NaOH and then with distilled water by holding with tweezers under a wash-bottle containing the relevant solution.

The grids were next floated, section-side down in a watch-glass containing freshly-filtered 4% uranyl acetate. The watch-glass was covered and the sections left for 1 hour to stain. The grids were finally removed and rinsed in distilled water by floating, section-side down on three watch-glasses in succession, each containing distilled water. The grids were then left to dry, section-side uppermost on filter paper in a petri-dish and were then ready for
inspection with the transmission electron microscope (2.6.7).

2.6.7. **Transmission Electron Microscopy**

Sections were studied using a Phillips transmission electron microscope (model AEI-EM6). The machine was operated according to the manufacturer's instructions.

The grids were first scanned quickly at low magnification (x 1500) in order to locate sections which were then studied at magnifications of (x 5000) to (x 20000). Sample fields and interesting structures were photographed on plate-negatives using the microscopes' built-in camera system.
Figure 2.4

(1) Orbital Mixer

Motor

(2) Coating of Grids

- dish
- ddH₂O
- paper ring
- grids
- collision ring

(5) Trimming of Blocks

(3) Knife-making

(4) Fitting Collars

- foil
- folds
- wax seal
2.7. **INDIRECT FLUORESCENT ASSAY (I.F.A.)**

The indirect immunofluorescent assay (see Review: Goding, 1983) was carried out in order to identify a suitable monoclonal antibody for use in the isolation of macroschizonts from lysed leukocytes. (3.6.3).

2.7.1. **Fixed Cell I.F.A.**

2.7.1.1. **Multispot Slide Preparation**

10ml of cultured cells in log phase of growth or freshly-prepared peripheral blood lymphocytes (2.1.3), were washed three times in PBS pH=7.2 as described (2.1.6). The cells were then resuspended in 1ml of PBS before addition of 1ml of chilled fixative (5% formalin, 10% foetal calf serum in PBS). The cell suspension was kept on ice for 10 minutes before removing the fixative by washing the cells three times with PBS, as before. The cells were resuspended to an appropriate density, and spotted onto the wells of multispot slides, (Hendley-Essex). They were then air dried at 37°C, for at least 4 hours, and finally stored at -20°C in resealable plastic bags containing silica gel, to remove moisture.

2.7.1.2. **I.F.A. on Fixed Cells.**

Multispot slides, prepared as in (2.7.1.1), were placed in a dessicator at room temperature for 30 minutes. 20ul of test mouse monoclonal antibody, (Shiels, et. al., 1985) was spotted onto each slide well and the slides placed in a humidified atmosphere for 30 minutes. Unbound antibody was then removed by pipetting of the excess and then washing the slides for 1, 5 and then 10 minutes in PBS pH=7.2. The area between the slide wells was then dried and 10ul of Fluorescein isocyanate (FITC) -labelled anti-mouse second antibody at 1/100 dilution was applied to the wells. (Riggs et. al., 1958). The incubation and washing steps were repeated as for the first antibody and then the slides were counter stained in 0.1% Evans Blue in PBS for 5 minutes. The slides were mounted with a few drops of 50% glycerol/water containing 2.5% w/v DABCO (1,4-
Diazabicyclo[2.2.2]octane to retard photobleaching - the fading of the fluorochrome.

Fluorescing cells were observed under a Leitz ortholux II fluorescence microscope fitted with a WOTAN 50w super pressure mercury lamp. Photographs were taken using a Leitz orthomat-w automatic microscope camera with Kodak 35mm slide film EES (ASA 400).

2.7.2. Surface I.P.A. of Macroschizonts

This assay was used on infected cells which had been lysed according to the method as described in (3.6.2) in order to release macroschizonts.

The cell lysate from 10ml of cultured cells was washed 3 times in PBS pH=7.2, and resuspended in 1ml PBS containing 10% foetal calf serum. 20ul aliquots of the suspension were placed in eppendorf tubes and 20ul antibody (ascites supernatant) was added to each, and incubated at 4°C for 10-45 minutes, with occasional mixing.

The lysate was then washed three times with PBS pH=7.2, spinning for 10 minutes in a microcentrifuge. The final pellet was resuspended in 20ul of cold PBS pH=7.2, 10% foetal calf serum, 5% formalin at 4°C for 10 minutes, and then washed three times in PBS pH=7.2 as above. The pellet was resuspended to an appropriate density, spotted onto multispot slides, dried at 37°C overnight and then anti-mouse FITC added and washed as in (2.6.1.2).
CHAPTER 3

RESULTS
3.1. GROWTH OF T. ANNULATA-INFECTED CELLS: A QUANTITATION OF TRANSFORMATION

3.1.1. Introduction

As already reviewed (1.3), the most prominent feature of T. annulata-infected leukocytes is their ability to proliferate indefinitely, in liquid culture conditions (see 2.1). This in itself raises a number of questions:

1. Do the transformed cells have altered serum requirements?

2. On infection of peripheral blood leukocytes with T. annulata, which subset(s) of leukocytes are transformed? (see 1.3).

3. What are the altered growth parameters of the transformed cells?

Answering these questions requires an alternative approach to the study of the T. annulata-induced transformation phenomenon which the project did not encompass. The following measurements are an initial attempt to quantitate the transformation events. Since some leukocytes from peripheral blood, suspended in culture medium at 37°C for several days, degenerate, while others remain viable and morphologically little changed, (Ling and Kay 1975), it becomes difficult to compare objectively, the proliferation of uninfected leukocytes (PBL110) and those infected with Theileria as an established cell line (such as TaH-PHL110). Measurement of cellular growth rate is based upon the relative numbers of active and inactive cells in a heterogeneous population and so perhaps the best comparison which could be made in order to measure the proliferative effect of Theileria infection, is between two purified cell-lines of the same origin, one of which has been infected with Theileria annulata.

The first experiment was undertaken to compare the rate of cell
division in the already transformed bovine lymphosarcoma cell line BL-20, with the same cell line 'super-transformed' with *T.annulata* (Hissar), and therefore determine whether *T. annulata* is able to further stimulate the cells to divide.

3.1.2. **Cell Division in BL-20 and TaH-BL-20**

BL-20 (---) and TaH-BL-20 (—), as indicated in figure (3.1) were seeded at two starting densities (1 x 10^5/ml) and (4 x 10^5/ml) in triplicate cultures, and incubated as previously described (see 2.1.2). The cell densities were measured over a period of time and the resulting growth curves are shown in figure (3.1). BL-20 cells, seeded at 1 x 10^5 did not proliferate, whereas TaH-BL-20 reached a density of 5 x 10^6 cells/ml within 138 hours - an average doubling time of 22 hours.

The cells seeded at 4 x 10^5/ml proliferated within 96 hours to 3.7 x 10^6/ml (BL-20) and 5 x 10^6/ml (TaH-BL-20). The average doubling times over this period (calculated from the graph by dividing the time taken to reach a cell density of 3.2 x 10^6/ml from 4 x 10^5/ml by 3, the number of doubling times involved) were 26 hours 20 minutes and 21 hours for BL-20 and TaH-BL-20 respectively; The error in these measurements are shown in figure (3.1) as error bars indicating +/- one standard deviation.

Therefore, the infected cell line TaH-BL-20 proliferates significantly faster than the uninfected BL-20 and shows a greater degree of independance from cell density.
Figure 3.1

PROLIFERATION OF THEILERIA ANNULATA—INFECTED AND UNINFECTED LEUKOCYTES.

Experiment carried out in triplicate. Error bars indicate ±1 standard deviation from mean.
3.1.3. Discussion

The results from these preliminary measurements were difficult to interpret due to the difficulty in obtaining base-line values from a good control cell line which was not transformed. Because of this, the proliferative effect of *T. annulata*-induced transformation could not be quantified. However, it was demonstrated that *Theileria* infection had an additive effect on the already transformed cell line BL-20, in that it further stimulated their proliferation by 25.4%. (This figure was obtained by comparison of mean doubling times over the period of measurement). In addition, the fact that TaH-BL-20 proliferated from a low density seeding, whereas BL-20 did not, suggested that TaH-BL-20 was less density dependant in its growth than BL-20. This could be due to TaH-BL-20 secreting some growth-stimulatory factor, which aids proliferation, whereas BL-20 does not. Since cells have been observed to lose their IL-2 dependency on infection with *Theileria parva* (see 1.3.3), then this is a reasonable hypothesis. Uninfected cells alternatively may not have expressed a suitable surface receptor for such a factor. It was also observed that TaH-BL-20 lymphoblasts formed aggregates in liquid culture, whereas BL-20 did not. This in itself may provide a clue to the mode of transformation by *T. annulata*. Formation of aggregates occurs after the addition of almost all mitogens to lymphocytes, but there is conflicting evidence about the importance of this for activation. Peters, (1972) prevented formation of aggregates in lymphocyte cultures stimulated by pokeweed mitogen, by agitation of the cultures and found that the response to the mitogen was greatly reduced. It has been suggested that some material initiating, or essential for activation may pass from cell-to-cell, and Husler, and Peters, (1972) have shown that low resistance junctions exist between bovine lymphocytes in the aggregates formed after addition of the mitogen PHA (phytohaemagglutinin). Intercellular communication was examined by using one microelectrode to record the membrane potential of a cell, while a second was inserted into a nearby cell and used for injecting pulses of current. No evidence was found for intercellular communication between unstimulated lymphocytes, even when agglutination was induced by addition of anti-pig lymphocyte serum which by itself did not stimulate the bovine lymphocytes. However, communication was observed between the cells in aggregates formed after PHA stimulation, even when the cell whose membrane
potential was being measured was separated from the cell to which the current pulse was being applied by several other cells. Thus it seems a reasonable hypothesis that lymphocyte stimulation may be a co-operative response, rather than the response of a single cell, although the nature and effect of this information transfer is, as yet, unknown.

Thus, cell contacts via agglutination may play a significant role in the stimulation of growth of T. annulata-transformed cells and may explain why these cells appear to be less dependant upon external factors for their growth in the culture medium. Alternatively, the results obtained (that the Theileria-infected cells are less dependant on cell density for their growth), may be explained by the infected cells secreting growth factors. These two hypothesised events are not exclusive and may both contribute to the ability of Theileria-infected cells to proliferate both at an increased rate and more independantly of growth factors already present in the culture medium.
3.2 ONCOGENE EXPRESSION

3.2.1. Introduction

As reviewed in Chapter (1.3), there is a strong association between cell transformation in general, and the expression of specific oncogenes, either as structurally altered cellular oncogene sequences or as altered levels of oncogene expression. Oncogenes code for proteins which are, in the main part, homologous to various components of the normal cellular growth control pathway(s) (see section 1.2.8). Therefore, it was decided to investigate whether, by using known oncogenes as probes, it was possible to detect the stimulation of expression of one or more components of the growth control pathways of the leukocyte after infection with Theileria annulata (Hissar). This would aid in the characterisation of some of the events either leading to the transformation event, or resulting from it.

In the following section (3.2), the expression of genes homologous to many of the oncogene probes which are available (listed in Table 2.1) was investigated in a number of leukocyte cell lines (2.1) - both uninfected and T. annulata (Hissar) - infected. Two techniques were utilised to assess the relative expression of oncogenes in infected and uninfected cells: the quick blot technique with total cell lysates (3.2.2) was used initially and the mRNA slot blot technique (3.2.3) was used subsequently to reduce likelihood of non-specific hybridisation of oncogene probes (discussed fully in 3.2.3.1). The two methods are discussed and the results are presented separately in sections (3.2.2) and (3.2.3), since the cell lines used and experimental controls were different in each case.

3.2.2.1. Quick-blot Technique

This technique described fully in section (2.2.1), was used to selectively immobilise messenger ribonucleic acid (mRNA) directly from a cell lysate onto nitrocellulose. Bresser et al., (1983) published results assessing this procedure, and concluded that transfer RNA (tRNA) and ribosomal RNA (rRNA) were not co-immobilised.
with mRNA in amounts which would cause non-specific binding of probes during experiments. In addition, virtually no DNA was seen to be co-immobilised, and since no baking of the nitrocellulose filters was required, any adventitiously adsorbed DNA remains double-stranded and unavailable for hybridisation. The technique of Hayashi et. al., (1978) was utilised to acetylate any proteins which do not flow through the nitrocellulose, so that they too, do not interfere with the subsequent hybridisation process. Fig. (3.3.a) shows the results of such an experiment: the cell lysates, (derived from equal numbers of cells for each cell line) prepared as described in (2.2.1) were dotted onto nitrocellulose in the relative dilutions of 100, 12.5 and 2.5. The filters were subsequently hybridised (see 2.2.4) with a series of 32P-labelled oncogene probes (2.2.15). The probes had been prepared as described, (2.2.14), and the appropriate fragments cut out of a low-melting point agarose gel (2.2.15) with the relevant restriction enzymes (see table 2.1, section 2.2.14).

3.2.2.2 Results

Hybridisation signal was obtained specifically on the mRNA isolated from T. annulata infected cell lines with oncogene probes - Ha-ras, Ki-ras and v-abl while c-B-lym hybridised only to TaH-PBL110 mRNA and not to mRNA from TaH-BL20 or the uninfected cells PBL110, ConA-PBL110 and BL20. No hybridisation was detected in any cell types using the probe v-erb A/B. This served as a negative control, demonstrating that the positive hybridisation signal was specific.

Leukocytes which were not infected with T. annulata (Hissar) did not exhibit strong hybridisation to any of the oncogene probes. However, the Ha-ras probe hybridised weakly to mRNA from both BL-20 and ConA-blasting PBL110 and the Ki-ras probe hybridised weakly to mRNA from BL-20.

The results are analysed below, for each probe, with reference to each oncogene product and its putative function in the normal cell.

(a) Ha-ras.

The Ha-ras gene plasmid contains a 0.45kb BglII - SalI restriction endonuclease fragment of the Harvey murine sarcoma virus.
cloned into the EcoRI site of pBR322. (For restriction map, see Fig. 3.2). The viral protein product is a transforming product (p21 ras), which, like its normal cellular product and the human ras oncogenes, binds guanosine triphosphate (GTP) with high affinity (see 1.2.8.3.1). The transforming gene differs by only 1 nucleotide from its normal homologue (Tabin et. al., 1982), and is localised (like p60 src and several other oncogene-encoded protein kinases) on the inner face of the plasma membrane. McGrath et. al., (1984) discovered that while normal p21 ras has a GTP-ase activity, this function is grossly impaired in the oncogenic protein. The guanine-nucleotide binding proteins (termed G- or N- proteins) to which p21 ras is homologous, are responsible for stimulation and inhibition of adenylate cyclase following the attachment of polypeptide growth hormones to specific cell surface receptors. (See Gilman, 1984 and Chapter 1.2.8.3.1). The ras protein is hypothesised to act like a G-protein (Newbold, 1984).

The 0.45kb EcoRI fragment of the Ha-ras gene plasmid did not hybridise at all with RNA from peripheral blood leukocytes from cow 110, but seemed to weakly hybridise to RNA from the same cells after they had been stimulated with the T-cell mitogen concanavalin A (see fig. 3.3.a). This suggests that there was a small increase in expression of a Ha-ras homologue in response to lymphocyte proliferation. The same cells, after infection with T. annulata (Missar) and subsequent growth in culture, appear to express a Ha-ras homologue at much increased levels, suggesting that its expression was stimulated by Theileria annulata infection. Similarly, while a low level of expression of a Ha-ras - like gene was indicated by weak hybridisation, in the (non-viral) lymphosarcoma line HL-20, the degree of hybridisation was several-fold higher after infection with Theileria (fig. 3.3.a). So, although expression of a Ha-ras homologue was not entirely Theileria-infection-specific, the apparent levels of transcription were much increased in infected cell lines.

(b) Ki-ras

The Ki-ras gene plasmid contains a 1.0kb HindII restriction endonuclease fragment (with EcoRI linkers) of the Kirsten murine sarcoma virus, cloned into the EcoRI site of pBR322. (For
restriction map, see Fig. 3.2). The 21 kilodalton (kD) viral protein product is structurally, immunologically and functionally closely related to the 21kd product of the Ha-ras gene (3.2.2.2.a) - (see Ellis et. al., 1981 and Tsuchida et. al., 1982).

The 310 base-pair (bp) SstII - XbaI restriction endonuclease fragment was used as a hybridisation probe (2.2.15). This small fragment was chosen in order to exclude the rat 30s ribosomal sequences which are included in the 1.0kb in plasmid insert (Fig. 3.2).

The hybridisation pattern using the Ki-ras fragment as a probe, was very similar to that seen when Ha-ras was used (3.2.2.2.a), in that RNA from all the Theileria- infected cell lines TaH-BL-20 and TaH-PBL110 demonstrated a stronger hybridisation signal than RNA from the uninfected cells PBL110, Con A-PBL110 or BL-20 (see fig. 3.3.a). The only uninfected cell line to show any hybridisation was the lymphosarcoma line BL-20.

It is not clear why the faint hybridisation visible on Con A - treated PBL110 cells using Ha-ras as the probe, was not seen with the Ki-ras probe (see fig. 3.3.a) - since the high degree of homology between the two probes would predict that identical results should be obtained. However, this may be due to the fact that the Ki-ras probe was not labelled to as high a specific activity as the Ha-ras probe (3.2 x 10^8 cpm/ug and 4.8 x 10^8 cpm/ug respectively). This explanation is consistent with the observation that hybridisation to RNA from the other cells (TaH-PBL110 and TaH-BL-20) with Ha-ras as the probe was also stronger than with the Ki-ras probe. Alternatively, the slight difference in hybridisation signal may be due to the fact that although the amino acid sequences of the protein products of Ha-ras and Ki-ras are 80% homologous, cross-react immunologically and have similar molecular weights, their DNA sequences hybridise with each other weakly, and only under non-stringent conditions. (Ellis et. al., 1981). Thus, the difference at the nucleotide sequence level may well account for the differing degree of hybridisation seen with the two probes, Ha-ras and Ki-ras: the two probes may be hybridising to different transcripts in this assay, or may be hybridising to the same transcript(s), but with differing hybrid stabilities.
Again, because there was hybridisation (though weak) to RNA from the BL-20 cell extract (see fig. 3.3.a), the expression of a Ki-ras gene homologue seemed not to be Theileria infection-specific, although the levels of transcription appeared to be increased in the infected cell lines.

(c) v-abl

The v-abl gene plasmid contains a 5kb Bam HI fragment of cellular DNA containing the complete provirus of Abelson murine leukaemia virus cloned at the Bam HI site of pAT153. (Srinivasan et. al., 1982; for map, see Fig. 3.2).

There are a family of gag fusion products, depending on the virus variant. The most commonly studied virus has a transformation product P120 gag-abi which has a tyrosine-specific protein kinase activity - an activity known to be an important regulator of cell growth, particularly associated with cell transformation (see 1.2.8.1.1).

The 5.0kb Bam HI fragment (fig. 3.2) was used as a hybridisation probe. The probe therefore contained the sequences for the complete gag-abi product and the 5' LTR of the provirus.

The v-abl probe did not hybridise detectably to either PHL110 or Con A - stimulated PBL110, but did hybridise to the RNA from TaH - PBL110, BL-20 and TaH - BL-20 (fig. 3.3.a). The hybridisation signal was stronger with the two Theileria infected cell line filtrates than with RNA from BL20 (fig. 3.3.a), again indicating that Theileria annulata (Hissar) infection is stimulating the transcription of an abl gene homologue. Concanavalin-A did not seem to increase the transcription of a v-abl -like gene in PHL110 cells, yet a low-level of transcription was detectable on BL-20 cells (see fig. 3.3.a). This suggests that although Theileria infection is not required for transcription of a v-abl homologue, it enhances it above the level detected in BL-20.
(d) Hu-B-lym-1

The Hu-B-lym-1 plasmid contains a 1kb Hind III fragment of DNA isolated from a Human Burkitt's lymphoma cell line (Diamond et. al., 1983). The clone was isolated because of its transforming activity and homology to a transforming gene Ch Blym-1, from chicken Bcell lymphoma DNA, (Goubin et. al., 1983). The protein product of the gene is located in the cell nucleus, and at present it's function has not been elucidated. A 0.95kb EcoRI fragment containing the active transforming gene, was used as a hybridisation probe (2.2.15).

The Hu-B-lym probe did not hybridise at all to RNA from the PBL 110 filtrate or to RNA from the same cells stimulated to divide with concanavalin A, or to RNA from the transformed cell-line BL-20 (see fig. 3.3.a). Thus, transcription of any Hu-B-lym homologue at a detectable level was not stimulated by cell proliferation, or by the transformation events in BL-20. However, hybridisation was seen with the RNA from infected cell filtrates TaH-PBL 110 (strong hybridisation signal) and TaH-BL-20 (weak hybridisation signal) (see fig. 3.3.a). Thus, expression of a Hu-B-lym homologue appeared to be Theileria-infection-specific.

(e) v-erb

The v-erb plasmid contains a 5.1kb EcoRI fragment of genomic DNA containing the permuted provirus of the avian erythroblastosis virus cloned at the EcoRI site of pBR322. (Vennstrom et. al., 1980). The viral transformation products are erb B (65-68kD) and gag-erb A (75kD), encoded by two distinct genes. The erb B protein is now well characterised: it is located within the plasma and cytoplasmic membranes and appears to be a truncated form of the epidermal growth factor (EGF) receptor - it lacks the receptor's EGF-binding region but retains the transmembrane segment and the catalytic protein kinase domain that extends inside the cell (Downward et. al., 1984 and section 1.2.8.2.2). There is now evidence that the erb B protein mimics the action of the EGF receptor, but in an unregulated fashion, thus constantly stimulating the cell to divide (see 1.2.8.2.2. and Beug et. al., 1986). The v-erb A oncogene unlike v-erb B is not itself capable of transforming erythroblasts, but does potentiate the
effects of \textit{v-erb} B by blocking the differentiation of erythroblasts at an immature stage. The \textit{erb} A protein bears statistically significant relatedness to carbonic anhydrases (Debuire et. al., 1984), enzymes which participate in the transport of carbon dioxide by erythrocytes, the precursors of which (erythroblasts) are main targets of avian erythroblastosis virus. Thus expression of a mutant homologue of carbonic anhydrase may be the explanation for the blocking of erythroblast differentiation. The recent demonstration of partial sequence homology of \textit{v-erb} A also with steroid hormone receptors (Beato, 1988), however, raises a possibility contradictory to that suggested by Debuire et. al., (1984) - that \textit{v-erb} A encodes an unregulated hormone receptor, similar to \textit{v-erb} B.

A 2.42kb \textit{Pvu II} fragment containing both \textit{v-erb} A and \textit{v-erb} B sequences and excluding viral LTR, gag and env sequences, was isolated and labelled as described (2.2.15), for use as a hybridisation probe.

No hybridisation signal was detected using the \textit{v-erb} probe, to the mRNA bound from any of the leukocyte cells or cell lines - both uninfected and \textit{Theileria}-infected (see fig. 3.3.a). This may be because the degree of homology with bovine leukocyte growth factor receptors and \textit{v-erb} B is not high enough for detection by this method. Similarly, \textit{v-erb} A may not have a homologue expressed at significant levels in leukocytes; its homologue carbonic anhydrase (Debuire et. al., 1984), has a function mainly in erythrocytes, and its receptor homologue (Beato, 1988) may not be expressed in leukocytes. However, the absence of hybridisation using this probe indicates that the hybridisation to the mRNA extracts using the other probes, which are all labelled to approximately the same specific activity (3.2-6.1 x 10^8 cpm/ug), must have been to specific sequences.

3.2.3.1. \textit{mRNA Slot-blot Technique}

A further series of hybridisations were carried out to compare expression of oncogene homologues in \textit{T. annulata} (Hissar) - infected, and uninfected leukocytes. However, in order to completely rule out the possibility of non-specific binding of the probes to residual protein, DNA, ribosomal or transfer RNA (which may, despite in-depth testing of the technique (Bresser et. al., 1983), be bound to the filters, an alternative method was utilised. This involved pre-
purification of mRNA from the leukocyte cells before applying to nitrocellulose. This mRNA extraction was carried out as described fully in (2.2.2). The mRNA was then applied to nitrocellulose using a slot-blot apparatus (2.2.3) and hybridised with a variety of oncogene probes, labelled with $^{32}P$ as before (see 2.2.15). The mRNA was extracted from the BL-20 lymphosarcoma cell line and the TaH-BL-20 cell line and was loaded in 3 concentrations per cell line: 30ug, 15ug, 5ug (as measured by optical absorbance at 260nm, see 2.2.2) for each of several replicate filters. To check that the loading of mRNA from BL-20 cells was comparable to that from TaH-BL-20 cells, hybridisation to one of the replicate filters carrying mRNA from both cell lines was carried out using $^{32}P$ labelled EcoRI-digested genomic DNA from BL-20 cells as a probe. The results (see Fig. 3.3.b) indicated, by stronger hybridisation to BL-20 than to TaH-BL-20 mRNA, a loading bias towards the uninfected cell line mRNA. Part of this bias may, however, have been due to a proportion of the mRNA from TaH-BL-20 belonging to the parasite and so effectively diluting the leukocyte mRNA.

3.2.3.2. Results (See Fig. 3.3.b)

The hybridisation signal was greater with mRNA from the infected cells compared to mRNA from BL-20 when several of the oncogene probes were used: - v-mos, v-fos, v-myc, Hu-B.lym. The v-sis oncogene hybridised with equal intensity to both BL-20 and TaH-BL-20, thus controlling the experiment internally. Thus any increase in intensity visualised by hybridisation of infected cell mRNA compared to uninfected cell mRNA with the other probes, was taken to be a real increase in expression of an oncogene homologue.

(a) v-sis

The v-sis oncogene-containing plasmid pAT/sis, includes a 1.3kb PstI fragment of the simian sarcoma virus (SSV) cloned into the PstI site of pAT 153. This fragment contains the v-sis gene and other viral sequences (see Fig. 3.2). Amino-acid sequence analysis of separated platelet-derived growth factor (PDGF) polypeptide chains (denoted A and B; Johnsson et. al., 1982) has shown that the N-
terminal 109 amino-acid residues of the B-chain are virtually identical with the predicted sequence of the transforming protein p28sis of simian sarcoma virus (residues 67-175) (Doolittle et al., 1983 and see 1.2.8.2.1.3). By inference from the structural homology between the p24sis dimer and PDGF, it is assumed that the viral gene product functions as a PDGF agonist - stimulating cell replication by interacting with the PDGF receptor (see 1.2.8.2.1.3). Indeed, SSV-transformed cells contain in their cytoplasm, a growth factor whose activity is neutralised by PDGF antibodies (Denel et al., 1983); and also a high level of v-sis related mRNA has been observed in human sarcoma and glioma cell lines (Eva et al., 1982).

