HRAS1-SELECTED CHROMOSOME MEDIATED GENE TRANSFER; MOLECULAR INSIGHTS INTO TUMORIGENICITY AND RECOMBINATION.

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1988
I declare that the work presented for this thesis is, unless otherwise stated, all my own work,

Mark C Hirst.

November 1988
The work presented in this thesis was carried out in the molecular genetics section of the Medical Research Council's Human Genetics Unit, Edinburgh.
The presence of a rearranged HRAS1 oncogene in the highly tumorigenic HRAS1-selected chromosome mediated gene transformant E65.5 has been investigated. I show that the elevated tumorigenicity in E65.5 is not reflected in elevated levels of HRAS1 expression. This lack of correlation between levels of HRAS1 expression and tumorigenicity extends to several other tumorigenic and non-tumorigenic bladder carcinoma cell lines. Secondary DNA transfer reveals no direct association between the novel DNA sequences contiguous to the rearranged HRAS1 gene and the characteristic in vitro anchorage independance and morphology of E65.5, suggesting that the molecular rearrangement does not directly contribute to these attributes. I conclude that the effect must reside in more distant cotransferred DNA sequences or reflect the epigenetic consequences of clonal variations in the C127 recipient cells. The mechanism of the DNA rearrangement in E65.5 has been investigated through molecular cloning and sequencing of the breakpoint region. I show that the rearrangement involves a translocation with human DNA, most probably originating from chromosome 6. This translocation does not structurally interrupt the HRAS1 transcription unit. Furthermore, characterisation of a molecular clone of the progenitor cellular DNA with which HRAS1 has rearranged reveals insights into possible mechanisms and suggests a role for small sequence motifs homologous to "minisatellite" core sequences in such recombination events. Global mapping of the human component of E65.5 provides insights into general processes of chromosome mediated gene transfer.
ACKNOWLEDGEMENTS

The research undertaken to produce the work embodied by this thesis and the production of the completed article would not have been possible without the great help, advice, criticisms and encouragement of great many people. In an attempt to redress the balance, I offer my thanks to everyone with whom I have worked during my three year stay in Edinburgh.

Most importantly this thesis could not have been produced without the continuous support, patience, kicks up the backside, chunky sandwiches, kind words, harsh words, warm duvet, love, care and attention given to me by Ella. Thank you.

The project has originated from and centred around the work and efforts of David. I thank him for all his patience in supervising, his understanding when things went wrong (as they frequently did), his guidance into the human genome, his unceasing help with writing this thesis, his foresight in creating and moulding such a fascinating project, but most of all for the friendship that has developed between us. My thanks go also to Rosie and "girls"; thank you for all the kindness and friendship you have offered to myself and Ella, the vegetable curry has oft created a clear mind and a haven from the pressures of lab life.

The many members of the Molecular Genetics section have all aided my research either directly by guiding me through new techniques and helping me interpret data, or indirectly through many hints of advice, borrowed solutions and enzymes or just by playing The Smiths! In particular I would like to thank Julia and John who have helped me with most things, Richard (M) for teaching me RNA work, Gwen for showing me how to Southern blot, Peter for hints with protein gels, Sheila for keeping me in line, Anne for endless pots of Por4 DNA, Alison and Liz for the aforementioned musical accompaniment and their unceasing appreciation of the Archers, Nick, Bob, Robin, Wendy, Veronica, "the Paul- las", Chris, John (M), Ian and Sally for encouragement and an interest in my work.
The production of this completed thesis and the posters I have presented over my three years would have been impossible without the dedication and expertise given by Norman, Sandy and Douglas in the photography suite. Many a fading autorad and an incomprehensible line has been transformed into a work of art. Let's hope the "powers that be" will learn to appreciate essential skills you bring to the unit.

Thanks must go also to Sheila and Helen in the library for all the help with many references and overheads, and to the computer section for allowing me to use and abuse the laser printing facilities.

I thank the Medical Research Council for financial support throughout my studentship, Professor H J Evans for smoothing the paths of travel money and offering ongoing encouragement and support, and BCL enzymes for their assistance.

Thanks would not be complete without a mention to some of those things peculiar to Edinburgh which have seen me through my stay. Halva from Herbies, Chapattis and spicy naan breads from Mrs Junis and of course the many pulses, nuts, grains and general goodies offered from Real Foods!

Thanks to all those mentioned and to all those I have absent-mindedly forgotten. My days at CAPCU will be remembered for many years to come!
ABBREVIATIONS

BCIG  5-Bromo-4-Chloro-3-Indoyl-Galactopyranaside, X-gal.
CMGT  