A 0.9kb XbaI -PstI fragment was isolated from pAT/sis, excising the v-sis gene from other viral sequences (see Fig. 3.2) and hybridised to one of the replicate filters. As can be seen from Fig. (3.3.b), the v-sis probe hybridises equally to mRNA from BL-20 and TaH-BL-20 cells. This could be interpreted in two ways: firstly an increase in expression of a v-sis homologue in the T. annulata-infected cells, since the control shows an mRNA loading bias in favour of BL-20; and secondly, as an equal expression of a v-sis homologue. (Assuming equal quantities of RNA were loaded in TaH-BL-20 and BL-20 slots, as indicated by O.D. measurements, an unknown proportion of TaH-BL-20 mRNA is parasite-derived, effectively diluting the infected cell RNA. Thus the equal loading of RNA by the optical density criterion, may have resulted in the loading bias towards more leukocyte-derived RNA in the uninfected cells, as indicated by hybridisation with BL-20 DNA).

Although it is likely that the control hybridisation with BL-20 DNA gave a true indication of loading of leukocyte-derived RNA, it was decided to err on the side of caution and take the hybridisation with the v-sis probe as an internal control for equal expression in BL-20 and TaH-BL-20.

(b) v-mos

The v-mos gene plasmid, pmos (pmos 31), contains a 1.0kb Hind III -XbaI fragment of Moloney murine sarcoma virus cloned at the PstI site of pBR322 by tailing (for restriction map, see fig. 3.2).
Although the mos oncogene is grouped with tyrosine kinase oncogenes because of sequence homology (Van Beveren et al., 1981), the v-mos transforming protein has no tyrosine kinase activity. However, it does have a serine kinase activity (Maxwell et al., 1985), although there is at present no link between this, or any other mos function and cell transformation.

The insert was cut from the plasmid using PstI which gave four insert fragments of: 100bp, 100bp, 460bp, 240bp. These were pooled and labelled for use as a hybridisation probe (2.2.15). The v-mos probe hybridised approximately three times more strongly with infected cell mRNA than uninfected cell mRNA (the 15ug slot of BL-20 has an equal intensity to the 5ug slot of TaH-BL-20; fig. 3.3.b). This indicates that T. annulata (Hissar) infection of BL-20 is associated with increased expression of a gene, or genes homologous to v-mos.

(c) v-fos.

The v-fos gene plasmid (designated pfos (p fos BS), contains a 760 nucleotide Bgl II - Sal I restriction endonuclease fragment from the FBJ murine osteosarcoma virus cloned into the Bam HI - Sal I sites of pAT 153 by substitution (Curran et al., 1982; see fig. 3.2. for restriction map). The viral gene products have no protein kinase activity, but the cellular gene product of c-fos has been located in the nucleus. Stimulation of proliferation of a variety of cell types, including the NIH3T3 mouse fibroblast cell line, (Miller et al., 1984) and stimulation of differentiation, including macrophages (see Miller, 1986) have been shown to result in an increase in expression of c-fos gene products. In fact, induction of c-fos is one of the earliest known effects on the control of gene expression exerted by growth factors (see Greenberg and Ziffi, 1984 and 1.2.8.4). The precise function of the fos protein is not known, although being a DNA-binding phosphoprotein (Armelin and Armelin, 1986), it is tempting to speculate that it has a regulatory role on gene expression governing growth and differentiation.

The complete insert was excised using MboI and Sal I restriction endonucleases. (MboI was utilised to cut at the BglII-BamHI fusion site which would no longer be recognised by BglII and BamHI.
restriction enzymes) and the 0.76kb fragment labelled for use in hybridisation. The v-fos probe hybridised 2-3 times more strongly with TaH-BL-20 mRNA than with BL-20 mRNA (Fig. 3.3.b), indicating a stimulation of expression of c-fos homologues, on infection with Theileria annulata.

(d) v-myc

The v-myc oncogene-containing plasmid (pSV v-myc) was constructed by Land et. al., (1983) and contains the complete provirus (see fig. 3.2 for restriction map) including the viral genes, gag, myc and long terminal repeats. The myc gene, as transduced by several retroviruses, transforms a variety of target cells of different histogenetic origins (Graf and Beug, 1978). In addition, amplification or abberant expression (sometimes as a result of chromosomal translocation - Dalla Favera et. al., 1982) of the c-myc gene has been implicated in human malignancies such as Burkitt's lymphoma (Taub et. al., 1982; Klein, 1983) and small cell carcinoma of the lung (Little et. al., 1983). Similar to c-fos (3.2.3.2.c), c-myc is a likely candidate for participation in the post-receptor pathway since addition of platelet-derived growth factor (PDGF) to stationary mouse (BALB/c) 3T3 fibroblasts induces a several-fold increase in the expression of c-myc mRNA, as does the addition of mitogens to mouse lymphocytes (Kelly et. al., 1983 and 1.2.8.4.1). Also, like c-fos, the myc product is a nuclear protein (Donner et. al., 1982), suggesting it has a functional role in the regulation of the expression of genes involved in the control of cell proliferation. Because of the wide variety of neoplasias with an abnormal expression of this particular oncogene, it is conceivable that the post-receptor pathways of several growth factors converge at the regulation of myc gene expression.

A 3.8kb KpnI - EcoRI fragment was cut from pSV v-myc and used in hybridisation to an mRNA slot-blot filter. This fragment included the complete gag-myc sequences, and omitted the viral L.T.R.'s. As can be seen from fig. (3.3.b), the v-myc probe hybridised approximately three times more strongly to the mRNA from the Theileria-infected BL-20 cells than to the uninfected cells. (The hybridisation signal intensity is equal on 15ug BL-20 mRNA and 5ug TaH-BL-20 mRNA). Thus
it appears that transcription of v-myc-like sequence(s) are stimulated by T. annulata (Hissar) infection.

(e) Hu-Blym-1

The Hu-Blym-1 probe [for a description of the oncogene and the isolation of the fragment used as a probe in the hybridisation, see 3.2.2.2.(d)] hybridised approximately twice as strongly to T.annulata (Hissar)-infected BL-20 cells as to the uninfected BL-20 cells (see fig. 3.3.b), indicating an infection-specific increase in transcription of Hu-Blym-1-like sequences. However, it should be noted that in the complementary experiment using this probe in the cell-blot technique (3.2.2.2.d), no hybridisation was detected to the uninfected cell lysate, whereas a weak hybridisation was detected to BL-20 mRNA in this assay. Thus, contrary to conclusions from the previous experiment, expression of B-lym-like sequences appears not to be Theileria-infection-specific, but to be stimulated on infection.
**Figure 3.2**

**Plasmid Insert Restriction Maps**

**Ha-ras** (pras(pBS9))
De Feo et al., (1981). 1 cm = 0.1 kb

![Ha-ras Diagram]

**Ki-ras** (pras(pHiHi3))
Ellis et al., (1981). 1 cm = 0.1 kb

![Ki-ras Diagram]

**abl** (pabl)
Srinivasan et al., (1982). 1 cm = 1.0 kb

![abl Diagram]

**λ HUBlym-1** (Diamond et al., 1983). 1 cm = 0.2 kb

![λ HUBlym-1 Diagram]

**erb** (perb)
Vennstrom et al., (1980). 1 cm = 0.5 kb

![erb Diagram]

**sis** (pAT/sis)
Eva et al., (1982). 1 cm = 0.2 kb

![sis Diagram]
figure 3.2 (cont’d from previous page).

mos [pmos(pmos31)]
Van Beveran et al., (1981). 1 cm = 0.1 kb

fos [pfos(pfosBS)]
Curran et al., (1982). 1 cm = 0.1 kb

myc (pSVv-myc)
Land et al., (1983). 1 cm = 1 kb

src [psrc(EcoRI-B)]
Karess et al., (1979). 1 cm = 0.5 kb

yes [pyes(pyes1)]
Kitamura et al., (1982). 1 cm = 0.5 kb

Key to Restriction Enzyme Nomenclature

A AccI  HII  HincII  Sa  SacI
B BamHI  K  KpnI  Sm  SmaI
Bg BglII  P  PstI  SII  SstII
E EcoRI  Pv  PvuII  X  XbaI
H HindIII  S  SalI  Xh  XhoI

136
Figure 3.3

(a)

Relative amount

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<th>Amount</th>
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<td></td>
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<td></td>
</tr>
<tr>
<td>2.5</td>
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</table>

Ha-ras
Ki-ras
v-abl
HuB-lym
v-erb

B = BL-20
T-B = Tah-BL-20
T = Tah-PBL 110
P = PBL 110
CA = ConA-PBL 110

(b)

mRNA (ug)

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</table>

v-sis
v-mos
v-fos
v-myc
HuB-lym
BL20 DNA

PROBE
3.2.4. Discussion

The results of these preliminary expression studies show that:

(1) Cell proliferation in general results in the increased expression of several genes which have homology to known oncogenes (Ha-ras, Ki-ras and abl, section 3.2.2). This may be expected since oncogenes have been homology to various components of the growth control pathways (reviewed in 1.2).

(2) Infection with *T. annulata* (Hissar) results in a further stimulation of expression of these genes and also results in elevated expression of other genes homologous to known oncogenes which included homologues of v-fos, v-mos and v-myc and Hu-B-lym. Expression of Hu-B-lym-like sequences, as described in 3.2.3, were not detected in mitogen stimulated leukocytes or the lymphosarcoma cells BL-20 in the cell blot analysis, but were detected at low levels in mRNA from BL-20, possibly as a result of increased sensitivity of the latter experiment. All of the probes used, which demonstrated hybridisation to *Theileria*-infected cell extract/mRNA, also hybridised to some extent to uninfected (BL-20) cell extract/mRNA, thus indicating stimulation of expression of host-encoded genes.

This type of slot-blot experiment does have limitations in that the specificity of hybridisation cannot be determined. For instance, hybridising under these non-stringent conditions may result in one probe hybridising to several different transcripts as a result of having some degree of homology with all of them - a kinase - encoding probe such as v-abl, for example, may hybridise with any of a number of protein kinase gene transcripts expressed in the cell. This problem, though certainly present, is obviously not resulting in totally non-homologous hybridisation, as can be seen from the lack of hybridisation of some probes with some cell lines. (Also, since Hu-B-lym-1 exhibits a consistently weaker hybridisation signal compared to the other probes in both experiments 3.2.2. and 3.2.3., this indicated some level of specificity for each probe - all were labelled to around the same-specific activity. If totally non-homologous hybridisation was occurring, all probes should have showed an equal hybridisation signal. In order to overcome this problem, it is necessary to run denaturing gels of mRNA in order to separate the
transcripts, and to northern blot and probe them. This would also give a size determination of transcripts. With the technical problems associated with such experiments using non-homologous probes, it was decided to wait until a later stage in the project, when a homologous probe was available, to study oncogene expression in more detail in T.annulata-induced transformation. In such a position, it would be feasible to carry out time-course experiments of oncogene expression after in vitro infection of leukocytes. This may reveal transient expression of oncogene sequences, as has been observed previously with c-myc and c-fos (3.2.3.2.d and 3.2.3.2.c, respectively), on stimulation with certain growth factors (see 1.2.8.4.1).

Until macroschizonts can be isolated pure and intact from infected leukocytes (see Chapter 3.6) in such quantities that it is feasible to prepare mRNA for northern gel blots, the presence of a transcript specifically in Theileria-infected cells cannot be attributed to the parasite or the host cell (if both host and parasite have genes homologous to the transcript of interest). Most, if not all of the results in this system are likely to be the stimulation of transcription of host cell genes as a result of Theileria infection and host cell transformation — since the infection-stimulated oncogene homologues demonstrated residual expression in uninfected leukocytes. Any direct causative events in the stimulation process by the parasite cannot be distinguished from expression of parasite oncogene homologues which do not interfere with the host cell and may be involved in the growth regulation of the parasite itself.
3.3. **T. annulata oncogene homologues**

3.3.1. **Introduction**

In order for the intracellular macroschizont to transform its host cell, the former must undergo some molecular interaction with the leukocyte. This may, as described more fully in the hypothesised models (section 1.3.6), involve either:-

(a) Transfer of parasite (or viral - see 1.3.6) DNA sequences into the host cell, possibly integrating into the host genome, leading to cellular transformation.

or (b) Secretion of a parasite (or viral - see 1.3.6) 'transformation' molecule (or molecules) which interacts with the host growth regulatory pathways.

or (c) Expression of antigens on the macroschizont or host cell surface, which interact with the host growth regulatory pathways.

In all cases, parasite (or viral) gene product(s) must be expressed, which have a growth regulatory function. The only way of understanding the initial causative events of the *T. annulata*-induced transformation is to detect these parasite/viral DNA sequences, or the RNA or proteins they encode.

In order to detect *T. annulata*-encoded sequences which are likely to be involved in the leukocyte transformation process, two strategies were considered:-

(1) Transfection of cloned *T. annulata* genomic DNA into mammalian cells and assaying for their transformation. The rationale is that active transforming genes, when expressed in suitable host cells, will result in loss of the constraints of normal growth and so can be detected by rapid proliferation - usually as a focus of cells on a plate. This approach has been taken to isolate activated transforming genes from a variety of different types of neoplastic
The genes being detected by their ability to efficiently transform NIH 3T3 (mouse fibroblast) cells upon transfection. (For review, see Cooper, 1982 and section 1.2.7). For example, the transforming gene Ch B lym-1 was isolated by Goubin et al., (1983) by constructing a genomic library from a chicken lymphoma cell line, dividing into pools of clones, and assaying each for transforming activity. Active pools were then sub-divided, re-assayed and the process repeated until a single clone was isolated which could effect transformation. This NIH 3T3 assay could similarly be used with T. annulata DNA sequences, or an alternative assay for transformation devised using freshly isolated bovine peripheral blood leukocytes (PBBL) instead of NIH 3T3 as the means of detecting transforming activity through stimulation of proliferation in culture (see also 4.3).

However, this technique of isolating transforming genes is not effective in every instance, and so the gene(s) of interest may not be detected. This may occur, for instance, if a transforming gene is not active within itself and relies on other sequences, neighbouring (e.g. a promoter or enhancer) or distant (e.g. a transcription factor gene) for its activation. This is normally the case where a gene transforms by a quantitative change in the levels of its expression, as opposed to a structural change. Such a gene may then be separated from the other sequences necessary for activating its transforming potential during the cloning procedure for transfection. Thus it would not be detected as a transforming gene using such an assay.

(2) Since the cellular homologues of transforming oncogenes appear to play a central part in growth and differentiation (reviewed in 1.2.8), these sequences have been highly conserved through evolution (see 1.2.5). In addition, in order for T. annulata to induce host cell transformation, the molecules which interact with the host cell growth regulatory pathways may mimic components of these host cell pathways. Thus, using the collection of mammalian oncogene probes available (table 2.1) parasite oncogene homologues may be detected by Southern blotting (2.2.13, 2.2.16), with piroplasm (the erythrocytic stage of the parasite life-cycle - see fig. 1.2) DNA. However, in order to finally prove if any of these are implicated in the transformation events, a functional test such as transfection (see paragraph 1) would have to be used. This method, although initially avoiding the
problem of missing genes which are not transformationally active in isolation, will miss any genes for which we do not have a probe - even if *T. annulata* does transform by means of a homologue of a known oncogene.

On the basis of the availability of the probes and the simplicity of approach, it was decided to initially adopt the latter procedure (2) in an attempt to identify *T. annulata* oncogene homologues.

### 3.3.2.1. Purity of *T. Annulata* (Hissar) DNA

DNA was extracted from the piroplasm (erythrocytic) stage of the parasite (see 2.2.10) in order to avoid contamination with leukocyte DNA. During the isolation of piroplasms, leukocyte contamination was checked as described previously (2.2.9). Contamination was kept below 1 leukocyte per several thousand erythrocytes. However, as the DNA content of the leukocyte is much greater than the piroplasm, the parasite DNA was checked for contamination. Figure (3.4.a) shows genomic DNA from the uninfected leukocytes (BL-20), the infected leukocytes (T-BL) and piroplasms (PIRO), cut with the restriction endonuclease *PcoRI*. The bovine DNA reveals a main satellite sequence about 1.4kb in length (described previously - see Singer, 1982). This prominent band does not appear in the DNA prepared from piroplasms (track marked PIRO), thus indicating that there is no major contamination with bovine sequences.

### 3.3.2.2. Southern Blotting

Piroplasm DNA was cut with restriction endonucleases (2.2.12) the fragments separated by agarose gel electrophoresis, (2.2.13), transferred to nitrocellulose (2.2.16) and hybridised with a series of oncogene probes using the protocol described in 2.2.16.2 for a non-homologous probe. The following probes did not hybridise detectably to the genomic DNA of *Theileria annulata* (Hissar):

- **Ha-ras (3.2.2.2.a)**; **Ki-ras (3.2.2.2.b)**; **v-abl (3.2.2.2.c)**; **Hu-Blym-1 (3.2.2.2.d)**; **v-erb (3.2.2.2.e)**; **v-sis (3.2.3.2.a)**; **v-fos (3.2.3.2.c)**; **v-myc (3.2.3.2.d)**; **v-src**. **[v-src probe was**
Figure 3.4

(a)

(b)
designated p-src (EcoRI-B) - see Fig. (3.2) and section (1.2.8.1).

However, hybridisation to T. annulata DNA preparations was observed with two oncogene probes: figure (3.4.b) shows the result from one such experiment, where the Theileria DNA was digested with the restriction endonuclease Hind III. The v-mos probe (3.2.3.2.b) hybridised weakly to a 4.3kb fragment of Theileria DNA. Hybridisation with a v-yes oncogene probe (see 3.3.2.4) showed strong hybridisation to two Hind III fragments of T. annulata (Hissar) DNA (3.35kb and 1.3kb) and also weak hybridisation to a 5.25kb fragment. This experiment indicates that the T. annulata genome contains a homologue of the v-yes oncogene. The 5.25kb weakly hybridising fragment later proved to be a bovine homologue of v-yes (3.3.2.3) while the 3.35kb and 1.30kb fragments were parasite encoded. The absence of hybridisation with several oncogene probes in this particular Southern blot (v-src, v-fos and Hu-B-lym; see fig. 3.4.b) served to act as a negative control in this experiment, indicating that the hybridisation with v-mos and v-yes was specific.

3.3.2.3. The yes Gene Homologue is Theileria Encoded.

Despite the fact that there was no gross contamination of piroplasm DNA by bovine sequences (3.3.2.1), it was still possible that the hybridisation of the v-yes oncogene probe to the piroplasm DNA preparation, was in fact due to contaminating bovine sequences. In order to check firstly that the hybridisation was repeatable and secondly whether the hybridising fragments were of parasite or bovine origin, a further series of Southern blot hybridisations were carried out.

Figure (3.5.a) shows the results of hybridising the $^{32}$P-labelled v-yes probe to piroplasm DNA preparations digested with restriction endonucleases MboI, Hind III and Pst I and to calf thymus DNA also cut with Hind III and Pst I. The yes probe hybridised to the following fragments of piroplasm DNA: 1.5kb MboI; 1.3 and 3.4kb Hind III (as in experiment 3.3.2.2); 1.95kb Pst I and to different sized fragments of bovine DNA: 5.25kb (relatively strong hybridisation) and 2.5kb (weak) Hind III; 5.1kb Pst I. Since the v-yes probe does not hybridise to the same size fragments of bovine DNA as Theileria
DNA, the strong hybridisation initially visualised (3.3.2.2) with the v-yes probe, was in fact to sequences encoded by the parasite T. annulata (Hissar), and not to contaminating bovine sequences. In fact, the weakly hybridising band originally visualised at 5.25kb with v-yes as the probe (Fig. 3.4.b) corresponds to the hybridisation seen here with calf thymus DNA, using Hind III as the restriction endonuclease. Thus, the hybridisation to the 3.35 and 1.3 kb Hind III fragments was both repeatable and was to parasite-encoded sequences.

3.3.2.4. The v-yes Onco gene

The v-yes onco gene probe which hybridised to T. annulata (Hissar) DNA, was a 1.6kb EcoRI - Xho I restriction endonuclease fragment of the plasmid p-yes. (see Fig. 3.2 for restriction map). The vector, plasmid (pBR322) contained a 4kb Pst I insert encoding the Y73 avian sarcoma virus (Yoshida et. al., 1980). By isolating the 1.6kb fragment, the v-yes sequences were purified from the retroviral LTR sequences.

The predicted amino acid sequence of the P90 gag-yes transforming protein (Kitamura et. al., 1982), shows a high degree of homology with pp60 v-src, the transforming protein of Rous sarcoma virus, in the car boxy-terminal regions. Both proteins have a tyrosine-kinase activity and the yes onco gene has now been assigned to the src family of protein kinases (Wagner and Miller, 1988), although the two transforming genes, surprisingly, have no homology as assessed by nucleic acid hybridisation (Kitamura et. al., 1982).

3.3.3. Isolation of the Theileria v-yes Homologue

Both the strength of the hybridisation signal of the v-yes gene probe on Theileria DNA and the fact that other related genes of the src family (i.e. src, erb, abl, - Wagner and Miller, 1988) did not hybridise with Theileria DNA, suggested a relatively high degree of specificity in the hybridisation between the v-yes gene and T. annulata (Hissar) DNA sequences. (One exception, however was the v-mos (a member of the src gene family) probe which did hybridise to Theileria DNA, but to different fragments compared with v-yes (Fig.
Thus, it was decided to concentrate on attempting to isolate the *T. annulata* homologue of the *yes* oncogene for the following reasons:-

1) In order to carry out detailed expression studies, a homologous probe was desirable. (See 3.2.4).

2) The exact degree of homology of the *Theileria* gene with *v-yes* and other transforming genes, kinase genes etc. could only be determined by cloning and sequencing the parasite homologue.

3) Any functional test of the ability of the *T. annulata yes* gene homologue to transform mammalian cells would require the use of the isolated *Theileria* gene (see 3.3.1.i).

3.3.3.1. **Screening the Lambda gt-11 Expression Library**

The unamplified lambda gt-11 expression library (2.2.17.1) was initially plated out as described (2.2.18) on 25cm x 25cm square agar plates in order to screen large numbers of plaques (>5000 per plate). Plaque lifts and subsequent hybridisation to the 1.6kb *v-yes* probe were also carried out according to the methods previously described (2.2.19.1 and 2.2.19.2).

There was a great deal of difficulty encountered in obtaining specific hybridisation using this non-homologous probe in plaque screening. The conditions of hybridisation outlined in (2.2.19.2) proved to be the most suitable - if the stringency was increased, no hybridisation was detected and if it was lowered, almost all of the plaques showed hybridisation. As it was, there were often large areas of non-specific hybridisation (see Fig. 3.5.b - top area) which were of no use in picking potential *yes* homologues. Plaques were thus picked which hybridised in an area of low non-specific background (e.g., see Fig. 3.5.b - lambda CL-12). These were picked from confluent plates using the blunt end of a pasteur pipette. These were then replated at lower density for rescreening, such that single plaques could then be picked for purifying (2.2.19.3).

Using this method, 12 plaques were eventually purified, each of which were candidates for being homologous to the *v-yes* oncogene by
Figure 3.5

(a) Bovine DNA (HindIII), TaH DNA (MboI), TaH DNA (HindIII)

- 5.25 kb
- 2.5 kb
- 1.5 kb

- 34 kb
- 1.95 kb
- 1.3 kb

(b) ACL-12
hybridising through each round of purification. Figure (3.5.b) shows one plaque (labelled lambda CL-12) which was subsequently picked and then purified by this criterion.

3.3.3.2. **CL12: A T. annulata yes Gene Homologue**

DNA was extracted from each of ten of these twelve clones (labelled CL1 - CL12), using the 'mini-preparation' method described in (2.2.20.2). It was then cut with the restriction enzyme EcoRI in order to release the cloned insert fragments (see figure 2.2a for lambda gt-11 restriction map) and run on a 1.5% agarose gel containing ethidium bromide to visualise DNA fragments (fig. 3.6.a). The bacteriophage arms which were cut, and those still containing the insert which did not cut, remained unresolved near the top of the gel. Inserts of varying sizes (200bp to 5.5kb) were visible in all clones apart from CL3 and CL9. Clones SuR II, 5, 8, 11 and 12 contained an EcoRI site within the insert DNA—as shown by presence of more than one band of insert DNA. Clones 1, 2, 7, 8, 11 and 12 all contained a 1kb EcoRI fragment as (part of) their insert. This implied that the same clone had been picked as separate plaques in the second round of plaque screening. SuR II was a clone donated by Susanne Williamson (Centre for Tropical Veterinary Medicine, University of Edinburgh) containing a fragment of the *Theileria* genome, also in lambda gt-11. This was included as a negative control for subsequent hybridisation experiments.

The DNA was then Southern blotted onto nitrocellulose and hybridised (2.2.16) with the 1.6kb fragment of the v-yes oncogene (3.3.2.4) which had been labelled with $^{32}$P (2.2.15). The resultant autoradiograph is shown in figure (3.6.b). A 1.0kb insert from CL12 hybridised with the v-yes oncogene probe. No other lambda sequences or other cloned *Theileria* inserts hybridised to the v-yes probe, showing the hybridisation to be specific. Thus, CL-12 contained sequences homologous to v-yes. Since the other clones (CL1, 2, 7, 8 and 11) which also contained a 1.0kb EcoRI fragment did not hybridise detectably with v-yes, then they are likely to contain different sequences to CL-12. Alternatively, they may have contained the same sequences, but not exhibited visible hybridisation with v-yes since there was less insert DNA available for hybridisation, as shown.
Figure 3.6

(a) 
\(\lambda Cl-1 \ -2 \ -3 \ -5 \ -6 \ -7 \ -8 \ -9 \ -11 \ -12 \ SuRII\)

(b) 
-12

-1.0 kb
by the higher intensity of ethidium bromide fluorescence of the lambda CL-12 1kb fragment (fig. 3.6.a).

3.3.3.3. Characterisation of CL-12

3.3.3.3.1. Preparation of CL-12 DNA

In order to prepare substantial amounts of CL-12 insert DNA for use as a probe in hybridisations, subcloning and eventual sequencing analyses, attempts were made to isolate CL-12 DNA using the liquid culture lysis method of amplification described fully in (2.2.20.1). Efficient lysis of the liquid culture of bacteria did not occur after inoculating with phage CL-12 harvested from 5-10 9cm diameter plates. Only after the product of 40 plates of CL-12 were used as the inoculum for 200ml host cells, did efficient lysis occur. Fig. (3.7.a) shows the density of bacterial host cells as O.D. 595 increasing for a time, and then being reduced after 300 minutes, by phage-induced lysis. This problem was partly due to the poor growth characteristics of phage lambda gt-11 (Young and Davis, 1983) itself. The effect of containing this particular insert may also have contributed to selection against the growth of CL-12 (Huynh, et.al., 1985; see 3.3.3.3.4, c).

DNA was then extracted from the bacteriophage following the protocol described in section (2.2.20.1), but only 2.5ug of DNA was recovered. A proportion of the phage recovered from liquid culture amplification, were kept at 4°C in S.M., with a few drops of chloroform.

3.3.3.4.2. Loss of EcoRI Restriction Site(s)

Due to the difficulties encountered in obtaining substantial amounts of DNA from CL-12, it was decided to subclone the EcoRI 1.0kb insert into a vector which could easily be grown in large quantities for DNA extraction. The plasmid vector pUC 18 was chosen because of its rapid growth rate and ease of handling. The selection system for recombinants is described in (2.2.17.3). 1.25ug of CL-12 DNA and 1.25ug of pUC18 DNA (prepared as in 2.2.14.3) were digested with EcoRI and then 500ng of each was checked for complete digestion of
agarose gel electrophoresis along with wild-type lambda gt-11 DNA, and pUC 18 DNA which served as markers. It was apparent from this gel (shown in figure 3.7.b) that the pUC 18 had been digested but CL-12 had not, as no insert could be detected. The result suggested that either the lambda CL-12 DNA preparation contained inhibitors of EcoRI or that the insert had been lost on amplification. However, it was possible that a proportion of the bacteriophage DNA had in fact been cut, but at such low levels that it could not be visualised. As this was the only sample of CL-12 and only one recombinant subclone was necessary, the restriction enzyme digestion was repeated, on the same DNA and the subcloning continued according to the method in (2.2.21.3-2.2.21.4). One recombinant colony was picked, grown up and DNA extracted according to (2.2.14.3). A sample of the DNA recovered was run on a 1.5% agarose gel beside wild type pUC 18 DNA (see fig. 3.7.c). From this gel, it appears that because the recombinant plasmid ran higher on the gel, it is larger and therefore carries insert DNA. However, as shown in fig. (3.7.d), when the plasmid was cut with EcoRI, the plasmid linearised, but did not release a 1kb insert. In order to check for the presence of the 1kb CL-12 insert, the gel was blotted and hybridised with the v-yes probe. No hybridisation was detected. This was in accordance with the recombinant plasmid being a pUC-18 -pUC 18 recombinant: a double plasmid.

The inability of EcoRI to excise the insert of lambda CL-12 (fig. 3.7,b) was further investigated by restriction enzyme site analysis of DNA mini-preparations (2.2.20.2) from the amplified lambda CL-12. Digesting lambda CL-12 with the restriction endonuclease EcoRI did not release any visible insert, suggesting that the EcoRI site(s) had been lost. In order to examine this possibility, double digests were carried out with the enzymes Kpn I and Sst I which flank the insertion site (see Fig. 2.2.a for restriction map). The results are shown in fig. (3.7.e). Wild type phage fragments were 5.0kb, 2.1kb and 1.5kb, as well as the phage arm fragments (approximately 20kb - not shown in fig. 3.17,e). Fragments were released from digestion of lambda CL-12 which by showing an increase in size compared with the 2.1kb wild-type fragment, suggested an insert of just over 2.6kb was present, itself containing a Kpn I or Sst I (see fig. 3.7.e). Both fragments of the 2.2/2.5kb doublet in CL-12 were larger than the single 2.1kb Kpn I -Sst I fragment of the wild-type lambda gt-11. If the doublet is due
Figure 3.7

(a) ABSORBANCE 5951 p

(b) (c) (d) (e)

(f) Marker Bam HI Hind II B/H Kpn I Sst I K/S Bgl II Pvu II 

wt 12 wt 12 wt 12 wt 12 wt 12 wt 12 wt 12 wt 12 wt 12

2.7 kb

2.1 kb

1.5 kb

(wt = wild type Agt11; 12 = ACL-12)
to a mixture of two phage (and not to a KpnI or SstI site in an inserted 2.6kb fragment) then neither of them are wild-type, since both must contain inserts to generate fragments larger than the 2.1kb wild-type KpnI - SstI fragment. If a mixture of two clones of bacteriophage lambda gt-11 were present, then the two bands in the (2.2/2.5kb) doublet, each unique to only one clone, should be less intense than the other phage bands common to both clones. However, as shown in fig. (3.7.e), the intensity of each band in the doublet was equal to that of the other phage DNA band of a roughly equivalent DNA content - the 1.6kb band. In addition, the two bands of the doublet themselves have an equal intensity to each other which is only possible if two clones were present in equal proportions. Therefore, it seems unlikely that there is a mixture of phage present. It appears that lambda CL-12 now contained a larger insert of 2.6kb which could no longer be excised by EcoRI digestion (EcoRI still excised inserts from other control bacteriophage such as lambda SuR II (fig. 3.6.a).

3.3.3.3.3. Loss of Hybridisation Between CL-12 and the v-yes Probe

In order to determine whether the 2.6kb insert (see 3.3.3.3.2) identified in CL-12 contained Theileria gene sequences, the bacteriophage DNA was labelled with 32P (2.2.15) and used to probe Southern transfers of restriction digests of piroplasm DNA and bovine leukocyte DNA. No hybridisation was detected, suggesting that the observed 2.6kb insert of CL-12 was not of either bovine or Theileria origin. In addition, Southern transfers of KpnI/SstI digested CL-12 DNA were probed with 32P-labelled v-yes probe, and showed no evidence of hybridisation. This suggested that the sequences originally cloned into this bacteriophage were no longer present.

3.3.3.3.4. Hypotheses as to the Events Leading to Loss of v-yes-like Sequences from CL-12

The following hypotheses were examined in order to explain the loss of v-yes-like sequences and the apparent increase in size of the insert (see 3.3.3.3.2) of lambda CL-12.

Hypothesis (a) There was a mix-up of the clones CL-1 to
CL-12 and the clone which hybridised to v-yes (3.3.3.2) was not the one which was picked and subsequently studied. The hypothesis was tested by repeating the experiment described in (3.3.2.3). Fresh DNA preparations of CL-1 - CL-12 were made (using liquid culture - amplified CL-12) according to the method described in (2.2.20.2). Inserts were cut out from the bacteriophage using the restriction enzyme EcoRI and the DNA run on a 1.2% agarose gel (no insert was seen in CL-12 but inserts were seen in other clones). The gel was then Southern blotted and hybridised with the 32P-labelled v-yes probe. No hybridisation was detected with any of the clones, indicating a genuine loss of cloned insert DNA from CL-12 and absence of these v-yes-like sequences from the other clones (which released insert DNA in sufficient quantities as to show intensely fluorescing bands when stained with ethidium bromide).

Hypothesis (b) CL-12 was not completely purified, and was outgrown on amplification by a faster growing bacteriophage. This was possible, since it is known that the bacteriophage lambda gt-11 with particular cloned inserts may grow slower than others due to some recombinants producing low levels of polypeptides inhibitory to phage or cell growth. This happens to some extent even in the presence of the lac I repressor which represses transcription from the B-galactosidase gene promoter. Thus, amplifying libraries cloned in lambda gt-11 creates a bias in favour of healthy recombinant phage (Huynh et. al., 1985). This will occur particularly with Y1090 host cells which do not express the lac repressor (Young and Davis, 1983). If this was the case, then there would still remain at least a small proportion of the original v-yes-hybridising CL-12 in the bacteriophage which had been amplified through liquid culture. So, in order to test this hypothesis, approximately 9000 bacteriophage from the liquid lysis culture were plated out and screened by filter hybridisation with the v-yes probe (2.2.18; 2.2.19). A few plaques were picked as tentatively positive, but on replating and re-screening did not result in a higher frequency of positive clones. Thus, the screen was considered negative, and unless there were less than 1 in (approximately) 9000 of the original CL-12 in the amplified culture, then this hypothesis was incorrect.

Hypothesis (c). Since the host cells Y1090 are not defective
for host-controlled restriction (and modification) enzyme activities, (Huynh et. al., 1985), insert DNA may have been lost through host cell restriction enzymes. However, to appear as positive clones originally, the insert must have been modified at this stage.

**Hypothesis (d).** Although the host cells, Y1090 are supposedly recombination deficient (rec A - ), there was the possibility of residual rec A activity, or reversion, depending upon the nature of the mutation in rec A. This may result in the ability of the phage to recombine with the host cell genome.

Favouring one of these latter two explanations (c and d) is the fact that there appears to be an unpublished history of recombinant lambda gt-11 losing restriction sites and inserts (Beck, Wellcome Unit of Molecular Parasitology, University of Glasgow, personal communication) and becoming scrambled, often with fragments of cloned DNA appearing in the vector arms (Carrington, Cambridge University, personal communication).

To investigate these possibilities further, a series of diagnostic restriction enzyme digestions were carried out on CL-12 DNA to reveal whether or not there was any gross rearrangement of restriction enzyme sites in either the left or right vector arms, or merely the central region. The samples were run on 1.2% agarose gels. The results from several gels are summarised in diagrammatic form in fig. (3.7.f). As the diagram shows, digestion with Bam HI, Hind III, Bam HI/Hind III, Bgl II, Pvu II, Kpn I, Sst I revealed no restriction fragments of detectably altered size from wild-type lambda gt-11. However as noted before, double digestion with Kpn I/Sst I showed an altered pattern (see 3.3.3.4). Thus, only the central region appeared to be altered and this was confirmed by the loss of an EcoRI site (see 3.3.3.3.2).

The most likely mechanism which can explain the observed alteration in restriction sites in the central region of CL-12 as well as the loss of sequences which hybridise to the v-yes probe, is shown in schematic form, in figure (3.8): recombination is postulated to occur between CL-12 and the E.coli chromosome due to homology between the insert DNA in CL-12 and a region of the E.coli genome. After recombination during the amplification of CL-12, the phage has lost
its original insert and contains a region of *E.coli* DNA. Although
this was the favoured hypothesis, it was not proven. This could be
done by hybridising $^{32}$P-labelled lambda *CL-12* DNA to restriction
digests of *E.coli* DNA, but it was considered more important to this
project to pursue the *Theileria v-yes* homologue.
POSSIBLE FATE OF YES CLONE IN \(\lambda\)gt11

**Figure 3.8**

**Wild Type**  |  **Yes +ve**  |  **Yes -ve**

<table>
<thead>
<tr>
<th>RESTRICTION PATTERNS (kb)</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>(Kpn/SstI DIGEST)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4.5 S-S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.0 K-S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.2 K-S</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.5 K-K</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
3.3.3.4. Library Screening

It was decided that little could be done with CL-12, and that a fresh attempt at isolating a clone containing sequences homologous to the v-yes probe should be made. Screening the unpurified lambda gt-11 mixtures of clones which were picked as potential positives and pooled during the initial screen (3.3.3.1) was avoided initially, due to the problems experienced (3.3.3.3) with this particular vector. This strategy (c) was only used after screening of other Theileria genomic libraries (a) and (b):-

(a) Lambda EMBL 3

An alternative T. annulata (Hissar) genomic library was available for screening. This library was constructed by Dr F. R. Hall, (Wellcome Unit of Molecular Parasitology, Glasgow) in lambda EMBL3 which accepts inserts of foreign DNA from 9-22kb. (see 2.2.17.2 for EMBL recombinant selection system). Therefore, this library had an advantage over the lambda gt-11 library, in that fewer recombinant bacteriophage needed to be screened in order to cover the whole genome. The library was plated out and screened as described in (2.2.18, 2.2.19), using the v-yes probe.

As before, (3.3.3.1) plaques which hybridised in an area of low non-specific background, were picked, pooled into 10 groups for ease of handling and re-screened. By the third round of screening and purification, the frequency of potentially positively hybridising plaques was no greater than the initial screen. This screening procedure was then abandoned in favour of constructing a mini-library specifically enriched for the T. annulata (Hissar) homologue of the v-yes gene.

(b) pUC 18

Due to the problems encountered with screening a large library with a non-homologous probe, it was decided to increase the proportion of clones carrying an insert of the Theileria yes gene homologue. This was achieved by cloning a 3-4kb fraction of Theileria Hind III-digested genomic DNA (A 3.35kb fragment of Hind III-digested Theileria
DNA hybridised to the v-yes probe - see 3.3.2.2) into the plasmid vector pUC 18. The procedures, and rationale for using pUC 18 as the vector are described fully in (2.2.21), and the selection system for identifying recombinants in (2.2.17.3). The proportion of insert DNA to vector DNA was found to be critical in obtaining recombinant plasmids in a reasonable proportion. The optimum was between 3:1 and 5:1 molar ratio of insert ends:vector ends (see fig. 3.9.a).

The resultant library was screened as described (2.2.21.5) for clones containing a Theileria homologue of the v-yes oncogene. No colonies were identified which hybridised with the v-yes oncogene probe.

(c) Lambda gt-11

Finally, the lambda gt-11 mixtures of clones which were picked and pooled as potential positives during the initial screening (3.3.3.1), but were unpurified, were rescreened using the v-yes probe. This was a final attempt, designed to isolate any yes-homologous clones which had not been picked as one of the clones CL-1–CL-12 (see 3.3.3.1). It was hoped that any clones isolated as having homology to v-yes would be more stable than lambda CL-12.

Screening the lambda gt-11 bacteriophage as before (3.3.3.1), yielded clones which showed a positive hybridisation signal with the v-yes oncogene probe. These were picked, replated and re-screened. Repeating this procedure resulted in the isolation of a pure clone (designated lambda-MD-2) to which the v-yes probe hybridised. The v-yes probe did not hybridise to control plaques of the wild-type phage lambda gt-11.

3.3.3.5. Characterisation of MD-2

3.3.3.5.1. Hybridisation of v-yes to MD-2

DNA was extracted from MD-2 as described in (2.2.20.2), and one sample digested with the restriction enzyme EcoRI to excise the
insert; another sample was digested with \textit{Kpn I}/\textit{Sst I} which flanked the insert; and two other samples digested with \textit{Bam HI} and \textit{Hind III} respectively. The restricted DNA was then run on a 1% agarose gel. The insert was not excised by cutting with \textit{EcoRI}, indicating a possible loss of the \textit{EcoRI} site(s). However, digesting with the enzymes \textit{Kpn I} and \textit{Sst I} which cut sites flanking the insert, revealed insertion of a foreign DNA fragment, 200 base pairs in size. This was visualised as a 200 base pair increase in size of the 2.1kb (wild-type) fragment to 2.3kb in lambda \textit{MD-2} - shown diagrammatically in fig. (3.9.b), first and last lanes. Southern blots of resolved fragments of wild-type lambda \textit{gt-11} digested with the same restriction enzymes (with the exception of \textit{EcoRI}) were then hybridised using $^{32}$P-labelled \textit{v-yes} oncogene probe. The \textit{v-yes} probe hybridised specifically with the 2.3kb \textit{Kpn I}/\textit{Sst I} fragment of \textit{MD-2} (fig. 3.9.b) which contained the 200bp insert of foreign DNA. It did not hybridise to any other fragments of the recombinant bacteriophage, nor did it hybridise to wild-type lambda \textit{gt-11}. Thus the insert of \textit{MD-2} has some degree of homology to the \textit{v-yes} oncogene.

3.3.3.5.2. Hybridisation of \textit{MD-2} to \textit{Theileria} Genomic DNA

In order to determine whether the insert from \textit{MD-2} was derived from the parasite or the host cell genome, $^{32}$P-labelled \textit{MD-2} DNA was used to probe a Southern blot of parasite (\textit{T. annulata} piroplasm) DNA digested with a series of restriction enzymes, namely \textit{Kpn I}/\textit{Sst I}, \textit{Bam HI}, \textit{Hind III} and a blot of bovine DNA digested with \textit{Hind III}. The results are shown in figure (3.9.c). \textit{MD-2} hybridised to specific fragments of parasite (\textit{T.annulata}) DNA, but not to bovine DNA. As well as hybridising to residual undigested \textit{Theileria} piroplasm DNA, \textit{MD-2} hybridised to a 3.4kb \textit{Hind III} fragment, a 4.0kb \textit{BamHI} fragment, and an 8-9kb fragment from the \textit{Kpn I}/\textit{Sst I} digest (see fig. 3.9.c). Since the initial Southern blot with the \textit{v-yes} probe on parasite DNA showed hybridisation to two \textit{Hind III} fragments of 3.35kb (which corresponds in size to the fragment which lambda \textit{MD-2} hybridises to) and 1.30kb, (3.3.2.2) it seems likely that \textit{MD-2} contains sequences from the larger parasite DNA fragment with which \textit{v-yes} originally showed homology (3.3.2.2).
In order to detect and compare expression of v-yes-like sequences in *Theileria*-infected and uninfected lymphoblast cells, northern blots of BL-20 and TaH-BL-20 RNA were probed with the v-yes oncogene probe, radiolabelled with $^{32}$P dCTP. Total, and poly A+ RNA was extracted from BL-20 and TaH-BL-20 as described in (2.2.2), electrophoresed on 1.2% agarose formaldehyde gels, blotted and hybridised at 62°C (2.2.5-2.2.7). The results from hybridisation with the v-yes oncogene probe are shown in figure (3.9.d). A band, at 3.0kb was detected in poly A+RNA from TaH-BL-20 (labelled T-BL), and not in BL-20. The band was most prominent in track 5, where 8.6ug of the TaH-BL-20 (labelled T-BL) poly A+ RNA was loaded, although an equivalent loading of BL-20 poly A+ RNA was not undertaken. However, the band is still, visible in track 4 (2ug TaH-BL-20 poly A+ RNA) and is absent from track 3 (2ug BL-20 poly A+ RNA), implying that a homologous transcript to v-yes was expressed as a result of *T. annulata* infection. No hybridisation was detected in tracks 1 and 2, which were loaded with total RNA from BL-20 and TaH-BL-20. The 3.0kb transcript which appeared to be specific to the *T. annulata*-infected BL-20, could not have arisen as a result of hybridisation to ribosomal RNA, since (a) no hybridisation was detected on total RNA and (b) the sizes of eukaryotic ribosomal RNA are 4.9kb and 2.2kb (Long and Dawid, 1980) and thus of different sizes to the transcript detected.
**Figure 3.9**

(a) Percent recombinant colonies

(b) Diagram of restriction enzyme digests

(c) Diagram of RNA gel electrophoresis

(d) RNA gel electrophoresis gels with different concentrations and types of RNA.
3.3.4. Discussion

_T. annulata_ (Hissar) DNA sequences which were homologous to the _v-yes_ oncogene probe were detected (see 3.3.2.4), while other probes encoding protein kinase products (_v-src_ and _v-abl_) did not show detectable hybridisation to _Theileria_ genomic DNA, thus indicating that the hybridisation was specific. In addition, a 3.0kb, _Theileria_-infection-specific RNA transcript was detected which was homologous to the _v-yes_ oncogene probe.

A cloned parasite DNA fragment (MD-2) was isolated which contained 200bp of insert DNA which, by hybridisation, showed homology to the _v-yes_ oncogene. This insert was shown to be of _T. annulata_ origin, and when lambda MD-2 was radio-labelled and used in a Southern blot analysis with _Theileria_ DNA, it hybridised to a fragment of the same size as that detected by Southern blots using _v-yes_ as a probe. Thus, MD-2 contains _Theileria annulata_ (Hissar) DNA sequences which are homologous to the _v-yes_ oncogene. Whether this cloned insert is part of the gene which gives rise to the infection-specific 3.0kb _yes_-homologous RNA transcript (3.3.3.5.3) remains unproven. However, the degree of homology with the viral transforming gene can only be determined accurately by sequencing MD-2. A functional assay must then be performed in order to assess whether the gene (of which MD-2 is likely to be only a fragment) has transforming activity and a role to play in _T. annulata_-induced host cell proliferation. Proposals for further investigation and possible significance of these findings are elaborated in the final discussion (Chapter 4).
3.4. PHOSPHO-PROTEIN PROFILES

3.4.1. Introduction

It is now well established that post-translational modification of vertebrate cellular proteins can occur by phosphorylation at serine, threonine and tyrosine (Swarup et al., 1983). Many serine- and threonine-specific protein kinases and their substrates have been extensively characterised, and in many instances, protein phosphorylation has been shown to play an important part in the regulation of cell metabolism through alteration of the properties of specific enzymes. For example, the active form of glycogen phosphorylase was found to be a phosphoprotein, and the protein kinase responsible, phosphorylase kinase, has been characterised (Krebs and Fischer, 1956). The regulation of phosphorylase kinase itself by phosphorylation has since been elucidated in detail (Cohen, 1978). In contrast however, tyrosine protein kinases were discovered before their substrates, and there is presently much ongoing research into identification of the protein substrates whose functions are modulated by tyrosine protein kinases. As reviewed in chapter (1.2.8), current models of cellular transformation incorporate abberant phosphorylation, particularly of tyrosine, and also of serine and threonine residues in certain key growth regulatory proteins.

Thus, it was decided to search for phosphoproteins specific to T. annulata (Hissar)-infected leukocytes. Such proteins, particularly if phosphorylated at tyrosine (see 1.2.8.1.2), would be key candidates for involvement in the Theileria-induced transformation process. Identification and characterisation of these infection-specific phosphoproteins would be the first steps towards purifying the proteins for functional assays or cloning the relevant genes. In addition, results from hybridisation to Southern blots of Theileria annulata (Hissar) genomic DNA (see 3.3.2.2) revealed sequences homologous to the v-yes oncogene which itself encodes a tyrosine protein kinase (3.3.2.4). Although there is no evidence as yet for involvement of these v-yes - like sequences in host cell transformation, this was certainly a possibility worthy of consideration in designing the following experimental approach.
The phospho-proteins produced by uninfected bovine leukocyte cells (lines) and the same cells infected with Theileria annulata (Hissar) were compared in profile (by labelling and separating in a gel matrix) to identify differences. The analysis was carried out in two ways. Firstly, inorganic phosphate, labelled with $^{32}$P was incorporated into living cells grown in phosphate-free medium, and the resultant labelled proteins extracted for analysis. (This was termed the \textit{in vivo} incorporation of $^{32}$P). Secondly, detergent extracts of infected and uninfected cells were labelled using $^{32}$P ATP - the equivalent \textit{in vitro} assay. This was carried out in order to see if the results of the \textit{in vivo} phosphoprotein profile were reproducible under various conditions, and to determine which conditions were optimal for detection of specific phosphoproteins. It was also hoped to reveal evidence of additional phosphorylation events not shown in the \textit{in vivo} situation, as a result of phosphorylation taking place in the altered (and controllable) ionic conditions \textit{in vitro}. However, since the relevance of findings in the artificial \textit{in vitro} conditions to the situation in an intact cell (\textit{in vivo}) is highly questionable (see discussion 3.4 for details), it was decided to first identify by molecular weight, phosphoproteins of interest in the \textit{in vivo} situation. These would then be characterised by \textit{in vitro} analysis.

3.4.2. \textbf{In vivo Phosphoprotein Profiles of T.annulata-infected and Uninfected Cells.}

In order to define those proteins which are phosphorylated \textit{in vivo}, specifically in macroschizont-infected cells, both Theileria-infected and uninfected cells were incubated in phosphate free medium containing radio-labelled inorganic phosphate according to the method described in (2.5.4). The period of incubation necessary for sufficient incorporation of $^{32}$P into cellular proteins was determined experimentally by monitoring the incorporation of radioactivity (2.5.5) over a period of 21 hours in all five cell lines which were to be used in this experiment. (For description of the cell lines, and rationale for their use in these experiments, see 1.3.7). The results were plotted in a graph as a time course of incorporation (fig. 3.10.a). Since the incorporation of phosphate was starting to reach a plateau after 8 to 24 hours of incubation in the case of BL-20 and TaH-PRL110 cells, the time chosen for incubation of all cells was
12 hours, even though the rate of incorporation was still steady in the other cell lines after 20 hours.

The levels of phosphate incorporation reached (see fig. 3.10.a) also provided strong evidence of increased phosphorylation activity over this 20 hour period (1.5 to 4 times) in all three infected cell lines TaH-PBL\textsubscript{110} (2 x 10\textsuperscript{7} cpm), TaH-PBL\textsubscript{46} (1.2 x 10\textsuperscript{7} cpm) TaH-BL-20 (9.8 x 10\textsuperscript{6} cpm) as compared to the uninfected BL-20 (5.2 x 10\textsuperscript{6} cpm) and Con A-stimulated PBL\textsubscript{110} (6.8 x 10\textsuperscript{6} cpm). These incorporations were measured on equal cell numbers for each cell line, as described in (2.5.4) and (2.5.5). (Cell lines are described in 2.1.1). Approximately two times as much labelled cellular protein from the uninfected cells had to be loaded on gels in order to have the same number of counts per track as the Theileria-infected cells. This increased specific activity of protein from infected cells implies an overall stimulation of phosphorylation in these cells by a factor of approximately two.

Cells were harvested after the twelve hour incubation, and the activity of incorporated radio-labelled \textsuperscript{32}P was measured by trichloroacetic acid precipitation and liquid scintillation counting (2.5.5). The cells were lysed in SDS sample buffer and equal counts per minute (cpm) per sample loaded onto a 7-17% linear gradient polyacrylamide gel for separation by SDS PAGE as described in (2.4.1). The separated proteins were then analysed by autoradiography (2.5.2).

The result of this analysis is shown in figure (3.10.b), and shows radiolabelled phosphoproteins as discrete bands ranging in molecular weight from <29kDa (kilodaltons) to >200kDa. There was a high background of radio-labelled phosphate in all tracks, most likely being caused by phosphate incorporation into DNA and RNA which smears down the tracks as the gels are run. Around sixteen discrete bands are visible in the Theileria-infected cells, and around twelve in the uninfected cells, although these figures are not absolute due to obscuring of the bands by the background. As a result of this high background, particularly in the high molecular weight area of the gel (>200kDa), many bands are obscured. Particularly prominent in all cell lysates (see fig. 3.10.b) is a phosphoprotein of 36kDa at approximately equal intensity in all tracks. (In the Con A-PBL\textsubscript{110} lysate, however, this band is not visible due to a high background in
Figure 3.10.a

INCORPORATION OF INORGANIC PHOSPHOROUS-32 INTO THEILERIA ANNULATA-INFECTED AND UNINFECTED LEUKOCYTES

TOTAL TCA COUNTS ( x10^6 )
IN 5x10^5 CELLS PER MIN.

Error bars indicate error in TCA counting.
this area of the track, and so it cannot be concluded to be present or absent). Three proteins were revealed which were clearly phosphorylated in all three infected cell lines and were absent (or at least, not phosphorylated) in the uninfected cells. These were, by this criteria, termed infection-specific phosphoproteins (ISPP), as were other phosphoproteins in subsequent assays which were either unique or increased in quantity in all Theileria-infected leukocytes studied. These three proteins, designated ISPP1, ISPP3 and ISPP 4, (see fig. 3.10.b), had molecular weights of 134, 50 and 200 kDa respectively.

In addition to infection-specific phosphoproteins (ISPP), another class of phosphoproteins was detected in this and subsequent assays, which were present in uninfected leukocytes, but absent (or present at reduced levels in Theileria-infected cells. These proteins were designated uninfected-cell-specific phosphoproteins (USPP); and thus appeared to be lost or dephosphorylated upon infection with T.annulata. Fig. (3.10.b) shows one such phosphoprotein, USPP 2, of 85kDa which is not present in the Theileria-infected cell lines.

Although uninfected cell-specific phosphoproteins (USPPs) were obviously leukocyte proteins, the infection-specific phosphoproteins (ISPPs) could be either host or parasite-encoded. Attempts to assign these proteins to either host or parasite, by molecular weight determination of in vivo-labelled phosphoproteins of isolated macroschizonts (isolation procedure as described in 3.6), were unsuccessful, most likely as a result of macroschizont disruption (see 3.6). In order to further characterise the phosphoproteins detected in this experiment, and to detect others which may be phosphorylated under different ionic conditions, an in vitro assay was utilised.
Figure 3.10.b

(In vivo)

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular Weight</th>
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<tbody>
<tr>
<td>ISPP4</td>
<td>200 kDa</td>
</tr>
<tr>
<td>ISPP1</td>
<td>134 kDa</td>
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<tr>
<td>USPP2</td>
<td>85 kDa</td>
</tr>
<tr>
<td>ISPP3</td>
<td>50 kDa</td>
</tr>
<tr>
<td>36 kDa</td>
<td></td>
</tr>
</tbody>
</table>

(kDa)
3.4.3. In vitro Phosphoprotein Profiles of T.annulata-infected and Uninfected Cells.

The main reasons for carrying out in vitro phosphoprotein assays were as follows:-

a) To see if the results obtained with the in vivo assay were reproducible under in vitro conditions. If so, this would aid in characterisation of the phosphoproteins through further in vitro analysis (see 4.3).

b) To reveal infection-specific phosphorylation events which occur in various ionic conditions in vitro, and were not detected in vivo. Although, as discussed fully in (3.4.4), the relevance of these events to the in vivo situation is not clear, they would represent genuine differences between infected and uninfected cells.

c) To determine which co-factors affect the Theileria-infection-specific phosphorylation events, and so to define the optimal conditions for each. Such well-defined assays would be useful in the future purification of these molecules.

In order to characterise the ionic requirements of various phosphorylation events (see 3.4.3.1. for rationale), the in vitro phosphorylation reaction was carried out in the presence of different ions at various concentrations. The cellular location of various phosphoproteins was then investigated by carrying out the phosphorylation reaction on sub-cellular fractions. The following experiments (3.4.3.1 - 3.4.3.3) are analysed firstly as individual experiments and then collectively (3.4.4). It may be helpful to refer throughout to fig. (3.16) and fig. (3.17), which summarise the main points of the results. The data was collected for these figures from all the experiments in (3.4.3), listing each phosphoprotein's various properties from all the experiments. Thus each polypeptide band was designated, using ISPPx (Infection-specific) or USPPx (Uninfected-cell-specific) according to its calculated molecular weight. Polypeptide bands which had identical molecular weights in two separate experiments were therefore given the same designation. However, this was only done if the properties defined for a particular
polypeptide band were consistent between experiments.

In all cases, unless otherwise stated, a crude cell lysate was prepared as described in (2.3.1), and the resultant lysate assayed for protein concentration using the Bradford assay (2.3.4). The labelling of cell extracts with \(^{32}\)-P ATP was carried out according to the protocol (2.5.1), additional ions being added to various concentrations as described in each experiment. Proteins were separated on 7-17% linear gradient SDS-PAGE gels (see 2.4.1), and the gels stained with Coomassie Blue (2.4.2.1) in order to visualise the markers for molecular weight calibration of each gel. Labelled phosphoproteins were then detected by autoradiography (2.5.2).

As described in (2.5.3), after initial exposure revealing all labelled phosphoproteins, gels were alkali-treated to specifically remove phosphate from serine and threonine residues. Subsequent re-exposure therefore showed those proteins which were phosphorylated at tyrosine. Because removal of phosphate from serine and threonine was incomplete, comparison with the original autoradiograph was necessary in order to determine the site of phosphorylation; in this investigation, proteins were defined as containing radio-labelled phosphotyrosine if they were specific to transformed cells, and were alkali-stable. (see 3.4.4. for full discussion on this criteria of selecting phosphotyrosine-containing proteins).

3.4.3.1. Ionic Titrations

The balance between phosphorylation of substrate proteins by protein kinases (and autophosphorylation events) and the dephosphorylation by phosphatases depends on the presence of divalent cations—notably magnesium, manganese and calcium. For example, phosphorylase kinase which phosphorylates glycogen synthetase at a serine residue (thus inactivating it), depends on Ca\(^{++}\) and a Mg-ATP complex for its activity (Stryer, 1981). Conversely, phosphorylase phosphatase which reverses the reaction is inhibited by Ca\(^{++}\) and Mg-ATP. All kinases require divalent cation(s) for their activity, although the preferred ions for a particular kinase may vary according to the substrate. However, all known tyrosine protein kinases work independently of Ca\(^{++}\) (Swarup et. al., 1983).
Thus it was decided to investigate the effects of the three principal cations which are described in the literature (Swarup et. al., 1983) and tables in figures 1.4.a and 1.6.a) as influencing protein kinase activity, namely Mg++, Mn++ and Ca++.

3.4.3.1.1. **Effect of Magnesium**

In this experiment, magnesium ions (as magnesium chloride in concentrations of 25mM, 10mM, 5mM) were added to each lysate (Con A-PBL110, TaH-PBL110, BL-20, TaH-BL-20) before the ATP incorporation was initiated by addition of ATP (2.5.1). To another series of lysates in which the reaction was carried out, no endogenous magnesium was added, and in two further series, EDTA was added to 1mM and 10mM respectively. This was designed to remove divalent cations, including magnesium from the lysate. The concentrations of magnesium added were designed to cover the range necessary for maximal kinase activity (Swarup et. al., 1983). Thus, a total of 24 reactions were carried out (4 cell lysates x 6 ionic conditions) as described (2.5.1) and analysed as in (3.4.3).

The results from this experiment are shown in figure (3.11.a) which shows all phosphorylation events and figure (3.11.b) which shows the gel after alkali treatment, enriched for phospho-tyrosine. The initial exposure of the gel was twelve hours, whereas after alkali-treatment, the gel was exposed for 1 week. The much-reduced intensity of the alkali-treated gel, despite longer exposure to autoradiograph film, indicates that only a small fraction of phosphorylation events are alkali-stable and hence occurring on tyrosine residues. The tracks are labelled: C(=Con A-PBL110); T(=TaH-PBL110); B(=BL-20); TB(=TaH-BL-20). The overall patterns of phosphoproteins were very similar in all four leukocyte lysates in the presence of exogenous Mg++ (see fig. 3.11), with the majority of labelled phosphoproteins in the molecular weight range 40kDa-150kDa. Phosphotyrosine-containing proteins were defined as described (3.4.3), as were ISPPs and USPPs (see 3.4.2).

This analysis firstly demonstrates that the concentration of the divalent cation, Mg++ has a profound effect on the overall level and
pattern of protein phosphorylation. For example, as the added magnesium concentration was decreased from 25mM to 0mM (see fig. 3.11.a, from left to right) the general level of phosphorylation dropped in all four cell lines from around 30 proteins, to predominantly one major infection-specific band, ISPP 1 at 134kDa, and a few very minor (less intense) bands. When endogenous divalent cations were removed by addition of 10mM EDTA, the total profile was essentially obliterated (fig. 3.11.a), demonstrating that almost every phosphorylation event in these experiments was dependant on the presence of some divalent cations. Interestingly, a 40kDa phosphoprotein appeared in all cell lysates as the primary phosphoprotein, on addition of 1mM EDTA. This could be explained by the relevant kinase and phosphatase which were responsible for the phosphorylation of this polypeptide requiring different divalent cations which were present in different endogenous concentrations in the lysate. Thus the phosphatase would require an ion present in small quantities for activity which would be largely removed in 1mM EDTA - hence the phosphorylation. Conversely in 10mM EDTA, the ion normally present in high concentrations which was required for the kinase activation, was also chelated, resulting in the inhibition of phosphorylation. This illustrates how the optimal conditions for phosphorylation may be controlled by the relative concentrations of more than one divalent cation.

ISPP 1. The most prominent infection-specific phosphoprotein ISPP1 (134kDa) was phosphorylated mainly on serine or threonine both in the presence of Mg++ and when no exogenous cations were added. This was seen from removal of this band by alkali-treatment, in conditions where 0-25mM Mg++ was added. However, when EDTA was present, the phosphoprotein remained labelled after alkali treatment, suggesting it to be phosphorylated on tyrosine. Thus, the phosphorylation site(s) of ISPP 1 appear to be altered to more alkali-stable sites (probably tyrosine) by removal of divalent cations. Alternatively, two different proteins of the same molecular weight may be present, one being phosphorylated on serine or threonine in the presence of Mg++ or when no ions were added, and the other on tyrosine in the presence of 1mM EDTA.

Examination of the phosphoprotein profiles in figures (3.11.a) and (3.11.b) revealed several other bands which were specific to
Figure 3.11

(a) 

exposure = 12 hours, except T = 48 hours

(b) Alkali-Treated

Exposure = 1 week
Theileria-infected leukocytes-ISPP's by the criteria outlined in (3.4.2):-

ISPP 2 (15kDa). This protein was phosphorylated at serine/threonine, since it was totally de-phosphorylated by alkali-treatment, and so was not visible in figure (3.11.b). It was only visible in the presence of 10-25mM Mg++, indicating a strong dependency on this cation.

ISPP 4 (200kDa). This phosphoprotein too, was detectable only with addition of 10-25mM Mg++, and was phosphorylated on tyrosine residues, as was shown by the fact that this band was enhanced above the background after alkali-treatment, whereas before alkali treatment it was obscured by other bands which were not stable to alkali-treatment.

ISPP 3 (50kDa). This protein was phosphorylated at tyrosine as seen by the enhancement of this band by alkali-treatment (fig. 3.11.b). It appeared to be present in all cell lines, both infected and uninfected, when Mg++ was added and absent from all cell lines when EDTA was added. However, in the absence of added Mg++ and EDTA, ISPP 3 was infection-specific.

USPP 1 An uninfected cell-specific phosphoprotein as defined in (3.4.2), of 20kDa, was phosphorylated on tyrosine residues as was apparent from the higher intensity of this band after alkali treatment. This phosphorylation event only took place in high concentrations of Mg++.

3.4.3.1.2. Comparative Effects of Magnesium and Manganese

In this experiment, the effect of adding manganese ions to the phosphorylation reactions was investigated and was compared to the effect of magnesium ions, with the aim of assigning the preferred ions to individual phosphorylation events. The phosphorylation assay was carried out under four different conditions: with added magnesium cations (25mM MgCl₂), with added manganese cations (2.5mM MnCl₂), with both magnesium cations (25mM) and manganese cations (2.5mM) added, and with no added ions. The concentrations of divalent cations added were as used previously by Swarup et. al. (1983), the
concentration of \([\text{Mn}^{++}]\) being one tenth of that of \([\text{Mg}^{++}]\). Lysates from the cell lines ConA-PBL\(_{110}\), TaH-PBL\(_{110}\), TaH-PBL\(_{46}\), BL-20 and TaH-BL-20 were used as described previously (3.4.3), and in fig. 3.12 were labelled \(T_{110}\), \(T_{46}\), \(B\), \(TB\) respectively. The results from this experiment are shown in figure (3.12a) which shows the initial 12 hour exposure of the gel and figure (3.12.b) which shows a 48 hours exposure of the gel after alkali treatment.

Firstly, comparing both the 25mM \(\text{Mg}^{++}\) and 'zero addition' conditions between this experiment and the previous one (2.4.3.1.1), similar patterns of phosphoproteins were evident (notably the presence of ISPP 2 and ISPP 4 in 25mM \(\text{Mg}^{++}\) and ISPP 1 with no added ions), indicating phosphorylation events to be consistent between experiments, under the same conditions. In addition, the overall patterns of phosphorylation in the presence of both magnesium and manganese (added separately; see fig. 3.12.a) were similar in that most (eg. the 56/58kDa band common to all 5 cell lysates) although not all of the polypeptide bands were observed under both conditions. Thus, Mn\(^{++}\) can replace Mg\(^{++}\) in most phosphorylation reactions (also see figs. 1.4 and 1.6). However, some events were preferentially phosphorylated in the presence of one or other of these ions. For example, the 56/58kDa bands were more intensely labelled in the presence of 2.5mM Mn\(^{++}\) than in the presence of 25mM Mg\(^{++}\), suggesting a preference of Mn\(^{++}\) as a divalent cation for the phosphorylation of these bands. These preferences for either Mg\(^{++}\) or Mn\(^{++}\) for different phosphorylation events have been previously reported (see figs. 1.4 and 1.6).

**ISPP 1** From figure (3.12.a), the most prominent infection-specific phosphoprotein (as defined in 3.4.2) is at 134kDa and so was termed ISPP1. This band was removed by alkali-treatment, showing it to be phosphorylated on serine/threonine when no exogenous ions were added. Under these conditions of no exogenous cation addition, ISPP1 was virtually the only phosphoprotein present. This phosphoprotein was visible also in the presence of either Mn\(^{++}\) or Mg\(^{++}\), but was no longer specific to Theileria-infected cells under these conditions. ISPP 1 was observed to have these same properties as defined in the previous experiment (3.4.3.1.1).

**ISPP 3** After alkali-treatment and a longer exposure (see fig.
Figure 3.12

(a) Exposure = 12 hours

(b) Alkali-treated Exposure = 48 hours
3.12.b), a 50kDa phosphoprotein (ISPP 3) was detected when no ions were added, as the main phosphoprotein band. It was shown to be infection-specific according to the definition in (3.4.2). This protein was again (as in 3.4.3.1.1) infection-specific only when no exogenous divalent cations were present as was shown by its presence in all cell lysates - from both infected and uninfected cells, when exogenous cations Mn$$^{++}$$ or Mg$$^{++}$$ were added. ISPP 3 was phosphorylated at tyrosine.

USPP 1 The uninfected cell-specific phosphoprotein of molecular weight 20 kDa was detected in both this and the previous experiment (3.4.3.1.1). This protein required the presence of exogenous divalent cations for its phosphorylation. Either magnesium or manganese stimulated this activity, although there was evidence for a slight preference for 25mM Mg$$^{++}$$, as compared to 2.5mM Mn$$^{++}$$ (shown by the slightly higher intensity of the band in the presence of Mg$$^{++}$$; see fig. 3.12.a/b). USPP 1 was alkali-stable, as demonstrated by its enhancement by alkali-treatment and longer exposure in fig. (3.12.b), and so is highly likely to be phosphorylated at tyrosine.

3.4.3.1.3. Titrations with Calcium

As discussed in (3.4.3.1), Ca$$^{++}$$ is one of the three principal divalent cations (along with Mg$$^{++}$$ and Mn$$^{++}$$) which are known to influence protein kinase activity, although tyrosine-specific protein kinases work independently of Ca$$^{++}$$ (see Swarup et al., 1983). The effect of Ca$$^{++}$$ on phosphorylation was therefore investigated in order to see which events were dependant and which were independant of Ca$$^{++}$$. In this experiment, calcium ions (calcium chloride) were added to the cell lysates before the radio-labelling reaction was commenced. The various conditions used were; 100uM Ca$$^{++}$$, 10uM Ca$$^{++}$$, 1uM Ca$$^{++}$$, 0.1uM Ca$$^{++}$$ and 0 Ca$$^{++}$$.

In addition, three other reactions were carried out in 0.1uM, 10uM and 1mM EGTA, which had the effect of removing the endogenous calcium ions from the lysate by chelation. Calcium was added in these lower concentrations (compared with Mg$$^{++}$$ and Mn$$^{++}$$ in previous experiments), since the effect of calcium ions on activation of protein kinases occurs at concentrations of the order of 10^{-7}M (eg. phosphorylase kinase - see Stryer, 1981).
Total cell lysates were assayed as previously described (2.5.1) and the proteins separated on 7-17% gradient polyacrylamide gels (2.4.1). Phosphoproteins were detected by autoradiography (2.5.2). The results from this experiment are shown in figures (3.13.a) and (3.13.b) which show all the phosphoproteins, and only those phosphorylated on alkali-stable residues, respectively.

The presence or absence of Ca\textsuperscript{++} had very little effect on the total phospho-protein profile; around 5 major phosphoproteins are present in Theileria-infected leukocyte lysates at approximately the same intensities at Ca\textsuperscript{++} concentrations ranging from 0 to 100\textmu M (fig. 3.13.a). However, when 10\textmu M EGTA is added, three bands (see fig. 3.13.a: ISPP 0, 105kDa; USPP 2, 85kDa; ISPP 3, 50kDa) decrease in intensity. The whole Ca\textsuperscript{++} profile was identical to that recorded in the previous two experiments (3.4.3.1.1) and (3.4.3.1.2) when no exogenous divalent cations were added to the reaction. However, there were two exceptions: the 85kDa phosphoprotein (USPP 2) which was present in the BL-20 lysate, but not in the TaH-BL-20 lysate in this experiment (fig. 3.13.a) was not detected in the previous two experiments; and ISPP 0 (105kDa) which was specific to infected cells in this experiment and was not previously detected. This may, however, have been due to the fact that all bands in this experiment are more intense than in the autoradiographs from the previous experiments, and so USPP 2 and ISPP 0 may have been detected if the gels had been exposed longer (3.4.3.1.1) and (3.4.3.1.2).

ISPP1, the 134kDa phosphoprotein was by far the most prominent phosphoprotein detected. It was also shown to be phosphorylated (at serine/threonine since it was removed by alkali-treatment - fig. 3.13.b) in uninfected BL-20 cells at levels which were significant, but were very much less than in TaH-BL-20. The extent of phosphorylation on ISPP 1 in the Theileria-infected cells compared with the uninfected cells was illustrated by exposing the gel to X-ray film for one tenth of the original exposure (see fig. 3.13.a). This phosphorylation event was independent of Ca\textsuperscript{++}. The presence of ISPP 1 in uninfected cells, indicates that it is a host cell-encoded protein.

ISPP 3 Similarly, as with previous experiments, the most prominent phosphoprotein band after alkali treatment and 1 week exposure of
Figure 3.13

(a)

Exposure = 15 hours

(b) Alkali-treated

Exposure = 1 week
gels, was at 50kDa and was infection-specific (ISPP 3: see fig. 3.13.b). The phosphorylation event on tyrosine residues was not further stimulated by addition of exogenous calcium but appeared to be inhibited in the presence of 10μM-1mM EGTA. This was unusual, since tyrosine kinases are not known to be dependant on calcium for their activity. However, it may be that either a tyrosine-phosphatase which had been inactivated by calcium, was now activated by EGTA addition, or that in such high concentrations of EGTA, other divalent cations as well as Ca^{++} are chelated and thus unavailable for the stimulation of kinase activity.

In addition, a serine/threonine-containing phosphoprotein which was specific to infected cells was detected at 105kDa (ISPP 0). This phosphoprotein’s dependance on calcium was not clearly defined — it was present when Ca^{++}, 0 ions, 0.1μM EGTA were added to the reaction, but was almost completely absent in the presence of 10μM EGTA.

The uninfected cell-specific phospho-serine/threonine-containing protein of 85kDa (USPP2) was also detected and was completely independant of the presence of Ca^{++}. Since none of these phosphoproteins were dependant on Ca^{++} for their phosphorylation, then the kinases responsible cannot be determined to be serine/threonine or tyrosine-specific by this criterion, as not only are all tyrosine kinases Ca^{++} independant, but so are many serine/threonine kinases (Swarup et. al., 1983).

### 3.4.3.2. Cellular Fractionation

It was decided to determine the cellular locations of phosphoproteins unique to both Theileria infected and uninfected leukocyte lysates which had been observed in previous experiments (3.4.3.1). This information may aid in future purification of these proteins and also may provide clues at to their potential functions.

An initial experiment was carried out, which was intended to locate the phosphoproteins observed in the previous experiments to either the nuclear-enriched fraction ("PELLET", in fig. 3.15) or the fraction which did not contain any nuclei ("SUPERNATANT"). This very
crude fractionation was carried out as described in (2.3.2). These fractions were then assayed for phosphoproteins by the ATP phosphorylation reaction (2.5.1) without the addition of exogenous divalent cations. The labelled extracts were then separated by SDS-PAGE (2.4.1) and analysed by autoradiography (2.5.2). The results are shown in figure (3.15.a; tracks 5-8) which shows all phosphoproteins and also in figure (3.15.b; tracks 9-16) which shows phosphoproteins after alkali-treatment of a concurrently run gel. The tracks were labelled BL (BL-20), TBL (TaH-BL-20), Con A (Con A-PBL110), 110 (TaH-PBL110). The overall appearance of the phosphoprotein profiles was identical to the profile observed in experiment (3.4.3.1.3) when no ions were added (as was also the case in this experiment). All of the major bands visible were located primarily in the non-nuclear fraction (supernatant). These included the previously described ISSP 1 (134kDa), ISPP 0 (105kDa), ISPP 3 (50kDa) and USPP 2 (85kDa), only the latter being phosphorylated on tyrosine (as shown by its persistence after alkali-treatment; fig. 3.15.b). Thus, it can be concluded that none of the above phosphoproteins are nuclear proteins.

It was decided to further fractionate the cells (both infected TaH-BL-20 and uninfected BL-20) in order to determine the cellular location of the phosphoproteins described in the previous experiments. It was particularly designed to see which were located within the plasma membrane. The procedures described in (2.3.3.1), (2.3.3.2) and (2.3.3.3) were used in order to prepare a nuclear fraction, a plasma membrane fraction, a fraction containing cellular organelles and a macroschizont fraction. The method used for the isolation of macroschizonts and infected cell ghosts is described fully in chapter (3.6). Unfortunately, not enough purified plasma membrane was obtained using the method in (2.3.3.1), and so only the crude membrane fraction was used, which contained plasma membrane plus cellular organelles (Standring and Williams, 1978). The purified cellular organelle fraction was run alongside, for comparison.

In an attempt to visualise as many phosphoproteins as possible, these phosphorylation reactions were carried out in the presence of: 25mM [Mg$^{++}$], 2.5mM [Mn$^{++}$] and 10μM [Ca$^{++}$] (which are known to stimulate protein kinase activity; Swarup et. al., 1983 and 3.4.3.1.1. to 3.4.3.1.3) - hence the different appearance of the profiles shown in fig. (3.14.b) and fig. (3.14.c) as compared to
previous experiments when no exogenous cations were added. The proteins of the various fractions were separated on a 10% polyacrylamide SDS-PAGE gel and stained with Coomassie Blue. Figure (3.14.a) shows that each fraction appears to contain its own subset of proteins, indicative of effective fractionation; fig. (3.14.a) shows the total lysate from Theileria-infected leukocytes TaH-BL-20 (T-BL-Tot.), containing a vast number (>100) of polypeptide bands. Crude membranes prepared from the same cells (labelled TBL Crude Memb.) contained around 35 polypeptide bands, while the organelle fraction contained a subset of only 6 or so of these. The nuclear fraction revealed around 50 polypeptide bands, while the macroschizont preparation showed around 12 discrete polypeptide bands.

Figure (3.4.b) shows the phosphoprotein profiles and figure (3.4.c) shows those phosphorylation events which are stable to alkali-treatment (2.5.3). Tracks are labelled BL (BL-20) and TBL (TaH-BL-20). The following infection-specific, and uninfected cell-specific phosphoproteins were detected.

ISPP 1 Unlike previous experiments (3.4.3.1.1 - 3.4.3.2), ISPP1 was not a major component of these phosphoprotein profiles, most likely due to the presence of high concentrations of the divalent cations Mg\(^{++}\) and Mn\(^{++}\) which were previously shown to stimulate many other phosphorylation events, thus obscuring ISPP 1 (compare with fig. 3.12). It was, however, located only in the crude membrane preparation and the infected cell ghost fraction.

ISPP3 (50kDa) Detected in previous experiments (3.4.3.1.1) to (3.4.3.1.3), ISPP 3 was not detected here, probably for the same reason given for ISPP 1 above.

ISPP 0 (105kDa; previously detected in 3.4.3.1.3). This polypeptide, phosphorylated mainly at serine/threonine (see 3.4.3.1.3), but not being completely dephosphorylated here by alkali treatment (fig. 3.14.c), was expressed in much higher levels in the infected cell crude membrane preparation than in the uninfected cell preparation. However, its presence in uninfected cells indicated it to be a host-encoded protein. This protein was also visible in the fraction containing TaH-BL-20 cell ghosts (see 3.6)
ISPP 5 (72kDa). This infection specific polypeptide (not recorded in previous experiments) was phosphorylated at tyrosine residues as shown by its alkali stability, and was located in the crude cell membrane preparation. It too was also visible in the TaH-BL-20 cell ghosts.

ISPP 6 (>200kDa). This high molecular weight infection-specific phosphoprotein appeared to be phosphorylated at tyrosine residues by its alkali stability and had not been recorded in previous experiments. It was located both in the crude membrane fraction, and also in the fraction containing cellular organelles.

ISPP 7 (53kDa) was another infection-specific phosphoprotein located mainly in the organelle fraction, and also showing faintly in the crude membrane preparation. It had not been recorded in previous experiments, and appeared to be phosphorylated at tyrosine by its enhancement compared to other bands after alkali-treatment (see fig. 3.14.c).

The following uninfected cell-specific phosphoproteins (as defined in 3.4.2) were detected in this experiment, all of which appeared to be phosphorylated at tyrosine and had not been recorded in the experiments in (3.4.3.1):

USPP 3 (55kDa). This prominent phosphoprotein was shown to be located in the crude membrane preparation and was almost entirely absent from the organelle fraction.

USPP 4 (50kDa). This phosphoprotein was most prominent in the nuclear preparation.

USPP 5 (35kDa). This protein, also phosphorylated at tyrosine residue(s), was located in the crude membrane preparation.

USPP 6 (46kDa). This phosphotyrosine-containing protein was unique to the membrane preparation.

The main phosphoproteins detected in the macroschizont preparation had molecular weights of 52, 55, 58, 60 and 81kDa and did not correspond to any of the infection-specific phosphoproteins.
detected in this assay by comparison of BL-20 and TaH-BL-20 lysates. (The 52kDa protein did have a similar molecular weight to ISPP 7 - 53kDa, but ISPP 7 was detected at low levels in uninfected BL-20 cell lysate and so could not be a macroschizont protein). This experiment therefore revealed several phosphoproteins, not detected in previous experiments (3.4.3.1) which were specific to Theileria- infected cell lysates, and also several which were unique to uninfected cells. The cellular locations of these phosphoproteins were defined and are summarised in fig. (3.17). Previously detected phosphoproteins ISPP 0 and ISPP 1 both appeared to be membrane-bound proteins.

3.4.3.3. Autophosphorylation

Many protein kinases themselves are known to be the substrates of phosphorylation (including all tyrosine kinases - see 1.2.8.1) and so some phosphoproteins detected may in fact be protein kinases. This experiment was designed to identify phosphoproteins which had the ability to autophosphorylate, thus having kinase activity and thereby distinguishing the kinase enzymes from their substrates. This experiment was made possible by the fact that the rate of substrate phosphorylation by a kinase which was carried out at 24°C in previous experiments, is reduced at 0°C (Dixon and Webb, 1964). The rate of autophosphorylation of a kinase, at 0°C, should not be reduced to the same extent since the energy necessary for the formation of a [substrate-enzyme] complex in the former situation, is not required for autophosphorylation. This experiment, therefore should detect proteins which are good candidates for being protein kinases since their level of phosphorylation at 0°C will not be reduced to the same extent as the substrate proteins. Thus, duplicate samples underwent the phosphorylation reaction at 24°C for comparison. No exogenous divalent cations were added at 0°C or 24°C. Total cell lysates were assayed as previously described (2.5.1) and the proteins separated on 7-17% gradient polyacrylamide gels (2.4.1). Phosphoproteins were detected by autoradiography (2.5.2). The results are shown in figure (3.15a, tracks 1-4) which shows all labelled phosphoproteins and in figure (3.15b, tracks 1-8) which shows alkali-stable phosphoproteins. Lysates are labelled BL (BL-20), TBL (TaH-BL-20), Con A (Con A-FHL110), 110 (TaH-FHL110).

The phosphoprotein profile, at 24°C (fig. 3.15) was identical to
those previously obtained in the experiments in (3.4.3.1.3) and (3.4.3.2.a).

ISPP 1 The most prominent phosphoprotein was the previously observed (3.4.3.1) 134kDa polypeptide (ISPP 1) which was greatly increased in intensity in the Theileria-infected cells (fig. 3.15.a; tracks 5-8). This protein was phosphorylated only very slightly at 0°C, indicating that it most likely cannot autophosphorylate. The residual activity is probably a result of the (non-auto) phosphorylation reaction still occurring, although at a reduced rate at 0°C.

ISPP 0 (105kDa), detected previously in section 3.4.3.1.3) was phosphorylated at serine/threonine as shown by its alkali lability (and hence absence from fig. 3.15.b) and did not undergo any detectable autophosphorylation, in that it was not phosphorylated at 0°C (fig. 3.15.a).

ISSP 3 The 50kDa infection-specific phosphotyrosine-containing protein, ISPP 3 (as previously described in 3.4.3.1 - 3.4.3.3.), was again the most prominent labelled band in the alkali-treated gel. It appeared to be equally well phosphorylated at 0°C as it was at 24°C, suggesting that the protein was indeed autophosphorylating at tyrosine and was therefore probably a protein kinase rather than only a substrate.

USPP 2 The previously detected (3.4.3.1.3) uninfected cell-specific phosphoprotein USPP 2 (85kDa) which appeared to be phosphorylated on serine/threonine (as shown by its absence from the alkali-treated gel; fig. 3.15.b), appeared to retain around 50% of its phosphorylated residues when incubated at 0°C. (This figure of 50% was estimated by visual comparison of tracks 1 and 3; fig. 3.15.a). Thus, the protein was considered to be capable of autophosphorylation on serine/threonine residues and is therefore probably a protein kinase.

Thus, as summarised in fig. (3.17), one phosphoprotein which is reproducibly detectable as being specific to Theileria-infected cells (ISPP 3) and one phosphoprotein which is specific to uninfected cells (USPP 2) were shown to have protein kinase activity by this assay.
Figure 3.15

(a) 

<table>
<thead>
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<th>24°C</th>
<th>0°C</th>
<th>SUPERNAT</th>
<th>PELLET</th>
</tr>
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<tbody>
<tr>
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<td>+0 ions</td>
<td>+0 ions</td>
<td>+0 ions</td>
</tr>
</tbody>
</table>

(kDa) BL TBL BL TBL BL TBL BL TBL

134 kD  
105 kD  
97.5 kD  
66 kD  
45 kD  
29 kD  

Exposure = 15 hours

(b) Alkali-treated

<table>
<thead>
<tr>
<th>24°C</th>
<th>0°C</th>
<th>Pellet</th>
<th>Supernatant</th>
</tr>
</thead>
<tbody>
<tr>
<td>+0 ions</td>
<td>+0 ions</td>
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</tbody>
</table>

(kDa) ConA 110 BL TBL ConA 110 BL TBL

Exposure = 1 week
3.4.4. Discussion

The Balance of Protein Kinase/Phosphatase Action  The action of phosphatases is now known to be regulated by hormone action in some cases, and may be modulated by the presence of Ca\textsuperscript{2+}, calmodulin, and cyclic AMP (for review, see Foulkes, 1983). Their action, like the protein kinases has been shown to be specific, in that tyrosine-, serine- and threonine-specific phosphatases are regulated by different ionic conditions, and have different sensitivities to inhibition by Zn\textsuperscript{2+} or vanadate (Gallis et al., 1981). Thus, in all systems which are regulated by phosphorylation (such as transformation), the outcome is the result of a dynamic equilibrium between phosphorylation and dephosphorylation. In fact, all viral tyrosine kinases themselves are phosphorylated on serine residues (Hunter and Sefton, 1981) and although the function of phosphoserine in these proteins remains to be determined, such a modification introduces the possibility that phosphoseryl protein phosphatases (and kinases) could regulate the level of phosphotyrosine in cells by modulating the activity of the tyrosine-protein kinases. Thus, if the availability of the substrate proteins is the same before and after Theileria infection, many of the changes in phosphorylation induced by Theileria infection may be a result of altered phosphatase activity as well as altered kinase activity.

The Detection of Phosphotyrosine  Detection of phosphotyrosine was carried out by alkali-treatment of the gels as described in section (2.5.3). This method relied upon the fact that phosphate-ester linkages are more stable to tyrosine than to serine or to threonine at high pH and raised temperature (Cooper et al., 1983). This relative stability of the phosphotyrosine bond has previously been used to detect phosphotyrosine-containing proteins in one- and two-dimensional polyacrylamide gels and in general assays for the phosphorylation of the tyrosine residues in proteins (Wang and Baltimore, 1983). However, not all phospho-tyrosyl residues are resistant to alkali-treatment due to the local environment in the protein, and as a result some are not detected. In addition, not all phosphothreonine residues are removed by alkali-treatment, and so alkali-treatment of a gel reveals not only phosphotyrosine-containing proteins, but also residual phosphothreonine. However, it was possible to determine the proteins with the most stable phosphate-
ester linkages (and hence those likely to contain phosphotyrosine) by comparing the strength of signal from the labelled proteins before and after alkali-treatment. Those proteins which retain most of their labelled phosphate, are most likely to contain phosphotyrosine. In addition, proteins whose phosphate content increases under conditions where total cellular phosphotyrosine levels are increased, are likely to contain phosphotyrosine (Cooper et. al., 1983). In the assays reported here, uninfected BL-20 cells contained approximately 0.03% alkali-stable phosphate groups, which is elevated to approximately 0.09% in *T. annulata*-infected BL-20. These figures were estimates obtained from comparison of the intensities of BL-20 and TaH-BL-20 tracks of *in vitro* radio-labelled phosphoproteins, before and after alkali-treatment from fig. (3.13). This is suggestive of tyrosine-protein kinase activation upon infection of these cells by *Theileria*. Thus, according to Cooper et. al., (1983), proteins whose phosphate content increases upon transformation (and hence also *T. annulata* infection), are likely to contain phosphotyrosine. Alkali-treatment of gels was used to facilitate this assay, and so alkali-stable phosphoproteins which were found exclusively, or at increased levels in *Theileria-annulata* transformed cells were considered highly likely to contain phosphotyrosyl residues. Using these criteria for detecting phosphotyrosine-containing proteins, Cooper and Hunter (1981) picked eight proteins from two-dimensional gels of which six were later proven to contain phosphotyrosyl residues. In the assays described in this thesis, eight phosphoproteins in all were found to be specific to *T. annulata*-infected cells/cell lysates - five of which contained phosphotyrosine by the above criteria.

The Relevance of the *In vitro* Assays For reasons fully discussed in section (3.5.4) with respect to protein kinase assays, many differences are likely to be seen between *in vivo* phosphoprotein profiles and *in vitro* phosphoprotein profiles. Firstly, the ionic conditions in which the *in vitro* assay takes place do not accurately reflect the conditions in *vivo*, and, since ionic concentrations have such a dramatic effect on phosphorylation (see results of 3.4.3.1.1. - 3.4.3.1.3) the phosphoprotein profiles may be drastically altered. Secondly, substrate specificity of the protein kinases may be altered as a result of loss of compartmentalisation and association of molecules by cell disruption and detergent action in the *in vitro* assay. So, as discussed in (3.4.1), more emphasis may be put on the results obtained in the *in vivo* assay as being relevant to leukocyte...
transformation by *T. annulata*. These phosphoproteins (such as ISPP 1, 3, 4 and USPP 2), subsequently identified in the *in vitro* assays (on the criterion of molecular weight) were thus characterised, whereas although interesting, the alterations in *Theileria*-infected cell lysates seen only in the *in vitro* situation were of questionable relevance to the real situation.

Substrate or Enzyme? The results of the experiment in section (3.4.3.3) provided evidence for two of the proteins which were seen to be altered in phosphorylation on infection by *T. annulata* (ISSP 3 and USPP 2) being protein kinases themselves. The evidence, however should be followed up by a more direct assay for detection of the protein kinase enzymes (see 3.5).

Parasite or Host? No phosphoproteins detected as being altered in *Theileria*-infected lysates were positively assigned to being macroschizont proteins; none of the molecular weights of the ISPPs and USPPs listed in fig. (3.16) and (3.17) correspond to the molecular weights of macroschizont phosphoproteins listed in (3.4.3.2.b). However, several were assigned to be host (bovine leukocyte) proteins, since phosphoproteins of the same molecular weight were detected (at much altered levels) in uninfected cell lysates. These included ISPP 0, 1, 3, 5 and USPP 2, 3, 4, 5, 6 and (see fig. 3.17).

Infection-specific and Uninfected Cell-Specific Phosphoproteins (see fig. 3.17). As already outlined in (3.4.1) and (3.4.2), the purpose of this part of the project was to define alterations in phosphoproteins caused by *T. annulata* infection and to characterise these alterations. As a result, many phosphoproteins detected in these assays have not been discussed since they were present in similar quantities in *Theileria*-infected and in uninfected cells/lysates. Four of the phosphoproteins altered in phosphorylation by *Theileria* infection, namely ISPP 0, 1, 3 and USPP 2 are discussed in detail by gathering information from all experiments in this section. These four phosphoproteins were chosen on the basis of being among the main substrates *in vitro*; being observed consistently throughout a number of independant experiments; and (apart from ISPP 0) being observed in the *in vivo* experiment (3.4.2), thus suggesting that they may have a role to play in *Theileria annulata*-induced transformation.

Figures (3.16) and (3.17), summarise the data on the various infection-specific and uninfected cell-specific phosphoproteins which
were detected both in the in vivo and in vitro experiments. The data was collected for each protein over all experiments as described fully in (3.4.3), and so proteins of the same molecular weight and phosphorylation properties were given the same designation, provided there was no contradictory information (i.e. the phosphoprotein band designated ISPP x, should display the properties expected, such as phosphorylation site and ionic dependancies as deduced for that protein from previous experiments).

As seen from these figures, eight infection-specific phosphoproteins, and seven uninfected cell-specific phosphoproteins were identified. The presence of uninfected cell-specific phosphoproteins (see figs. 3.16 and 3.17) was particularly interesting. They could be a result of: loss of specific protein kinase activity; expression of specific phosphatases on infection by T. annulata (Hissar); or unavailability of the substrate, possibly through not being expressed or by competition from a new Theileria infection-specific substrate. The most interesting uninfected cell-specific phosphoprotein (by the above criteria) revealed by this study was USPP 2 since, being detected in the in vivo assay it was most likely to be relevant to Theileria infection. It was also observed in the in vitro situation and was apparently phosphorylated at serine/threonine residues. It also appeared to autophosphorylate at 0°C, suggesting that it had protein kinase activity. Thus, USPP 2 may tentatively be a protein kinase, which is dephosphorylated at serine/threonine residues, on infection with T. annulata (Hissar). This may be through the action of a serine/threonine phosphatase.

The most interesting infection-specific phosphoproteins (by the criteria outlined above) were:-

a) ISPP 1. This was the primary substrate for serine/threonine kinase activity in the in vitro assays, when no exogenous cations were added, but appeared to be the major tyrosine kinase substrate in the presence of EDTA. This observation can be interpreted as either the specificity of one kinase changing with the ionic environment, or that the activities of more than one kinase, at least one serine/threonine-specific, and another tyrosine, were changing relative to each other (if, indeed the band labelled ISPP 1 consists of one polypeptide). From carrying out the assay at 0°C, it does not appear likely that
ISPP 1 has autophosphorylation activity and so is unlikely to be a kinase. This infection-specific phosphoprotein was observed in the \textit{in vivo} situation at the same molecular weight, and is not a parasite protein but a host protein stimulated in phosphorylation/expression by \textit{Theileria} infection. ISPP 1 appeared to be associated with the plasma membrane, as shown by cell fractionation experiments (3.4.3.2).

b) ISPP 0. The phosphorylation of ISPP 0 at serine/threonine (see 3.4.3.1.3) in \textit{Theileria}-infected cells was independent of the presence of either Mg\(^{++}\), Ca\(^{++}\) or Mn\(^{++}\). There was no suggestion of this phosphoprotein having any ability to autophosphorylate. ISPP 0 was not detected in the \textit{in vivo} experiment, raising the question of relevance of infection-specific phosphorylations observed \textit{in vitro}. Although differences \textit{in vitro} do reflect real changes which have occurred as a result of \textit{T. annulata} infection, nothing can be gleaned about the nature of the changes \textit{in vivo}, since the ionic conditions, and compartmentalisation are so different, both of which have an obvious effect on patterns of phosphorylation and dephosphorylation (see also 3.5.4 and above).

c) ISPP 3. This phosphotyrosine-containing protein (see 3.4.3.1.1) was only infection specific \textit{in vitro} if no exogenous divalent cations, or EDTA were added. However, under these conditions, it was the major substrate of tyrosine kinase(s) in \textit{T. annulata} (Hissar) - infected cells. An infection-specific phosphoprotein of the same molecular weight was observed in the \textit{in vivo} experiment, suggesting this phosphorylation event may be of some relevance to \textit{Theileria}-infection in the \textit{in vivo} situation. ISPP 3 was phosphorylated at 0\(^{\circ}\)C, suggesting it had autophosphorylation potential at tyrosine residue(s) and so may be a tyrosine kinase. In accordance with being phosphorylated at tyrosine, this event was not inhibited by removal of calcium ions by (0.1\text{uM EGTA}). However, at higher concentrations of EGTA -10\text{uM} and 1\text{mM}, the amount of phosphorylation of ISPP 3 was reduced. It was not clear whether this meant that the event was in fact dependant upon very small concentrations of endogenous calcium, or if addition of EGTA at these relatively high concentrations had another effect apart from removal of Ca\(^{++}\), such as removal of another cation.
**Figure 3.16**

<table>
<thead>
<tr>
<th>INFECTION-SPECIFIC PHOSPHOPROTEINS</th>
<th>PHOSPHOPROTEINS UNIQUE TO LEUKOCYTES NOT INFECTED WITH <em>T. ANNULATA.</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>ISPP 6 ———&gt;200</td>
<td></td>
</tr>
<tr>
<td>ISPP 4 —— 200</td>
<td></td>
</tr>
<tr>
<td>ISPP 1 —— 134</td>
<td></td>
</tr>
<tr>
<td>ISPP 0 —— 105</td>
<td>USRPP0—— 112</td>
</tr>
<tr>
<td>ISPP 5 —— 72</td>
<td>USRPP2—— 85</td>
</tr>
<tr>
<td>ISPP 7 —— 53</td>
<td>USRPP3—— 55</td>
</tr>
<tr>
<td>ISPP 3 —— 50</td>
<td>USRPP4—— 50</td>
</tr>
<tr>
<td>ISPP 2 —— 15</td>
<td>USRPP6—— 46</td>
</tr>
<tr>
<td>ISPP 1 —— 20</td>
<td>USRPP5—— 35</td>
</tr>
<tr>
<td></td>
<td>USRPP 1—— 20</td>
</tr>
</tbody>
</table>

**MOL.WT. (kDa.)**

- 200
- 150
- 100
- 50
- 0
**PROPERTIES OF PHOSPHOPROTEINS**

<table>
<thead>
<tr>
<th>PROTEIN</th>
<th>MOL WT (KD)</th>
<th>PHOSPHORYLATION SITE</th>
<th>CATION-DEPENDENCIES</th>
<th>IN VIVO</th>
<th>AUTO-PHOSPHORYLATION</th>
<th>CELLULAR LOCATION</th>
<th>FREQUENCY OBSERVED</th>
<th>HOST PROTEIN</th>
</tr>
</thead>
<tbody>
<tr>
<td>ISPP 6</td>
<td>&gt;200</td>
<td>Tyr</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>Crude Membrane</td>
<td>1</td>
<td>?</td>
</tr>
<tr>
<td>ISPP 4</td>
<td>200</td>
<td>Tyr</td>
<td>Dependant Inhibited</td>
<td>?</td>
<td>√</td>
<td>?</td>
<td>2</td>
<td>?</td>
</tr>
<tr>
<td>ISPP 1</td>
<td>134</td>
<td>Ser, Thr</td>
<td>It add Mg, Mn or O ions</td>
<td>Independent</td>
<td>√ + –</td>
<td>Crude Membrane</td>
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<td>√</td>
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<tr>
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<td>?</td>
<td>Independent</td>
<td>– NO</td>
<td>Crude Membrane</td>
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<td>√</td>
</tr>
<tr>
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<td>Tyr</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>Crude Membrane</td>
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<td>√</td>
</tr>
<tr>
<td>ISPP 7</td>
<td>53</td>
<td>Tyr</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>Organelles</td>
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<td>Mg, Mn – Minor, All Cells</td>
<td>Dependant?</td>
<td>√ + + +</td>
<td>Sup &gt; Pellet</td>
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<td>√</td>
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<td>?</td>
<td>-</td>
<td>?</td>
<td>2</td>
<td>?</td>
</tr>
<tr>
<td>USPP 0</td>
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<td>Ser, Thr</td>
<td>Mg, Mn – Dependant</td>
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<td>?</td>
<td>?</td>
<td>1</td>
<td>?</td>
</tr>
<tr>
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<td>?</td>
<td>Independent</td>
<td>√ + –</td>
<td>Sup &gt; Pellet</td>
<td>3</td>
<td>√</td>
</tr>
<tr>
<td>USPP 3</td>
<td>55</td>
<td>Tyr</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>Crude Membrane</td>
<td>1</td>
<td>√</td>
</tr>
<tr>
<td>USPP 4</td>
<td>50</td>
<td>Tyr</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>Nucleus + Memb.</td>
<td>1</td>
<td>√</td>
</tr>
<tr>
<td>USPP 6</td>
<td>46</td>
<td>Tyr</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>Crude Membrane</td>
<td>1</td>
<td>√</td>
</tr>
<tr>
<td>USPP 5</td>
<td>35</td>
<td>Tyr</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>Crude Membrane</td>
<td>1</td>
<td>√</td>
</tr>
<tr>
<td>USPP 1</td>
<td>20</td>
<td>Tyr</td>
<td>Mg, Mn – Dependant</td>
<td>?</td>
<td>-</td>
<td>?</td>
<td>2</td>
<td>?</td>
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</tbody>
</table>
3.5. **PROTEIN KINASE ASSAYS**

3.5.1. **Introduction**

The results obtained from the phosphoprotein assays (Chapter 3.4) revealed several infection-specific phosphoproteins, while the results from the in vivo $^{32}$p labelling of cells demonstrated a gross increase in phosphorylation activity in *T. annulata* (Hissar) - infected cells (see fig. 3.4.2). From these lines of evidence, it was clear that on *Theileria*-infection, there were changes in protein kinase activity. Furthermore, carrying out the phosphorylation assays at $0^\circ$C (3.4.3.3), revealed two phosphoproteins (ISPP 3 and USPP 2) which appeared to autophosphorylate, thus suggesting that these proteins have protein kinase activity.

It was decided to further investigate the activities of protein kinases in *Theileria*-infected cells (the enzymes responsible, along with phosphatases, for the infection-specific differences seen in the phosphoprotein profiles). A method was chosen which allowed simultaneous direct comparison of activities of several protein kinases present in cell lysates. This was achieved by separation of cell lysate proteins by non-denaturing gel electrophoresis followed by an in situ assay for kinase activity on the resolved proteins within the gel matrix. Initially, the method of Grove and Mastro, (1987) (see 2.5.6.1) was employed for assaying the protein kinase activities of proteins separated on native polyacrylamide gels (PAGE) (2.4.3). However, because the resolution of different protein kinases was poor, an attempt was made to improve upon this by the use of agarose gel iso-electric focusing (2.4.5).

3.5.2. **In Situ Gel Assay of Protein Kinase Activity of Cellular Proteins Resolved by PAGE.**

Crude cell extracts from TaH-PHL$_{110}$, TaH-PHL$_{46}$, HL20 and TaH-HL-20, were prepared for the assay as described in (2.5.6.1). Proteins were separated on 4% polyacrylamide gels under non-denaturing conditions (2.4.3) and the in situ kinase assay using histone as a substrate carried out as described in (2.5.6.1). Tracks are labelled
TaH 46 (TaH-PBL 46), TaH 110 (TaH-PBL 110), BL-20 and T-EL (TaH-EL-20). The results (fig. 3.18.a) showed that kinase activity was detected by this method, but that the resolution of individual bands of activity was very poor. However, analysis of the kinase activity patterns suggest that there may be differences between the uninfected cell line BL20 and Theileria-infected cells (TaH-EL-20, TaH-PBL 46, TaH-PBL 110). The BL20 cells show only one area of kinase activity, (Band 1), while the Theileria-infected cell extracts all appear to show an additional band (Band 2) above this main unresolved region of kinase activity. The TaH-EL-20 cell extract also exhibited a third region of kinase activity below Band 1. Molecular weight estimation of the Band 2 activity, using the method described in (2.4.4) showed this band to have a molecular weight of 180kDa. (see Fig. 2.3).

However, no definite conclusions could be made from these assays due to lack of resolution. This was not entirely unexpected, since the published results of Grove and Mastro, (1987) also exhibited poor resolution.

3.5.3. In Situ Gel Assay of Protein Kinase Activity of Cellular Proteins Resolved by Agarose Gel I.E.F.

Cell extracts were prepared and 50ug total protein per sample loaded and separated by agarose gel iso-electro-focusing (I.E.F), as described in (2.4.5). The in situ kinase gel assay was then carried out essentially using the method of Grove and Mastro, (1987) but with several modifications (see 2.5.6.2). After completion of the assay, the gels were dried down onto the gel bond support at 37°C for 1 hour, and then exposed to X-ray film (2.5.2). The results, with extracts of BL-20, TaH-EL-20, PBL 110 and TaH-PBL 110, are labelled BL-20, T-EL, PBL and TaH 110 respectively in figure (3.18.b). Reduced exposure of the gel to autoradiography allowed identification of the top, more intensely-labelled region of the gel, which resolved into distinct bands.

In total, 10 bands of protein kinase activity were identified (in Theileria-infected leukocyte extracts), ranging in pI from 4.8 to 7.8 (see fig. 3.18.b). Two of these protein kinases, ISPK 1 (pI = 6.1) and ISPK 2 (pI = 6.55) were much more active in phosphorylating
histone in the *Theileria*-infected cells TaH-BL20 and TaH-PBL110 than in the uninfected cells BL-20 and PBL110 (as shown by the increased intensities of these bands in infected cells in fig. 3.18.b). This may be due either to increased expression, or to activation of the kinases, on infection. A further two kinases ISPK 3 (pI = 7.2) and ISPK 4 (pI = 7.8) were detected; these appeared to be specific to the *T.annulata*-infected cell line and may therefore be of parasite origin. In addition, a protein kinase (USPK 1; see fig. 3.18.b) was identified which appeared to be more active in phosphorylating histone in the uninfected cell lines than those which were *Theileria*-infected. This activity may be in part responsible for the phosphoproteins which were detected at elevated levels in uninfected cells (see 3.4), although phosphatase activity may also be contributing to these observations (see 3.4.4).

Although the kinases present in the PBL110 cells and the BL-20 cells appeared to be the same species, the proliferating cells of the lymphosarcoma cell line revealed a higher intensity of all bands per ug of protein loaded than extracts from fresh bovine peripheral blood leukocytes. This may be explained in two ways:-

1) As a result of proliferation in the lymphosarcoma cell line BL-20, all the protein kinases visualised were either increased in expression, or were more active than in PBL110.

2) Protein kinase activity may be normally higher in lymphocytes (BL 20 is a lymphocyte cell line) than in other white cells. Since the preparations of PBL contain all leukocytes including granular cells and monocytes, it is possible that only a subset of these cells may express protein kinase at high levels. Thus, the total kinase activity of PBL extracts would be effectively reduced, and a higher intensity of all bands of kinase activity may be expected in BL-20 than in PBL110. The best comparison between *Theileria*-infected and uninfected lysates in this experiment is therefore between cell lines BL-20 and TaH-BL-20.

3.5.3.2. **Quantitative Analysis of Protein Kinase Activity**

A quantitative comparison of kinase activity between *Theileria*-
infected and uninfected cell lines TaH-BL-20 and BL-20 was undertaken by measuring the density of each band in autoradiograph tracks from the kinase assay shown in figure (3.18.b), tracks 1 and 2. This was undertaken by use of a laser densitometer, as described in 2.5.7. The results from a scan of BL-20 and TaH-BL-20 tracks, are displayed in figure (3.19). Since the area under each peak (given as units², or u²) of this plot gives a measure of the relative activity of each protein kinase, the densitometer output (tabulated in fig. 3.19.b) revealed that the total activity of the topmost of six bands (peaks 1-6 on the graphs in fig. 3.19.a) was comparable in both cell lines (289 760u² for BL-20 and 248 634u² for TaH-BL-20). This similarity in kinase activity between BL-20 and TaH-BL-20 was also reflected in the profiles of the graphs of kinase activity of peaks 1-6 (fig. 3.19.a). However, bands 7 (ISPK 1) and 8 (ISPK 2) are much more prominent in the Theileria-infected cell line, with the relative activity of band 7 comprising 16% of the total kinase activity in TaH-BL-20 compared with 1.2% of the total kinase activity in BL-20. Band 8, similarly contained 15% of the kinase activity of TaH-BL-20 and only 0.8% in BL-20. Bands 9 and 10 which were unique to Theileria-infected cells, comprised 1% and 0.4% respectively of the total kinase activity of TaH-BL-20.

3.5.3.3. Analysis of Infection-Specific Protein Kinases

Some of the properties of the two infection-specific protein kinases (ISPK 3 and ISPK 4) and the two kinases which were increased in activity in Theileria-infected cells (ISPK 1 and ISPK 2) were further investigated.

3.5.3.3.1. Autophosphorylation and Molecular Weight Estimation

The bands of agarose each containing ISPK 1, ISPK 2, ISPK 3 or ISPK 4 from TaH-BL-20, were cut out from iso-electric focusing gels and the protein eluted from the agarose by boiling for 20 minutes in 5% SDS, 2.5M urea. SDS sample buffer (2.4.1) was added to a concentration of 1x, and the eluted samples run on a 10% SDS-PAGE gel. The gel was then analysed by autoradiography. The result is shown in figure (3.18.c).
The vast majority of the phosphorylation was shown to co-migrate with the substrate histone (H-2A), which ran near the bromophenol blue dye front. However, a further band of $^{32}$P labelled protein was also detected in the tracks where ISPK 1 and ISPK 2 were loaded. Both eluted bands showed low levels of phosphorylation and had molecular weights of 47-50kDa (see fig. 3.18.c). This demonstrated that these two kinases had some autophosphorylation activity since no other substrate was available apart from histone (14kDa). ISPK 3 and ISPK 4 were not detected on these gels, most likely since less radio-labelled protein (approx. one fifth of the activity of ISPK 1 and 2) was available for loading onto the gel and so was not detectable. Alternatively, ISPK 3 and 4 do not autophosphorylate. It was not possible to determine which was the correct explanation without increasing the gel loading.

3.5.3.3.2. Phosphorylation Sites

In order to determine whether ISPK 1-4 phosphorylated the substrate protein histone on serine/threonine, or on tyrosine residues, or both, the SDS-PAGE gel, after initial exposure (2 weeks), was alkali-treated as described in (2.5.3) to remove phosphate groups by hydrolysis, preferentially from serine/threonine residues. The gel was subsequently analysed by autoradiography (Fig. 3.18.d). Unfortunately, since the previous exposure had been for 2 weeks in order to detect the autophosphorylation of the kinases ISPK 1 and 2 by autoradiography, it was not possible to detect whether autophosphorylation was occurring on serine/threonine or tyrosine residues. This was because the $^{32}$P had already decayed to such a low level that no length of exposure would be able to detect these faint bands, if present. However, the amount of label due to histone substrate was much greater than that due to autophosphorylating kinases, and so the labelled histone substrate should still be detectable after the initial two week exposure. Fig. (3.18.d) therefore shows that since most of the label has been removed by alkali-treatment from the histone substrate of all four infection-specific protein kinases (ISPK 1 to 4), the phosphorylation of histone primarily occurred at an alkali labile sites-serine or threonine in all four cases.
Figure 3.18

(a) BL-20 T-BL TaH46 TaH110

(b) Bl-20 TBL PBL TaH110

exposure (hours) 6 6 24 6

(c) ISPK-

Exposure = 2 weeks

(d) ISPK-

Exposure = 4 weeks

Alkali-treated

- Band 2 180 kD
- Band 1

pKi 4.85 5.65 5.92 6.45 7.3 8.3 10.6

- USPK 1

- Histone 2A (14 kD)

- H-2A
Figure 3.19

Densitometer scan of autoradiograph (see figure 3.18b, tracks BL-20 and TaH-BL20, from X to Y)

<table>
<thead>
<tr>
<th>PROTEIN KINASE</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>TOTAL UNITS</th>
</tr>
</thead>
<tbody>
<tr>
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<td>1.7</td>
<td>0.4</td>
<td>24</td>
<td>37</td>
<td>26</td>
<td>0.9</td>
<td>1.2</td>
<td>0.8</td>
<td>308</td>
<td>228</td>
</tr>
<tr>
<td>T-BL</td>
<td>1.0</td>
<td>1.2</td>
<td>14</td>
<td>26.4</td>
<td>22.7</td>
<td>2.3</td>
<td>16</td>
<td>15</td>
<td>1</td>
<td>0.4</td>
<td>376</td>
</tr>
</tbody>
</table>

DISTANCE UNITS

PROTEIN KINASE 1 2 3 4 5 6 7 8 9 10 TOTAL UNITS

% TOTAL CELL KINASE ACTIVITY

BL 20 | 1.7 | 0.4 | 24 | 37 | 26 | 0.9 | 1.2 | 0.8 | 308 | 228 |
T-BL | 1.0 | 1.2 | 14 | 26.4 | 22.7 | 2.3 | 16 | 15 | 1 | 0.4 | 376 | 791 |
3.5.4. DISCUSSION

As a result of this improved technique for identifying protein kinase activity on proteins separated on a gel matrix, it was possible to identify two protein kinases (ISPK 1 and ISPK 2) which were increased in activity in leukocytes as a result of *T. annulata* (Hissar) infection, and two which were specific to infected cells (ISPK 3 and ISPK 4). All four phosphorylated the substrate histone primarily at serine/threonine residues. ISPK 1 and 2 were shown to autophosphorylate, but the sites of phosphorylation were not determined, while the autophosphorylation abilities of ISPK 3 and 4 were not determined. ISPK 1 and ISPK 2 both had very similar molecular weights of 47-50kD, but differed in pI being 7.2 and 7.8 respectively.

The results from both the *in vitro* and *in vivo* phosphoprotein profiles in section (3.4) suggested that such differences in protein kinase activity between Theileria-infected and uninfected cells exist. The results presented here, confirm this, but the relevance of the protein kinases detected in this assay to the *in vivo* situation is indeterminable at present. (A full discussion is given in (4.2.3) of the relevance of these findings to the mechanism of transformation of leukocytes by *T. annulata*, comparing these results of protein kinases with those of section (3.4) of their substrates).

The ionic conditions in which the protein kinase assay was carried out, were unlikely to accurately reflect the ionic conditions *in vivo*. As was seen from the phosphoprotein profiles in section (3.4), small changes in availability of certain divalent cations (Mg$^{++}$ and Mn$^{++}$) can have large effects on the activity of protein kinases and phosphatases. In addition, the activity of the kinases detected in this assay on the histone substrate may not be the same as would occur *in vivo* with the natural substrates of the kinases. This specificity of protein kinases for particular substrates has already been documented to differ between *in vivo* and *in vitro*, cell-free reactions: in practice, tyrosine protein kinases can phosphorylate many proteins *in vitro* which do not appear to be substrates *in vivo*. There is no evidence, for example, that histones are phosphorylated at tyrosine under any condition *in vivo*, yet they are good substrates for the EGF receptor-associated tyrosine protein kinase *in vitro* (Cohen
et. al., 1980). Authentic in vivo substrates may also be phosphorylated artifactually in vitro. For example, the EGF receptor and AMuLV (Avian Murine Leukaemia Virus) P120 gag-abl appear to be phosphorylated at only one or two tyrosine residues in vivo, but can autophosphorylate at many tyrosines when incubated in vitro with ATP (Hunter and Cooper, 1981; Hunter and Cooper, 1983; Witte et. al., 1980; Sefton et. al., 1981).

Although these discrepancies between in vivo and in vitro observations could be due to the insensitivity of the in vivo measurements, or differential phosphatase activities under the different conditions, it seems more likely that the substrate specificity (but not tyrosine specificity) of tyrosine protein kinases is lost when they are extracted from a cell. Fidelity may normally be ensured by combined effects of compartmentalisation and by association of other molecules with kinase or substrate.

Thus, the activity of protein kinases assayed here in cell-free extracts using histone as a substrate, although reflecting genuine differences between Theileria-infected and uninfected leukocytes, have an unknown physiological relevance to the in vivo situation. Purification of the infection-specific protein kinases or cloning of the genes encoding them would be necessary before an appropriate study could be carried out to determine their role, if any, in Theileria-induced leukocyte transformation. (see Chapter 4.3).

In an attempt to localise ISPK 1-4 within the cell and to see if ISPK 3 or 4 were possibly parasite protein kinases, the in situ gel assay of protein kinase activity was carried out on both purified plasma membranes and on preparations of macroschizonts. Membranes were prepared as described in (2.3.3.1) and macroschizonts as in Chapter (3.6). Unfortunately, kinase activity was not detected in these extracts, but this was most likely to have been due to loss of enzymatic activity during the processing of this material.
3.6. ISOLATION OF MACROSCIZONTS FROM INFECTED LEUKOCYTES

3.6.1. INTRODUCTION

3.6.1.1. The Need for Purified Macroschizonts

In attempting to elucidate the mechanism of transformation of white blood cells by *T. annulata*, one problem persists throughout a number of approaches taken by the researcher when examining *Theileria*-infected cells; that of being unable to distinguish molecular events induced by the parasite but taking place in the host cell, from those which are taking place in the macroschizont itself. For example, comparison of phospho-protein profiles (3.4), kinase activities (3.5) and oncogene expression (3.2) between *T. annulata*-infected and uninfected cells, all revealed alterations which were specific to the infected cells. None of these events could be localised to either the host cell or the parasite, without prior isolation of the parasite from the host cell on which to carry out the relevant assay. Isolation of the intracellular macroschizont would be an important feature of most biochemical characterisations of *T. annulata* and in particular, construction of a cDNA library from macroschizont mRNA, would greatly aid in any study of *Theileria* gene expression.

3.6.1.2. Existing Methods

Two methods already existed for the isolation of intracellular macroschizonts from host leukocytes; that of Tait, Wellcome Unit of Molecular Parasitology, Glasgow, (personal communication) designed for *T. annulata*, and that of Sugimoto et. al., (in press) designed for use with *T. parva*.

The method of Tait involved selective lysis of a concentrated suspension of host cells in 2% Tween 40 detergent in Tris-saline, by stirring at 0°C. The resultant lysate was homogenised, layered onto a prespun continuous Percoll gradient (40% isotonic Percoll in tris-saline centrifuged first at 20000g in an MSE Ultra Centrifuge for 15 minutes) and then at 800g (MSE Chillspin) for 15 minutes in order to
resolve the lysate into 3 distinct bands. The top band consisted largely of macroschizonts (as detected by Giemsa stained smears or cytospins). However, these macroschizont preparations were contaminated with host cell nuclei, and because of the relatively large size DNA and RNA content of the host nuclei, the macroschizont preparations were of limited use.

The method of Sugimoto, on the other hand, made use of the cytolytic toxins, aerolysin and Ah-1 hemolysin, produced by *Aeromonas hydrophila*, to induce lysis of the host cells of *T. parva* macroschizonts without lysing the macroschizonts themselves. Macroschizonts were then separated from the lysate by making the mixture up to 65% Percoll (isotonic), overlaying with 45% Percoll and subjecting this step gradient to centrifugation. The separated macroschizonts retained their normal morphology and were essentially free from host cell components, as revealed by light and electron microscopic examination. The macroschizonts also retained their antigenicity as determined by their reactivity with anti-macroschizont monoclonal antibodies. However, there were two disadvantages of this method; firstly, the recovery of macroschizonts was estimated to be only 18% at best, and secondly, the bacterial toxins aerolysin and hemolysin were not commercially available. Culturing of *A. hydrophila* (strains Ah-65 and Ah-1, respectively) would therefore be necessary in order to purify the toxins and the microbiological facilities for culturing such toxic bacteria were not available in our laboratory.

A third method has in fact been used, involving the use of a Stansted cell disrupter, selectively lysing host cell nuclei and then using ion-exchange chromatography with DE52 cellulose to remove chromatin (Nyormoi, et. al., 1981). However, the effectiveness of the method was not well characterised, and also requires a cell disrupter which was not available.

Thus it was decided to investigate the possibilities for an alternative method of purifying macroschizonts from *T. annulata*-infected cells.
3.6.2. MACROSCHIZONT SEPARATION: VARIOUS METHODS ATTEMPTED

3.6.2.1. Use of Magnetic Beads With Anti-macroschizont Monoclonal Antibodies

Several monoclonal antibodies were available, which recognised antigens of the *T. annulata* macroschizont within the host leukocyte (Shiels et al., 1986). An attempt was made to separate macroschizonts from *Theileria*-infected cells lysed according to the method of Tait, (see 3.6.1.2) by reacting monoclonal antibodies with magnetic beads attached to macroschizont surface antigens. The beads, together with the parasites would then be separated from the lysate by means of a magnet.

3.6.2.1.1. Optimisation of Cell Lysis

In order to disrupt host cells with as a little damage as possible to macroschizonts, lysis was monitored over a period of time, using different concentrations of the detergent Tween 40, which Tait had found to be the most suitable for minimising macroschizont damage. 8 x 10⁷ infected cells (TAH-BL-20) were harvested and washed twice in PBS as described in (2.1.6), for every lysis condition. Cells were then resuspended to a total volume of 0.5 ml in 10% Ficoll-400, 1 x Tris-saline. (Tris-saline = 25mM Tris.H-Cl pH=7.4, 150mM NaCl, 10mM Na azide, 1mM MgSO₄, 2mM CaCl₂). Addition of up to 5% Ficoll 400 in the lysis solution was reported by Sugimoto et al., (in press) to increase the yield of macroschizonts and decrease the contamination by host nuclear debris. The favourable effect of Ficoll 400 for macroschizont preparation is not understood, but its protective effect on subcellular organelles (Rickwood, 1984) may also apply to macroschizonts. 0.5ml of different concentrations of Tween 40 in tris-saline was then added to give the required final concentration of Tween 40. The cells were immediately placed in a 10ml beaker with a magnetic stirrer and stirred on ice. Lysis was monitored every 5 minutes by pipetting out 20-50ul onto a slide and examining under an inverted microscope in order to estimate the percentage of cells which had lost their outer membrane without being broken up. Cells which appeared to lose their sharp outline were considered suitable for
isolating intact macroschizonts. Giemsa stains (2.1.4) of smeared samples were also taken for examination. Fig. (3.20.a) shows a TaH-BL-20 cell which was considered to be at the correct stage of lysis for harvesting (i.e. for terminating lysis and separating macroschizonts). Lysis was monitored over a period of 80 minutes, using Tween 40 at final concentrations of 0.1%, 0.5%, 1%, 2% and 5%. The results, showing the percentage cells at a suitable state of lysis for harvesting at each time point, are displayed in figure (3.20.b). The percentages were calculated by examination of 100 cells under an inverted microscope, and counting the number which appear to be at a suitable stage of lysis as outlined above.

The use of Tween 40 at 2% was the most effective, giving 92% of the cells in a suitable state for harvesting after 45 minutes of lysis (see fig. 3.20.a). The time for the most efficient lysis, however, was found to vary inexplicably and so lysis had to be monitored on each occasion. Factors involved in this variation may have included cell size, speed of stirring, exact cell concentration, temperature etc.

3.6.2.1.2. Identification of a Suitable Monoclonal Antibody

Eight monoclonal antibodies were available, which identified antigens in, or on the intracellular macroschizont of T. annulata (Shiels et al., 1986). These were first checked for intensity of reaction by carrying out IFA (Indirect Fluorescent Assay) on fixed T. annulata-infected leukocytes, as described in (2.6.1). The monoclonal antibodies which showed the most intense reaction with the macroschizont had been previously designated 4A4, 1E11 and 2G2. A typical fluorescence pattern is shown in figure (3.20.d). However, it was then necessary to identify a monoclonal antibody which recognised an antigen located specifically on the surface of the macroschizont, for use in isolation of the parasite. Infected cells were lysed as described in (3.6.2.1.1), and surface IFA was carried out on this lysate with the three monoclonal antibodies 4A4, 1E11 and 2G2, as outlined in (2.6.2). Chromatin was stained with DAPI, as described in (2.6), thus allowing the fluorescing multinucleate macroschizonts to be identified using a suitable filter in the fluorescence microscope. Once macroschizonts were identified in a
field, the filter to allow observation of fluorescine was moved into place in order to determine if the monoclonal antibody (attached to FITC) in question recognised the macroschizont surface. Figure (3.20.e) shows a macroschizont fluorescing firstly with DAPI and then with FITC using monoclonal antibody 2G2.

Monoclonal antibody 2G2 seemed to react strongly with approximately 80% of the macroschizonts; 1E11 recognised 50%, relatively weakly; and 4A4 recognised less than 10% of macroschizonts. Thus 2G2 was chosen as the best candidate for use in isolation of macroschizonts. It also appeared that the macroschizont surface may have been damaged, or antigens altered in some way, during the Tween 40 lysis, since only a percentage of parasites reacted with the monoclonal antibodies.

3.6.2.1.3. Separation of Macroschizonts from Lysate Using Dynabeads (M-450)

Two methods were examined in order to effect isolation of macroschizonts from a lysate of TaH-BL-20 cells both involving the use of magnetic beads, (Dynabeads M-450, from Bio-Services). They are magnetic beads coated with sheep anti-mouse antibody, which had previously been used in applications involving separation of subsets of cells from mixed populations (eg. Butturini et.al., 1986).

The first method involved incubating the mouse monoclonal antibody 2G2 with a lysate of TaH-BL-20 cells, prepared as in (3.6.2.1.1) and diluted 1:3 with tris-saline to prevent further detergent action. EDTA was added to 5mM to prevent aggregation of particulate material. The incubation was carried out for 1 hour in order to allow binding of the antibody to macroschizonts. The mixture was then diluted further, to 5ml with Tris-saline, 5mM EDTA in order to be sure of preventing detergent action. Dynabeads coated with sheep anti-mouse 2nd antibody were then added in a ratio of 2 beads: 1 parasite. The suspension was rocked gently for 2 hours before collecting the Dynabeads with a magnet. Smears of the resultant pellet were then giemsa-stained (2.1.4) for inspection.

The second method involved pre-attachment of monoclonal antibody
2G2 to Dynabeads (according to the supplier's instructions), and then incubating these beads with an infected cell lysate, as before. This incubation was carried out for various periods of time (30 minutes-12 hours).

In both methods, Dynabeads attached in a non-specific manner both to nuclei and nuclear fragments as well as to macroschizonts. Many macroschizonts were still apparently intact, yet only 30% of these had any Dynabeads attached. It seemed that the specificity of the antigen-antibody reaction was lost, possibly due to presence, or effect of the detergent, Tween 40. The antigenicity of the macroschizont surface may well have been altered by changes in protein conformation or detergent action. This method was not investigated further.

3.6.2.2. Effect of Cytochalasins on Theileria-infected Cells.

The cytochalasins - a group of mould metabolites - have been shown to inhibit movement and cytoplasmic cleavage in cultured mammalian cells, and at higher doses to cause nuclear extrusion within a few minutes, often leading to total enucleation (Carter, 1967). It was decided to investigate the latter property of cytochalasins A and B (donated by Dr G. G. Selman, Dept. of Animal Genetics, University of Edinburgh), on Theileria annulata-infected leukocytes. This was done with a view to isolating macroschizonts, which would be facilitated by either the loss of leukocyte nuclei, or the specific extrusion of macroschizonts.

Cytochalasins A and B were added to cultures of BL-20, TaH-BL-20 and TaH-PBL110 cell lines in microtitre wells, at final concentrations varying from 1ug/ml to 100ug/ml. The cells were incubated as described in (2.12) and 50ul samples were then taken at time 0, 5 minutes, 10 minutes, 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 8 hours, 20 hours, for preparation of giemsa-stained smears as described in (2.1.4). Both cytochalasin A and B caused nuclear extrusion in T. annulata (Hissar)-infected cells, as well as in BL-20, at all concentrations above 10ug/ml and macroschizonts were not extruded. This was observed in more than 90% of cells only after eight hours incubation, by which time most cells had become multi-
**Figure 3.20**

(a) *Tween 40 Lysis (x 2000)*

(b) *Tween 40 Lysis*

(c) *Effect of Cytochalasin (x 500)*

(d) *Fixed cell IFA: 2G2 Mab*

(e)(i) *DAPI*

(e)(ii) *Schizont surface IFA 2G2*
nucleate as a result of inhibition of cytoplasmic cleavage, (see Carter, 1967). Often, one or more nuclei were extruded, but there were always one or more nuclei remaining in each cell, accompanying the macroschizonts (see Fig.3.20.c). Thus cytochalasins, under these conditions, were of no use the isolation of macroschizonts from T.annulata-infected leukocytes.

3.6.2.3. **Tween 40 Lysis/Percoll Step Gradient Separation**

Because the Tween 40 lysis method, described in detail in (3.6.2.1.1), appeared to be an efficient way of releasing apparently intact macroschizonts from host cells, it was decided to attempt to improve on the subsequent separation of macroschizonts. Tait, as previously described (3.6.1.2), had used a continuous Percoll gradient to bring about partial purification of macroschizonts. Sugimoto et. al., (in press) however, (although using a different lysis method - see 3.6.1.2) achieved a more efficient separation of the parasites from host cell material by flotation, up through a Percoll step gradient.

Thus cell lysis was carried out exactly as described in (3.6.2.1.1) with TaH-BL-20 cells in a volume of 1ml. When more than 90% of the cells had lost their sharp outline without complete disruption, lysis was stopped by addition of 1ml of tris-saline pH = 7.4, 5mM EDTA and 5.2ml of 90% isotonic Percoll (9 parts Percoll:1 part 1.5M NaCl) was then added to the cells to give 7.2ml of 65% Percoll. This was divided evenly between two 10ml polycarbonate centrifuge tubes and overlayed with 3.5-4ml of 45% isotonic Percoll and centrifuged at 25 000g for 30 minutes using a swingout rotor. The results from a typical preparation are illustrated in figure (3.21). Five distinct bands were observed and each was collected in the minimum possible volume, diluted with tris-saline, pH = 7.4 and centrifuged for 5 minutes in a microfuge. Pellets were then resuspended in 50-200ul tris-saline. Samples were spotted onto slides, or dried and giemsa-stained as described in (2.1.4).

The giemsa-stained slides revealed that each of the five bands separated during the Percoll centrifugation, contained distinct components (see Fig.3.21). The top band contained macroschizonts
which appeared essentially free of host cell nuclei (less than 1 leukocyte nucleus per 1000 macroschizonts) - fig. (3.21). The second band, also shown in figure (3.21), consisted largely of host cell nuclei and a few intact cells. The third band contained a mixture of very broken macroschizonts, host nuclei, and other non-chromatin components. The fourth band did not contain chromatin and could not be identified, while the lower band contained what appeared to be leukocyte ghosts - disrupted cells which contain no apparent nuclei or cytoplasm (see Fig.3.21).

This separation was consistent, and so the purity and recovery of macroschizonts isolated using this method was investigated, and the state of damage to the macroschizonts assessed.
Figure 3.21

1. Hazy band schizonts
2. Host cell nuclei
3. Hazy band broken schizonts + nuclei
4. Unidentified, non-chromatin
5. Host cell ghosts

(x 1000)

(x 1500)

(x 1500)

(x 500)
3.6.3. **Assessment of Macroschizont Preparation**

3.6.3.1. **Yield**

The percentage recovery of macroschizonts prepared using the method described in (3.6.2.3) was estimated by assuming every host cell contained an average of one macroschizont. The number of macroschizonts recovered was then measured by taking a small aliquot of the final suspension and (a) counting the macroschizonts in a haemocytometer, and (b) dropping them onto a microscope slide, air drying and giemsa-staining to facilitate counting. Positive identification was more difficult with unstained macroschizonts, but giemsa-staining had the disadvantage of loss of some material from the slide during processing. Both methods were used in parallel, and an average of the two was taken to give an estimate of percentage recovery for each preparation. The percentage recovery varied from 7% to 22%.

3.6.3.2. **Purity**

3.6.3.2.1 **Giemsa-stains**

Preliminary results from examination of giemsa-stained macroschizont preparations (see fig.3.21), showed contamination by host cell nuclei to be very low in most preparations, although some variation from one preparation to the next was encountered. As little as one host cell nucleus per 1000 macroschizonts was frequently observed, but in some preparations, 20-30 host nuclei per 1000 macroschizonts was recorded.

3.6.3.2.2. **Analysis of Protein Content**

**Total Protein Profile** Macroschizont preparations were lysed in SDS sample buffer and proteins separated on a 7%-17% gradient polyacrylamide SDS-PAGE gel (2.4.1) and compared with total lysates...
Figure 3.22

(a) 

(b)
from BL-20 and TaH-BL-20 cells. Proteins were stained with Coomassie Blue (2.4.2.1), as shown in (fig.3.22.a). The polypeptide profile of the macroschizont preparation was completely different to that of the host cell lysate. None of the major bovine leukocyte bands were detected on the macroschizont preparation. High molecular weight proteins were not observed in the macroschizonts, possibly indicating some degradation.

**In Vitro Phosphoprotein Profile** A phosphoprotein profile of a macroschizont preparation was carried out as described in (3.4.3.2). The results, displayed in figure (3.22.b) show that the macroschizont phosphoprotein profile is quite different to that of the host cells, carried out under the same conditions, at the same time. Thus, the macroschizont preparation appears to be largely free (by these criteria) of contaminating host cell proteins.

### 3.6.3.2.3. Analysis of DNA

DNA was extracted from macroschizonts using the method described in (2.2.10). Macroschizont DNA, piroplasm DNA and bovine DNA were digested with the restriction endonuclease EcoRI, yielding a prominent satellite band at 1.4kb with bovine DNA, when fragments were separated by agarose gel electrophoresis (see 3.3.2.1). Figure (3.23.a) shows such a gel, stained with ethidium bromide, as described in (2.2.13). The 1.4kb bovine satellite DNA was clearly visible in the calf thymus DNA track, but was absent from both the piroplasm and macroschizont tracks.

In order to increase the sensitivity of detection of contaminating bovine sequences, the 1.4kb EcoRI bovine repetitive DNA fragment was excised from an agarose gel, purified as described in (2.2.21.1), labelled with $^{32}$P dCTP (2.2.15) and used as a probe in hybridisation to a Southern blot of EcoRI-digested *Theileria* piroplasm (2ug), *Theilera* macroschizont (2ug) and bovine (0.1ug) genomic DNA. Less bovine DNA than *Theileria* DNA was used so that the probe would not hybridise excessively to bovine DNA, thus reducing the
concentration of probe available for hybridisation to any contaminating DNA in the parasite preparations. The result of this experiment is shown in figure (3.23.c). Strong hybridisation occurred between the bovine repetitive DNA probe and calf thymus DNA, particularly at the 1.4kb region. This band was not detected in either the *Theileria* piroplasm or the *Theileria* macroschizont DNA, indicating contamination with bovine DNA sequences to be minimal.

The converse experiment was also carried out, in order to prove that genomic DNA extracted from *Theileria* macroschizonts was not degraded and that a *Theileria* probe would hybridise to discrete fragments of restriction-endonuclease digested macroschizont DNA. *Theileria* -piroplasm (0.2ug), *Theileria* -macroschizont (0.2ug) and calf thymus DNA (5ug) were digested with restriction enzyme EcoRI, run on an agarose gel, Southern blotted and hybridised to the probe, as described in (2.2.15) to (2.2.16). The probe used here was a $^{32p}$ dCTP-labelled lambda gt11 recombinant bacteriophage (RH-14) containing insert DNA known to be of *Theileria* (piroplasm) origin. The probe was donated by Dr R. Hall, Wellcome Unit of Molecular Parasitology, University of Glasgow. The results from the hybridisation experiment are shown in figure (3.23.b). As expected, the probe did not hybridise to bovine DNA, but did hybridise with both *Theileria* piroplasm and *Theileria* macroschizont DNA. Two bands which hybridised to the probe were visible with piroplasm DNA at 7.0 kb and 2.0 kb, the smaller band being considerably fainter. Hybridisation of the probe with the 7 kb macroschizont DNA band was weaker and the smaller, 2.0 kb band present in the piroplasm DNA was not detected. This may have been due to uneven transfer of the DNA fragments onto the filter.

Thus, hybridisation of a discrete fragment of macroschizont DNA to a cloned fragment of *Theileria* DNA showed the macroschizont DNA to be intact, while lack of hybridisation of macroschizont DNA to a bovine DNA probe revealed it to be relatively free from contaminating host cell DNA.
Figure 3.23

(a) TaH Piroplasm EcoRI TaH Schizont EcoRI Bovine EcoRI

(b) Probe = Piroplasm cloned DNA RH-14

- 7.0 kb
- 2.0 kb

(c) Piroplasm EcoRI Bovine EcoRI Schizont EcoRI

- 1.4 kb

Probe = 1.4 kb bovine EcoRI-EcoRI fragment
3.6.3.4. Integrity of Macroschizonts

In order to assess the integrity of the purified macroschizonts, they were first examined by giemsa staining. Of particular interest was the condition of the macroschizont membrane which, from reactivity with monoclonal antibodies (see 3.6.2.1.2), appeared to have lost some of its antigenic properties in a proportion of the macroschizonts. Photographs were taken of giemsa-stained macroschizonts (shown at a magnification of x 1500 in fig.3.21) showing apparently intact macroschizonts. In order to assess their integrity more critically, electron microscopy was carried out (as described in detail in Chapter 2.5), to visualise the macroschizont membrane.

Figure (3.24.a) shows the appearance of the macroschizont within the host cell (TaH-BL-20). Characteristic 'gaps' were seen in the parasites, and in addition, the macroschizont nuclei and the outer membrane were also visible. However, after detergent isolation of macroschizonts, their morphology as revealed by electron microscopy, was very much altered in most cases. Fig. (3.24.b) shows a macroschizont which appears to be identical to those seen in sectioned infected cells; the outer membrane is still intact and the characteristic gaps apparent. Most other macroschizonts (>95% by counting) however, appear to be disrupted (see fig.3.24.c). They are less electron-dense and have lost the characteristic gaps.
Figure 3.24

(a) TaH-BL-20 (x7500)

(b) Schizont (x15,000)

(c) Disrupted Schizont (x7500)
3.6.4. Discussion

The results described here, show that the Tween-40 lysis/Percoll step gradient method of isolating macroschizonts is capable of isolating macroschizonts essentially free from contaminating host cell material - as assessed by polypeptide profiles and analysis of restriction digests of DNA. However, macroschizont recovery appeared to be dependant upon harvesting the cells at a suitable point of lysis: if the host cell nuclei were disrupted, the released chromatin trapped macroschizonts in a sticky mass. The yield was low (7% to 22%), but was comparable to other previously published methods, such as that of Sugimoto et.al., (in press) who reported optimal recovery of macroschizonts to be about 18%.

It was important that the cell disruption method used caused minimal damage to the parasite, if various biological studies were to be carried out on macroschizonts. The Tween-40 detergent method appeared to lyse many macroschizonts, and although structural features such as nuclei were visible in all macroschizonts, the parasite membrane was nearly always disrupted and much of the ultra-structural detail (as defined by electron microscopy) had been lost.

The method was still useful in some respects: antigenic reactivity was only partially lost (as revealed by monoclonal antibody studies - see (3.6.2.1.2)), macroschizont proteins were not degraded (fig.3.22) and genomic DNA was intact (fig.3.23). In addition, band 5 (see fig.3.21) which was recovered from the Percoll density centrifugation containing host cell ghosts, may be a useful source of cell membranes although this would have to be analysed in more detail before being used as a standard method.

Many other methods of cell lysis which have been used in the past in an attempt to isolate macroschizonts from host cells, have resulted in damaged macroschizonts. Methods may also have to be modified between T.parva and T.annulata in order to obtain optimum lysis conditions for each. These differences, reflected in lysis properties, were noted by Sugimoto et.al.,(in press), who recommended different concentrations of Ficoll 400 for parasite
protection during lysis. Sugimoto et al. (in press) also reports that osmotic shock and the use of anti-lymphocyte serum plus complement were both unsatisfactory in releasing *T. parva* macroschizonts, undamaged from the host cells. However, contrary to the latter report, Preston et al., (1986) published findings which indicated that lysis of *T. annulata*-infected lymphoblast cell lines Tah-PBL46 and Tah-BL-20 in presence of diluted rabbit serum (as a source of complement) and monoclonal antibody 4H5 (which recognises antigen expressed on the surface of infected cells) released macroschizonts which appeared to be intact. However, this was only assessed by examination of giemsa-stained cytocentrifuge smears and electron microscopy would be necessary to investigate the integrity of the macroschizonts further. Complement lysis, therefore may be worth considering as a possible method for attempting to isolate macroschizonts, from *T. annulata*-infected lymphoblasts. However, it should be noted that the Tween 40 lysis method also appeared to release macroschizonts in relatively good condition and it was only closer examination with the electron microscope which revealed structural damage.
CHAPTER 4

DISCUSSION
4.1. Summary of Results

The experiments described in this thesis demonstrated a variety of features unique to bovine leukocytes which had been infected with *Theileria annulata*:

1. *T. annulata* infection of a transformed bovine leukocyte cell line (BL-20) resulted in a decrease in doubling time of approximately 25% (see 3.1).

2. *T. annulata*-infected BL-20 cells were capable of proliferation from a lower seeding density than their uninfected counterparts (see 3.1).

3. *T. annulata*-infected leukocytes (both TaH-BL20 and TaH-PBL 110) tended to form aggregates in liquid culture, whereas uninfected BL-20 did not.

4. Cell proliferation, as indicated by comparison of peripheral blood leukocytes (PBL 110) with Con-A treated PBL 110 and BL-20, resulted in increased expression of several genes which were homologous to known oncogenes - (Ha-ras, Ki-ras, v-abl).

5. *T. annulata*-infected leukocytes (TaH-BL-20, TaH-PBL46 or TaH-PBL110) showed a further increase in expression of the genes homologous to the three oncogenes listed above (4). In addition an increased expression of genes homologous to the Hu B-lym, v-mos, v-fos and v-myc oncogene probes was observed, suggesting that the increased expression of these genes was a direct result of *Theileria* infection. Expression of the homologues of v-erb B were not detected, while expression of the v-sis homologue although detected, appeared unaffected by *T. annulata* infection.
(6) *T. annulata* DNA sequences were detected which exhibited specific hybridisation to the v-yes oncogene probe on Southern blot analysis.

(7) A clone was isolated from a genomic library of *T. annulata* DNA sequences which hybridised to the v-yes oncogene probe. The cloned DNA appeared to be part of the 3.35-3.4kb*Hind III* fragment of *T. annulata* DNA to which the v-yes probe originally hybridised (in Southern blot analysis).

(8) A gene homologous to the v-yes oncogene was expressed as a 3.0kb transcript as a direct result of *T. annulata* infection. It was not determined if the transcript was of host or parasite origin.

(9) Analysis of phosphoprotein profiles in infected and uninfected cell extracts showed that eight polypeptides were phosphorylated and seven dephosphorylated as a direct result of *Theileria* infection. Four phosphoproteins of identical molecular weight to some of these were also detected in an *in vivo* assay. Several of these proteins were alkali-stable and so were likely to contain phosphotyrosine (see fig. 3.17 for details). These phosphoproteins were characterised in terms of their ionic requirements, cellular location, and ability to autophosphorylate, (see section 3.4 and figure 3.17). Particularly prominent were the infection-specific phosphoproteins designated ISPP1 and ISPP3 which were major substrates for protein kinases under most ionic conditions (see section 3.4.3.1). All of the prominent phosphoproteins which were specific to either *T. annulata* infected, or uninfected cells, appeared to be host-cell-encoded since they were visible in altered levels in uninfected cell lysates.

(10) Development of an *in situ* assay for protein kinases separated by isoelectric focusing led to detection of ten protein kinases in *T. annulata* infected cells and eight protein kinases in the uninfected cell line BL-20. Thus two protein kinases (ISPK3 & ISPK4) were only active in *Theileria* infected cells and in addition, a further two kinases (ISPK1 & ISPK2) were increased in activity in these cells and shown to be capable of autophosphorylation. One of the kinases detected (USPK1) was decreased in activity as a result of *Theileria* infection.
These findings begin to characterise some of the basic biochemical and cell biological parameters of the transformation events induced by *Theileria* infection and provide important new leads into the understanding of the mechanism by which the host cell is induced to proliferate.
increase in presence/activity of protein.

If decrease "loss" increase in transcription of "disappear"
only the cellular location of the known oncogene product is shown (see 12.8)
4.2. RELEVANCE OF FINDINGS TO THE UNDERSTANDING OF TRANSFORMATION

4.2.1. Growth Characteristics of T. annulata - Transformed Leukocytes

The stimulation of the already proliferating BL-20 cells to an increased proliferation rate by infection with Theileria annulata, may be induced through further stimulation of the pathways responsible for the maintenance of the BL-20 transformed state, or through novel pathways. Activation of different pathways appears to be at least partly responsible, since cellular aggregation occurs after T. annulata infection which does not occur in BL-20 transformed cells. As discussed in section (3.1.4), this phenomenon appears to be necessary for lymphocyte stimulation by a number of different mitogens. In addition, Peters (1972) demonstrated what appeared to be intercellular communication between mitogen-stimulated agglutinated lymphocytes (reviewed in section 3.1.4). Thus, the agglutination observed in T. annulata-transformed leukocytes may, by allowing communication between cells through cell-cell contacts, play an important part in the stimulation of proliferation. The importance of intercellular communication in T. annulata-induced leukocyte transformation may then also form the basis of an explanation for the observation that in lymphoid tissues of infected cattle, both T. annulata-infected and uninfected leukocytes are stimulated to proliferate (De Martini and Moulton, 1973) and the observation that incubation of Theileria (parva)-infected lymphoblastoid cells with normal peripheral blood lymphocytes (PBL) from the same, or unrelated cattle induced DNA synthesis in the PBL to a greater extent than a classical mixed lymphocyte reaction (see 1.3.4) (Pearson, et al., 1979). Whether or not cell-cell contacts are necessary for maintaining the transformed state of T. annulata-transformed leukocytes could be determined simply by gentle agitation of the liquid cultures to disrupt aggregates and measuring the growth rate of the culture to see if it is reduced.

Studies of the effect of seeding density showed that a lower density was necessary for the establishment of a liquid cell culture with T. annulata-infected BL-20 cells than with uninfected BL-20 cells suggesting that the infected cells could produce diffusible growth stimulators. This is in accordance with the work of Williams et al., (1988) who provided evidence for growth factor secretion in T. parva-
infected cells (reviewed in section 1.3.3).

Further work on this more physiological aspect of Theileria-induced transformation, although not within the scope of this thesis project, is likely to yield useful information about the mechanism of maintenance of the transformed state of *T. annulata*-infected leukocytes (see 4.3).

4.2.2. Expression of Oncogene Homologues in *T. annulata* Transformed Leukocytes

Increased expression of homologues of several known oncogenes were detected in *T. annulata*-transformed leukocytes (see 3.2). These oncogenes encode protein products which are good candidates for participation in the mitogenic pathways: the ras and abl oncogenes encode G-like proteins (see 1.2.8.3.1) and a tyrosine protein kinase (see 1.2.8.1.1 and 1.2.8.2.3) respectively - both the gene products have been shown in other systems to be located at the plasma membrane; mos encodes a cytoplasmic serine/threonine protein kinase (see 1.2.8.1.1); and B-lym, fos and myc all encode nuclear-located proteins of unknown function, although fos and myc have DNA-binding potential and so may have a function in regulating gene expression (see 1.2.8.4). Figure (4.1) shows the cellular locations of these c-onc protein products. The precise degree of homology between these oncogene probes and the transcripts detected was not known, and so it cannot be concluded that these genes which showed an increase in expression on infection with *T. annulata*, were proto-oncogenes. Since some degree of specificity was indicated (see 3.2.4), it is likely that the transcripts detected have domains in common with the probes, and by inference may have a similar function. Thus, for example, the v-abl probe may be detecting several different protein kinase transcripts by hybridising to conserved functional regions, although other parts of the sequence such as substrate recognition domains may differ substantially. Similarly, the fos, and myc probes may be detecting several different transcripts, each of which encodes a DNA-binding domain, but differs in other domains such as substrate recognition sequences and other functional units.

Because the transcripts show some homology with the oncogene
probes, it is probable that the proteins they encode have functions similar to components of the mitogenic pathways and so may be involved either directly, or indirectly with growth control. The fact that they are increased in expression in *T. annulata*-transformed cells compared with homologous uninfected cells, is an indication that these transcripts encode proteins which are involved in some way with the transformation process. Since none of these transcripts (with the exception of the *mos* oncogene) were detected as genomic sequences in the parasite DNA, then it can be concluded that the observed stimulation of ras-, abl-, B-lym-, fos- and myc-like gene expression involved increased steady state levels of host-encoded genes. Furthermore, expression was also detected, but at a lower level in uninfected cells. It is most likely, therefore, that these oncogene homologues are stimulated in expression as a result of *T. annulata* infection and play some role in the maintenance of the transformed state. This may be via direct involvement in the mitogenic pathways, or be via indirect regulation of them. Alternatively they may be involved in a secondary function - one of the consequences of transformation.

The increased transcription of the *mos* oncogene homologue(s) in *T. annulata*-infected cells however, may be in part due to expression of parasite-encoded DNA sequences since sequences showing some homology to v-mos were detected in Theileria DNA preparations (see 3.3.2.2). However, since (a lower level of) transcription of sequences homologous to v-mos were also detected in uninfected cells, the increase in expression could be attributed to either host cell or parasite-encoded sequences, or both. In order to determine the source of infection-specific *mos*-like transcripts, it would be necessary to screen RNA prepared from isolated macroschizonts. Unfortunately, an efficient, reliable method was not available for isolating intact macroschizonts (see 3.6.1).

4.2.3. **Protein Kinase Activity and Substrates**

Analysis of the phosphoprotein profiles of *T. annulata* infected and uninfected cells revealed a considerable number of differences and a high proportion (10 out of 15) of the altered phosphoproteins were alkali stable and so probably phosphorylated on tyrosine residues.
Since only 0.1---2.0 percent of phosphorylation events in transformed cells result in tyrosine residue phosphorylation, (Cooper and Hunter, 1983), this result is surprising. However, this finding is comparable to other, similar studies; for example Cooper and Hunter, (1981), demonstrated that 7 out of 10 of the phosphoproteins which contained more phosphate in RSV (Rous sarcoma virus)-transformed chick embryo cells, compared with the same cells uninfected with the virus, contained phospho-tyrosine. As with the RSV-transformed cells, this high proportion of infection-specific phosphoproteins which appeared to contain phosphotyrosine in *T. annulata*-infected cells, was indicative of involvement of tyrosine-protein kinases in the transformation mechanism. In the chick embryo cells transformed by RSV, the agent responsible for induction of the transformed state was a viral src oncogene, encoding a tyrosine kinase (see section 1.2.8.1.1.2), so it is a reasonable hypothesis that *Theileria annulata* may also transform by a similar mechanism.

As indicated by *in vivo* incorporation of $^{32}$P phosphorous into cellular proteins (see section 3.4.2) the overall phosphorylation activity as a result of *T. annulata* infection was around two fold that of uninfected cells. Although the phosphotyrosine-containing proteins were increased threefold after infection, this only accounted for a small proportion of the increase in total phosphorylation activity, phosphotyrosine being such a small component of the phospho-amino acid pool in the cells. Thus, it may be expected that tyrosine protein kinase activity would be more difficult to detect in the *in situ* gel kinase assay, since alkali-treatment of the agarose gels was not feasible and so tyrosine-specific protein kinases could not be selected for. However, some of the protein kinases detected may be specific for tyrosine-residues, since two of the alkali-stable, infection-specific phosphoproteins which were detected as major substrates for protein kinases *in vitro* were also detected in the *in vivo* situation, suggesting that they are of importance to the *Theileria*-transformed cells, and possibly to the transformation itself. Both these substrates (ISPP1 and ISPP3) appear to be host-encoded proteins and ISPP1 is membrane associated, while the cellular location of ISPP3 was not determined (although it was mainly detected in the supernatant after brief centrifugation of the cell lysate). Since these two proteins appeared to be highly phosphorylated at tyrosine residues at least *in vitro*, then it is likely that tyrosine-
protein kinase activity would be detectable by the in situ kinase gel assay.

4.2.3.1. **Identification of Protein Kinases**

The protein kinases detected by the in situ gel assay were not fully characterised (for suggested methods of characterisation and identification of protein kinases, see section 4.3). However, clues as to the possible identity of some of these activities were deduced from both the literature, and also from the phosphoprotein profiles:

Mitogenic stimulation of lymphocytes is thought to involve stimulation of two pathways following the binding of the mitogen to the lymphocytes. These two pathways involve the hydrolysis of phosphatidylinositol 4, 5 bisphosphate into diacylglycerol and inositol 1, 4, 5 trisphosphate. These two molecules are second messengers in the initiation of two pathways: one leading to the activation of protein kinase C by diacylglycerol and the other causing the mobilisation of calcium by inositol 1, 4, 5 trisphosphate (reviewed by Grove and Mastro, 1987; see also 1.2.9). Both protein kinase C (a Ca$^{2+}$-activated phospholipid-dependant enzyme) and cyclic AMP-dependant protein kinases are abundant in lymphocytes, and are considered potentially important in lymphocyte stimulation (Grove and Mastro, 1987).

Grove and Mastro (1987), detected six protein kinases in extracts of bovine lymphocytes. They used an in situ gel kinase assay upon which the method described in this thesis was based, with the primary difference that they separated proteins on a non-denaturing polyacrylamide gel system whereas the method described here used iso-electric focusing to separate the proteins. The method used in this thesis project resulted in improved resolution and eight protein kinases were detected in bovine leukocytes, with an additional two in cells which were infected with T. annulata. Since the Grove and Mastro method separates on the basis of size whereas iso-electric focusing in agarose separates on the basis of charge, the migration of the kinases in the two gel systems could not be directly compared.
Grove and Mastro, (1987) by characterising various properties of these bands of protein kinase activity, identified three of the four most active protein kinase bands as protein kinase C, cyclic AMP-dependant protein kinase I and cyclic AMP-dependant protein kinase II. A less active band was identified as a third cyclic AMP-dependant protein kinase. These four protein kinases should therefore represent four of the protein kinases visualised in the assay described in this thesis. It is most likely, that the most active bands in both assays correspond: hence, bands 2, 3, 4 and 5 (see fig. 3.18.b) are likely to include protein kinase C and cyclic AMP-dependant protein kinases I and II. None of these four bands were increased significantly in activity in T. annulata-infected cells. However, this does not exclude their involvement in transformation, as Grove and Mastro (1987) showed that following treatment of bovine lymphocytes with the mitogen concanavalin A, protein kinase C shows no overall change in activity, but increases in activity within a few minutes by a factor of (approximately) three in a particulate fraction of the cell lysate, while decreasing activity in the cytosol fraction. This is suggestive of translocation of protein kinase C, but was observed to be only temporary, returning to normal proportions in cytosol and particulate fractions after 10 mins. Such transient increases or decreases in activity, or translocation of protein kinases would not be detected in the preliminary assay described in this thesis, even though such events may be relevant to the transformation of bovine leukocytes by T. annulata. Band 5 in the protein kinase assay described here (see figure 3.18.b) is the one most likely to correspond to protein kinase C, since the isoelectric point of band 5 was estimated at (approximately) pI = 5.55 which is very close to the known value for rat protein kinase C (pI = 5.6; Kikkawa, et al., 1983).

There was also some indication, although speculative, as to the identification of a subunit of cyclic AMP-dependant protein kinase I in the assays described in this thesis. Cyclic AMP-dependant protein kinase is composed of regulatory (R) subunits and catalytic subunits (C). In its inactive form, in the absence of cyclic AMP, the enzyme takes the form of an R₂C₂ complex (see figure 4.2). On binding of cyclic AMP however, this complex dissociates to an R₂ unit and two activated catalytic subunits which can then phosphorylate substrate proteins at serine or threonine residues. The regulatory subunit of
Cyclic AMP activates protein kinases by dissociating the complex of regulatory and catalytic subunits.

(adapted from Stryer, 1981).
protein kinase I (RI), was shown by Grove and Mastro (1987) to comprise 94% of the regulatory subunits of c-AMP-dependant protein kinases in bovine lymphocytes. This RI subunit has a molecular weight of 50kDa - (the same for all R subunits of c-AMP-dependant protein kinases) - which is the same size as a protein kinases ISPK1 and ISPK2 which were greatly enhanced in activity specifically in T.annulata-infected leukocytes. In addition, ISPK1 and 2 were shown to phosphorylate at alkaline-labile residues, which is in accordance with the C subunit of c-AMP-dependant protein kinase being specific for serine/threonine residues (Stryer, 1981). So, it can tentatively be suggested that the protein kinases ISPK1 and 2 which were stimulated on infection of bovine leukocytes by T. annulata by factors of 15.5 and 23 respectively, may both regulatory (R) subunits of c-AMP-dependant protein kinase.

Interestingly, phosphoprotein analysis also revealed a 50kDa T.annulata-infection specific protein (ISPP3) which, by its apparent ability to autophosphorylate seemed likely to be a protein kinase. However, ISPP3 appeared to autophosphorylate at tyrosine residues (since the phosphorylated residues were alkali-stable) and so is unlikely to be a c-AMP-dependant protein kinase. Although three protein kinases detected in the in situ gel assay may represent protein kinase C (band 5), and c-AMP-dependant protein kinase C subunits (ISPK 1, band 7 and ISPK 2, band 8), it is necessary to further characterise these proteins in order to substantiate these conclusions and to identify the other kinases-particularly ISPK 3 and 4 which were active only in Theileria-infected leukocytes (see 4.3). ISPK 1 and 2, since they were also detected (at reduced activities) in uninfected cells must be host cell protein kinases, whereas ISPK 3 and 4 which were unique to infected cells may be parasite protein kinases.

The fact that such large increases in kinase activity of ISPK 1 to 4 occur on infection of bovine leukocytes with T. annulata, is strongly indicative that the activation of these protein kinases plays a central role in the maintenance of the transformed phenotype of T. annulata-transformed cells. (For a review of the role of protein kinases in transformation, see section 1.2.8). In fact, it is
possible that one of these Theileria-infection-specific protein kinases is directly causative in the leukocyte transformation (see 4.2.3.3).

4.2.3.2. Identification of Phosphoproteins

As reviewed in section (1.2.8.1.2), the search for substrate proteins of (tyrosine) kinases has not revealed any such phosphoproteins which are relevant to cellular transformation in a physiologically determined manner. However, several candidate proteins have been described, which are phosphorylated upon cellular transformation and may be involved in determining particular characteristics of the transformed cell. Several others are known either by only their electrophoretic properties, or are themselves protein kinases (see table in figure 1.6). In addition, cells transformed by retroviruses carrying oncogenes of the tyrosine kinase class appear to phosphorylate the same subset of cellular proteins (Swarup, et.al., 1983).

Several of the phosphoproteins which were found to be present in bovine leukocytes as a result of T. annulata infection were phosphorylated on alkali-stable sites, most likely at tyrosine residues (see 3.4.4) and so were primary candidates for involvement in transformation. Although these phosphoproteins were partially characterised, no experiments were carried out which yielded information as to their potential function. However, it was possible to suggest potential functions for some of these proteins by looking for proteins described in the literature with similar properties. The four phosphoproteins considered most likely to be relevant to T.annulata-induced leukocyte transformation were ISPP 1, ISPP 3, ISPP 4 and USPP 2, since they were (a) specific to T. annulata-transformed cells (or specific to uninfected cells in the case of USPP 2) (b) detected in vivo as well as in in vitro assays (c) were major substrates for protein kinases in in vitro assays under a variety of ionic conditions and in some cases (notably ISPP 1 and ISPP 3) were the most predominant species of phosphoprotein and (d) in the cases of ISPP 1, ISPP 3 and ISPP 4, were likely to be phosphorylated at tyrosine residues at least under some ionic conditions. Of these four phosphoproteins, two (ISPP 1 and ISPP 3) have the same molecular
weights as proteins which have been reported in the literature as having potential roles in transformation and have altered phosphorylation patterns in transformed cells.

(1) ISPP 1 has an estimated molecular weight of 134kDa, which is very similar to that of one of the few proteins of known function which has an altered tyrosine-phosphorylation pattern in transformed cells - vinculin (130kDa). Vinculin is a cytoskeletal protein which was found to contain phosphotyrosine in RSV-transformed chicken embryo cells during a survey of cytoskeletal proteins undertaken by Sefton, et.al., (1981). In these RSV-transformed cells, 20% of phosphate linked to vinculin was phosphotyrosine. The fact that vinculin is phosphorylated not only at tyrosine, was reflected in the 134kDa protein in in vitro assays (see 3.4.3.1). Many other transformed cells have now been shown to contain vinculin which is phosphorylated at tyrosine, including cells transformed by Y73, the avian sarcoma virus which carries the yes transforming gene (see 3.3.2.4); Sefton, et.al., 1981). Very low levels of phosphotyrosine have been detected in vinculin of normal cells; less than 1/20th of the levels in RSV transformed cells, suggesting that this protein is a substrate for tyrosine protein kinases of normal cells as well as that of virally-transduced protein kinases such as v-src and v-yes (Sefton, et. al., 1981). The discovery that vinculin phosphorylation accompanied transformation by some retroviruses, initiated speculation that this phosphorylation may be responsible for causing the cytoskeletal changes often seen in transformed cells. About 20% of cellular vinculin in normal cells is concentrated in adhesion plaques, where the outer surface of the membrane is bound to the extracellular matrix, and the inner surface provides an anchorage point for stress fibres composed of actin microfilament bundles. There are many reports of reorganisation of both the total cellular microfilament system and the adhesion plaques in transformed cells (see Cooper and Hunter, 1983 for review). Shriver and Rohrschneider (1981) found both pp60 v-src and vinculin together in the vestiges of adhesion plaques of RSV-transformed cells, suggesting that pp60 v-src is in a position to phosphorylate vinculin. It is hypothesised that the greatly increased phosphorylation of tyrosine in vinculin may reduce its ability as a linker and lead to the release of actin filaments, which are indeed radically disorganised in many types of transformed cells (Hunter, 1984). It has, however, been suggested that vinculin
phosphorylation alone may not be enough to cause changes in cell shape via cytoskeletal alterations (Cooper and Hunter, 1983). Phosphorylation of other, as yet unidentified proteins in the adhesion plaque may also be necessary.

Thus although speculative, ISPP 1 in *T. annulata*-transformed leukocytes may represent the abberantly phosphorylated vinculin protein reported in some virally-transformed cells. Both proteins have similar molecular weight and ISPP 1 is found in the membrane fraction, as would be expected of vinculin. In addition, ISPP 1 is phosphorylated independently of the presence of Ca$^{2+}$ions, which is compatible with being phosphorylated by a tyrosine kinase (see 1.2.8.1.1). Particularly suggestive is the fact that vinculin is known to be phosphorylated in cells transformed by Y73 - the v-yes oncogene-transducing virus. The viral yes protein is thought to directly phosphorylate vinculin (Sefton, et al., 1981), and so the presence and expression of *T. annulata* v-yes-like sequences in Theileria-transformed leukocytes (3.3) may be responsible for phosphorylating vinculin (tentatively ISPP 1).

(2) ISPP 3, as well as being a candidate for the RI subunit of c-AMP-dependant protein kinase (see 4.2.3.1), is also the same molecular weight as a transformation-associated phosphoprotein. Unlike the RI subunit, both ISPP 3 and the transformation-associated protein appear to contain phosphotyrosine, indicating a stronger relationship of ISPP3 with the transformation associated phosphoprotein, than with the RI subunit. ISPP 3 and the transformation-associated phosphoprotein (pp50) share the same molecular weight of 50kDa. The transformation associated phosphoprotein pp50 was first isolated from RSV-transformed chicken embryo cells and has since been found in cells transformed by several other viruses including Y73 - the v-yes-transducing retrovirus. pp50 is a cellular protein which is phosphorylated at a single serine and a single tyrosine site in transformed cells, and is complexed with viral tyrosine protein kinases, including Y73 p90 gag-yes along with a 90kD phospho-serine-containing protein. This suggests that pp50 is a substrate for its associated viral tyrosine kinase. Since both pp50 and pp90 have been detected, bound to newly synthesised pp60 v-src and pp90 v-yes, but not to the membrane-bound proteins, it has been postulated that they (pp50 and pp90) could mediate the transport of transforming proteins such as pp90 v-yes from
the ribosomal synthesis sites to the plasma membrane (for review of pp50 - see Cooper and Hunter, 1983). It is not known whether pp50 associates with all tyrosine kinases, or whether it associates with any transforming proteins which are not tyrosine kinases.

In accordance with the hypothesis that ISPP 3 and the pp50 protein described in the literature are identical, is the fact that ISPP 3 was located in a cytoplasmic cellular fraction. This would be expected of a protein involved in transport between the ribosomes and plasma membrane. Also, ISPP 3 was phosphorylated at alkali-stable sites (most likely tyrosine) primarily and may also have been phosphorylated at serine/threonine - a pattern also reported with pp50 (Cooper and Hunter, 1983). However the fact that ISPP 3 appeared to autophosphorylate (it was phosphorylated in vitro at 0°C) indicates that this protein has kinase activity, thus making it an unlikely candidate for a transport protein. However, with the close association known to exist between pp50 and viral tyrosine kinases, then the apparent autophosphorylation of ISPP 3 could be explained by its association with a protein kinase. Thus, the features of ISPP 3 which have so far been investigated are in accordance with ISPP 3 being the protein pp50. The detection of a parasite encoded v-yes homologue and its transcript in T.annulata-infected cells, allows one to speculate that the yes encoded kinase may be associated with ISPP 3 and the latter may effect the transport of the kinase to the plasma membrane.

(3) A 36kDa phosphoprotein was detected in the in vivo assay (see figure 3.10.b) which was present in all proliferating cell lines, including those mitogenically stimulated, a lymphosarcoma cell line and T. annulata-infected leukocytes. A phosphoprotein also of 36kDa (pp36) had previously been identified in cells transformed by various retroviruses and growth factor stimulated cells (see Cooper and Hunter, 1983 for review). However, the relationship between these two phosphoproteins is doubtful since pp36 has been previously detected only at very low levels in transformed lymphoblasts (less than 1% of the levels present in transformed fibroblasts) - and Sefton, et.al. (1983) suggested that presence of pp36 is not essential for lymphoid proliferation or transformation, although it correlated well with fibroblast transformation. The function of pp36 is not known, although many experiments have been carried out to determine
its cellular location and activity (see Cooper and Hunter, 1983 for review).

(4) A protein doublet of 53kDa and 56kDa was reported by Swarup, et al., (1983) to be present in a variety of normal cell types, including T-lymphocytes (where there was a size variation to 56kDa and 58kDa). Both proteins contained phosphotyrosine and depended on the presence of Mg$^{2+}$ or Mn$^{2+}$ ions for their phosphorylation. In this project, a doublet of similar size, phosphorylated on an alkali-stable site, probably tyrosine, was detected in an in vitro assay in the presence of either Mg$^{2+}$ or Mn$^{2+}$ (see fig. 3.12). Although this doublet was not T. annulata-infection specific, or specific to proliferating cells, its identification (if indeed it is the same phosphotyrosine-containing protein as previously identified) would be significant, since identification of all phosphotyrosine-containing proteins is of interest. There is no indication of these proteins involvement in growth regulation or transformation, since they have previously been found in normal, unstimulated cells, and in this project were not found to be altered in phosphorylation pattern in T. annulata-transformed cells.

The phosphoproteins discussed above are the only ones with similarities to previously described phosphotyrosine-containing proteins, and further characterisation must be undertaken in order to define the basic properties and role of these proteins in transformation.

A further conclusion that can be made from the phosphoprotein results concerns the protein kinase (USPK 1- see fig.3.18.b) which is apparently inactivated after infection of leukocytes by T.annulata. Analysis of in vitro phosphoprotein profiles also identified a polypeptide (USPP 2) which was absent or not phosphorylated on infection with T. annulata but was present and phosphorylated in uninfected cells. Since this polypeptide (USPP 2) showed autophosphorylation activity, it is likely to be a protein kinase and may therefore be USPK 1 in view of its similar properties (i.e. lack of phosphorylation) in relation to Theileria-infection. However, without further information, it is also possible that USPP 2 is a substrate for USPK 1. In either case these results suggest that deactivation of protein kinase(s) may also be involved in the
stimulation of leukocytes by Theileria-infection. Such an event is reminiscent of the activity of suppressor genes which in normal cells have a negative growth regulatory capacity. Thus, on their deactivation, transformation may result (see 1.2.10, paragraph 4 for review).

So, as yet, the role of these and the other proteins which were detected as being altered in phosphorylation pattern by T. annulata transformation, remains speculative. These proteins are certainly relevant to the transformation process, and although parallels have been drawn here with some previously detected transformation-associated phosphoproteins, further work is necessary to define how these proteins are functionally involved in bringing about the transformed phenotype of T. annulata-transformed leukocytes, (see 4.3).

4.2.4. T. annulata yes Homologue

The presence in the T. annulata genome of v-yes-like sequences and expression of these sequences in T. annulata-infected bovine leukocytes, leads to speculation on the likelihood of the protein product of these sequences (see 3.3.2.4), being responsible for induction of transformation in this system. This may occur in a manner similar to models (1) or (2) (see section 1.3.6), where the macroschizont is envisaged as secreting a molecule(s) into the leukocyte cytoplasm which proceeds to interact with the host cell growth regulatory pathways in such a way as to induce transformation. This could be achieved by mimicking a component of the host cell pathways (model 1) or by producing a novel protein product, unlike any component of the host cell pathways, but able to interact with these pathways at some point to bring about proliferation (model 2). Since a cellular homologue of the v-yes gene has been characterised and the corresponding protein products of 62kDa and 59kDa identified (Sudol and Hanafusa, 1986), then it is at least possible that the T.annulata yes-like gene product mimics a component of the normal cellular pathway leading to unregulated growth (model 1). Since the c-yes products have been shown to be tyrosine kinase(s) (Kornbluth, et al., 1987), then the parasite yes-like product(s) may interfere with host cell growth regulation by phosphorylating c-yes protein substrates in an unregulated, aberrant manner. Indeed, cells which have been
transformed by v-yes - transducing retroviruses have also been shown to have an increased phosphotyrosine content (Swarup, et al., 1983), which is also evident in T. annulata-transformed leukocytes (see 4.2.3). Kornbluth, et al., (1987) reported that in cells transformed with polyoma middle-T antigen, the c-yes protein is found associated with the middle-T antigen which it phosphorylates. This is similar to the association between the v-yes protein and pp50 (see 4.2.3.2 paragraph 2) in which the latter transports the viral yes gene product to the host cell plasma membrane. A similar process can be envisaged as occurring with the parasite-encoded yes-like gene product and the phosphoprotein substrate ISPP 3). Once transported to the membrane, one of its substrate proteins may include vinculin which is thought to result in altered cell morphology associated with transformation. The evidence that pp50 and vinculin may be proteins both phosphorylated as a result of T. annulata infection has been reviewed in (4.2.3.2) paragraphs (1) and (2). The other substrates of the putative Theileria yes-like protein may then include component(s) of the leukocyte growth regulatory pathways. This could result in transformation (see fig.4.3).

If this hypothesis is correct, then the putative parasite yes-like protein (p-yes) would function as a tyrosine kinase, and so should be detectable in the in situ gel assay. The two kinases which were detected only in T. annulata-infected cells - ISPK 3 and ISPK 4 are then possible candidates for p-yes gene product. Also, since all tyrosine kinases so far characterised, are themselves phosphorylated at tyrosine (1.2.8.1.1.2), then p-yes should also be detectable as a Tannulata-infection specific phosphotyrosine containing protein. No infection-specific phosphoprotein of the same molecular weight as either the gag-yes 90kDa viral protein or the 59 and 62kDa c-yes proteins was detected. However the p-yes gene product may be quite different in molecular weight to the gene products of either the v-yes or c-yes oncogenes. Therefore, any one of the infection-specific phosphotyrosine-containing proteins may be a p-yes gene product although from knowledge of tyrosine protein kinases in general and the v-yes gene product specifically, it is likely to be located in the membrane fraction and to autophosphorylate independantly of calcium. ISPP 1, 4, 5 and 6 have such properties (although their abilities to autophosphorylate have not been confirmed), but further research is necessary to positively identify the p-yes gene product.
In addition, comparing the intensity of hybridisation signal of the v-yes gene between uninfected bovine leukocyte DNA and Theileria DNA (fig. 3.5.a) indicated that the parasite gene was more homologous to the viral yes gene than to the cellular yes gene. This may however, be at least partly due to loading more 'gene equivalents' in Theileria DNA tracks as a result of the lower complexity of the parasite genome compared with the bovine genome (Alsopp and Alsopp, 1988). The homology between the viral yes gene and the parasite sequences raises the possibility that the T. annulata yes-like gene was derived from a retrovirally-transduced yes gene or, indeed that T. annulata carries a virus which encodes a yes-like gene (see model 4, section 1.3.6).

In conclusion, possession of parasite DNA sequences which are specific to the yes oncogene, and expression of such sequences in T.annulata-infected cells may represent the mode of transformation of bovine leukocytes by this parasite. This may be via several mechanisms as outlined in models 1, 2 or 4 in section (1.3.6). Although an attractive hypothesis because of its simplicity - T.annulata acting as, (or carrying) a transforming retrovirus, there is as yet no conclusive evidence to prove this correct or incorrect. As described in section 4.3., the only way in which this hypothesis can be ultimately tested is to carry out a functional assay of the transformation ability of the Theileria yes gene on bovine leukocytes and to characterise its gene product in detail.
Jneighbouring cell

growth factor receptor

extracellular matrix vinculin (ISPP1?)

is phosphorylated

ULI

cAMP dependent protein kinase C

pp50 (ISPP3?) transports pyes protein (tyrosine kinase)

in cellular phosphotyrosine

Macroschizont

*pyes induces host cell transformation through phosphorylation and stimulation of host autocrine mechanism.
4.3. DIRECTIONS OF FUTURE WORK

Although, as summarised in section (4.1), the work described in this thesis project defined a number of molecular features unique to *T. annulata*-transformed leukocytes which were relevant to the transformation process, the actual mechanism of initiation of transformation was not unambiguously determined. Further work must be carried out both in order to elucidate whether or not the *T. annulata* v-yes-like gene product is the causative agent of leukocyte transformation and if not, to determine the causal events. Furthermore, the functional relevance to transformation of the various features which were detected here as unique to *T. annulata* transformed cells needs to be determined.

In order to determine the precise relationship between the viral yes oncogene and the homologous sequences detected in the genome of *T. annulata*, the entire parasite gene must be cloned and sequenced. Computer-aided comparisons with known sequences would then reveal any homology with the v-yes gene, and other tyrosine kinases. Assuming the homology to be significant, a functional test would have to be devised in order to determine whether the parasite yes gene product is in fact capable of bringing about transformation. Transfection of the cloned parasite yes gene (p yes) into host NIH 3T3 cells, or bovine peripheral blood leukocytes, and assays for increased proliferation would form the basis of such a test. Transfection of DNA into NIH 3T3 cells by calcium chloride precipitation and assaying for transformed cells is a well-established technique (for review, see section 3.3.1). However, many alternative types of assay could be used for introducing biologically active DNA into bovine leukocytes as well as calcium chloride precipitation; electroporation, microinjection or use of retroviral vectors - all of which have been used previously, with success. Alternatively, purified protein derived from recombinant clones of p-yes could be micro-injected directly into bovine leukocytes to see if transformation could be induced - this has been achieved previously by micro-injection of mutant ras protein into rat PC12 cells (see Bar-Sagl and Feramisco, 1985).

If such a test proved the p-yes gene product to be capable of inducing host cell transformation, a plethora of experiments could be
devised to study the temporal expression of the gene and the function of the gene product, so that the mechanism by which it brings about the leukocyte transformation could be elucidated. Alternatively, if the p-yes protein was not capable of inducing leukocyte transformation, the techniques listed above for introducing foreign DNA into NIH 3T3 or leukocytes could be considered for use in screening *T. annulata* genomic DNA for alternative 'transforming genes'. This approach is outlined in more detail in section (3.3.1), but is not capable of detecting transforming genes if two or more co-operating parasite genes are involved in induction of leukocyte transformation.

In addition to the above approach, it is important to further characterise the various protein kinases and phosphoproteins which were unique to either *T. annulata*-transformed leukocytes, or to uninfected cells. Most importantly, the protein kinases which were detected to be specific to/altered in activity in *T. annulata* infected leukocytes, should be positively identified. This could be achieved by firstly defining the characteristics of the protein kinases, such as substrate phosphorylation site(s), ionic dependency, cyclic nucleotide dependency and molecular weight. Some kinases may be identified on the basis of this information, and those that are not, may be purified by existing methods (e.g. see Grove and Mastro, 1987). The characterisation of the kinases would then be important in the designing of the particular purification protocol for each protein kinase. Purified protein kinase may then be further analysed in a number of ways including amino acid sequencing, in vitro assays and also assays for transforming potential by micro-injecting into leukocytes (see Bar-Sagi and Feramisco, 1985). The genes encoding the protein kinases could subsequently be isolated from an expression library of *Theileria*-infected leukocyte DNA using antibody recognising the kinase(s), and studied.

Phosphoproteins which appeared particularly relevant to *T.annulata*-induced leukocyte transformation may, if radio-labelled be unambiguously tested for phosphotyrosine content by cutting out the appropriate proteins, hydrolysing and separating the phospho-amino acids by thin layer electrophoresis (Cooper, et.al., 1983). The phosphoproteins may also be purified from two-dimensional gels and could be used to raise antibodies, which in turn would be a means of
isolating the genes encoding the phosphoproteins from a DNA expression library of bovine origin. Both antibodies and DNA sequence data would then be useful in elucidating the functional role of these phosphoproteins in T. annulata-infected leukocytes.

In addition to following up the results from this thesis project, alternative strategies may be taken in order to determine the mechanism by which T. annulata induces leukocyte transformation. Firstly, in order to determine whether or not T. annulata may transform by the action of a "passenger" virus (as outlined in transformation model 4; section 1.3.6), viral sequences could be searched for. Comparison of BL-20 and TaH-BL-20 therefore would indicate whether or not presence of viral sequences was associated with T. annulata macroschizonts. A suitable probe may be retroviral long terminal repeat sequences (LTR's - see 1.2.2) or genes encoding viral coat proteins. Any viruses detected would then be isolated and assayed for transforming potential as described for T.annulata genomic DNA and the p-yes gene (see above). Secondly, involvement of autocrine mechanisms could be assayed for, by means of conditioned medium experiments: medium in which Theileria-transformed cells have been growing can be taken and uninfected cells added (peripheral blood leukocytes) to see if any molecules secreted by the infected cells will induce the proliferation of the uninfected cells. An alternative means of screening for secreted transformation - associated growth factors would be to label medium by biotinylation (see Forsters et.al., 1985) in which (a) BL-20 and (b) TaH-BL-20 had been growing. Subsequent addition of the same cells back to their appropriate, labelled medium would allow the now-labelled growth factors to bind to the cell surface. Washed cells would then simply be subjected to SDS-PAGE, blotted onto a suitable membrane and 'developed' (see Forsters et.al., 1985) to visualise the growth factors. Again, comparison of BL-20 and TaH-BL-20 would reveal any growth factors binding specifically to T. annulata-infected leukocytes. Finally, the antigens specific to/increased in expression in, Theileria-transformed leukocytes, which were detected by Shiels, et. al., (1986) (see 1.3 for review) are currently being investigated by Dr R. Hall, Wellcome Unit of Molecular Parasitology, Glasgow University. Some of these proteins may represent components of the host cell mitogenic pathways which are stimulated on infection, while others, parasite-encoded, may be involved in the initiation of transformation. Since some of the
genes encoding these antigens have been isolated (Hall R, personal communication), they may be tested for transformation ability by DNA transfection into suitable cells and assaying for their transformation as described previously (this section).

Clearly, there are many approaches which could be taken in order to continue the investigation into the mechanism of T. annulata-induced transformation of bovine leukocytes. Indeed, the information gathered during such investigations may also be of value in understanding cellular growth control and transformation in general. In addition, it will be interesting to see if the pathological and target cell differences between the closely-related parasites T. annulata and T. parva (see 1.1) are reflected in the manner in which they transform their host cells.
4.4. ACHIEVEMENTS

The work described in this thesis did not achieve the ultimate long-term aim of this project - that is, to positively determine the mechanism of initiation of transformation of bovine leukocytes by Theileria annulata. However, with the complexity of growth regulatory systems and the fact that such mechanisms of control are not entirely understood in normal cells, then this was to be expected.

This work did succeed in defining in molecular terms, a variety of features which are relevant to the transformed state of T. annulata-infected leukocytes. These are summarised in section (4.1) and must be further characterised in order to define their functions in transformation. Analysis of these results has led to the tentative hypothesis that the parasite may bring about host cell transformation in a manner similar to some mammalian retroviruses - by supplying a tyrosine-specific protein kinase - a-yes-like protein. Although based only on circumstantial evidence, this provides an attractive working hypothesis because of its relative simplicity. Care must, however, be exercised in not placing too much emphasis on this hypothesis until it has been properly tested.

In addition to the results relevant to the transformation process, an improved technique for assaying the activity of several protein kinases simultaneously, by an in situ gel assay, and a method for isolating macroschizonts (though not intact) from Theileria-infected leukocytes, were developed. Both of these techniques may prove useful to researchers in the future.

Indeed the one, general problem which this study set out to investigate - that of how T. annulata transforms bovine leukocytes - has now been successfully broken down into a number of more specific questions by the results described here. These questions include that of the relevance to transformation of the various protein kinases and their substrate proteins which are altered by T. annulata infection, and of the presence of parasite-encoded v-yes-like DNA sequence. Thus, the results described here, though inconclusive as to the mechanism of T.annulata-induced leukocyte transformation, both provide a description of some of the events involved, and provide a good platform for future investigation.
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Control of Lymphoproliferation
by Theileria annulata

M. Dyer and A. Tait

The economic importance of bovine theilerioses has prompted several new approaches to understanding the diseases in the hope of developing more efficient methods of control. Most Theileria species that infect cattle cause a lymphoproliferative disease. Sporozoites, injected into the host bloodstream by the tick vectors, rapidly invade host lymphocytes and stimulate rapid division of infected cells. As these rupture, merozoites are released which invade red blood cells ready to infect feeding ticks again.

The process by which Theileria parasites can control host lymphocytes, and induce them to divide in synchrony with the parasites themselves, is poorly understood but seems to be the key to pathogenesis. In this article, Michael Dyer and Andrew Tait discuss the possible mechanisms of cellular control in the light of recent work revealing sequences homologous to oncogenes in the DNA of T. annulata.

Theileria annulata is transmitted by tick vectors of the genus Hyalomma, and causes tropical theileriosis of cattle and domestic buffalo, which is endemic in a geographical belt extending from North Africa, through the Middle East, southern USSR to India and China (Fig. I). Around 200 million cattle are at risk to infection, but estimates of the actual numbers contracting theileriosis and the consequent loss of productivity are likely to be underestimates due to a lack of accurate data. Exotic cattle (Bos taurus) are particularly susceptible to this and related Theileria, and mortality is around 40–60%; in contrast to indigenous cattle (B. indicus) where mortality is much lower (about 5%) and confined mainly to calves.

Lymphoproliferation

Infected ticks inject sporozoites which rapidly invade host lymphocytes. Maturation of the parasite to a multinucleate macroschizont is accompanied by a transformation of host cells to become large lymphoblastoid cells. As the schizont multiplies rapidly, there is a synchronous division of the host cell, with at least one parasite being distributed to each daughter cell, such that a huge population of parasitized lymphocytes results. Eventually, after maturation, the host cell ruptures to release merozoites which invade red blood cells to form the stage infective for the tick vector. The life cycle is completed through a further complex series of developmental stages in the tick, after it feeds on an infected animal.

The diseases produced by T. annulata and the related T. parvo (which causes East Coast fever in East Africa) are highly pathogenic often fatal lymphoproliferative disorders, first manifest as a swelling of the lymph nodes draining the site where the infected ticks fed. However, in tropical theileriosis, anaemia from phagocytosis and destruction of infected erythrocytes also plays a major role in the pathogenesis.

As the cells of the lymphoid organ and parasitized lymphocytes are stimulated to a massive proliferation, fever develops and the animal loses condition. The appearance of killer cells in the peripheral blood coincides with the onset of extensive destruction of parasitized and nonparasitized cells in the lymphoid tissues during the terminal stages of the disease. Death, if it occurs, is usually 3–4 weeks after infection.

Control of Lymphoproliferation

Phenotypically, the rapid proliferation of lymphocytes that results from parasite invasion can be considered as cell transformation. The mechanism of normal cellular growth control (Box I) is somehow disrupted by the interaction between host and parasite. Three main mechanisms for this can be postulated:

1. The parasite produces a molecule(s) which mimics component(s) of the host cell regulatory pathway in a manner similar to a retrovirally transduced oncogene, thus stimulating cell division.

2. The parasite produces a molecule which is unlike any component of the pathway, but interferes with it. This may be through stimulating transcription of a positive regulatory component of the pathway, or by destroying a negative regulatory component.

3. Insertion of parasite DNA sequences into or near a host gene encoding a component of the pathway may result in either altered activity in the case of the former, or altered regulation in the latter. Both could lead to transformation.

Fig. I. The distribution of tropical theileriosis (adapted from Purnell).
Mechanism (3) is often found in Burkitt's lymphoma, which is associated with a chromosomal translocation resulting in the myc proto-oncogene being placed under the influence of the immunoglobulin enhancer. Since B-cells express immunoglobulin at high levels, the myc oncogene is also transcribed excessively, and transformation occurs. Similarly, Theileria may supply a DNA promoter of gene expression to a host proto-oncogene.

However, at least one type of experiment with T. parva suggests that the lymphocyte transformation requires the continued presence of the parasite. The transformed phenotype is lost in progeny cells which no longer contain schizonts, when infected cloned cells are treated with cytochalasin B in vitro (Ref. 7). Similarly, treatment of natural infections with naphthoquinones will kill the parasite and stop lymphocyte transformation. Thus the parasite appears to be continually supplying some molecule(s) (which we term PSF, proliferation stimulatory factor) from within the lymphocytes that has the effect of inducing the host cell to divide. Thus model (3) seems unlikely, since it involves a process which is not readily reversible.

### Oncogene Homologies

In contrast, we have already shown that several components of the host regulatory mechanism are stimulated by T. annulata infection in vitro (unpublished data), and we are now attempting to distinguish which of the two most likely models is operating. The gene by which the parasite transforms the host cell may be similar to a host proto-oncogene (model 1), in which case it is likely that an oncogene probe will be available for it. Alternatively, if it is an unknown gene (model 2), one of several protocols may be used for isolating parasite gene(s) capable of inducing transformation. To date, a preparation of parasite DNA has been shown by Southern blot analysis8 to contain sequences homologous to the yes oncogene, which is a retroviral transforming gene encoding a tyrosine kinase9 (Table I; Box I). The association between cell transformation and altered protein-kinase activity, particularly at tyrosine residues, has been well established by several lines of inference9 so it is tempting to speculate that a parasite yes oncogene is altering tyrosine kinase activity in the host cell – thus stimulating the lymphocyte to divide (Fig. 3). Such

### Table I. Oncogene products and their normal cellular homologues

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Protein Product</th>
<th>Cellular Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>sis</td>
<td>PDGF (Growth factor)</td>
<td>Extracellular</td>
</tr>
<tr>
<td>erb-B</td>
<td>EGF receptor</td>
<td></td>
</tr>
<tr>
<td>Ki-ras</td>
<td>GTPase (G Protein)</td>
<td>Membrane-associated</td>
</tr>
<tr>
<td>Ha-ras</td>
<td>Tyrosine-kinase activity</td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>Receptor-like glycoprotein</td>
<td></td>
</tr>
<tr>
<td>src</td>
<td>Serine, threonine kinase</td>
<td>Cytoplasmic (free)</td>
</tr>
<tr>
<td>abl</td>
<td>?</td>
<td>Nuclear</td>
</tr>
<tr>
<td>fms</td>
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<td>myb</td>
<td></td>
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<tr>
<td>fos</td>
<td></td>
<td></td>
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<tr>
<td>Blym</td>
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</tbody>
</table>
Activation of signal pathway

Tyr

Absence of normal stimulus

Nucleus

signal to divide

yes-like protein

Tyrosine kinase activity

Fig. 3. A model for lymphocyte transformation by T. annulata. The yes gene may activate the growth control pathway in the absence of an external stimulus. This fits the observation that in-vitro infected T-cells lose their interleukin-2 (IL-2) growth hormone dependence.5

Activity is well documented for the control of cell division (Box 1). In this context, preliminary results from phospho-protein assays of labelled cell extracts show the presence of at least four kinase substrates (two are phosphorylated at tyrosine) specific to T. annulata-infected lymphocytes. A fifth appears to be dephosphorylated in parasitized cells.

But although the phosphorylation pattern of infected cells seems to be altered, and the parasite appears to contain a homologue of a transforming phosphorylating enzyme (a tyrosine kinase), it remains to be seen if the parasite yes homologue is a causative agent of Theileria-induced lymphoproliferation. The gene has been cloned from a parasite gene library constructed by Dr R. Hall, and experiments are underway to characterize the gene and the activity of its product and to determine its role in transformation. Ultimately, the only significant functional test is to attempt to mimic a Theileria infection with our cloned gene in a retroviral vector.

The implications of these findings for other Theileria species such as T. parva are as yet difficult to assess, since the host cell targets for infection seem to differ. T. parva seems preferentially to invade T-cells, whereas T. annulata appears to prefer B-cells and macrophages (MHC class II positive cells; R. Spooner, pers. commun.). Dr R. Williams (Kernforschungszentrum, Karlsruhe, FRG) is currently analysing the process of transformation in T. parva infections, and as the two mechanisms are elucidated, it will be interesting to see if the pathological and target cell differences between the two related parasites are reflected in the manner in which they transform their host cells.

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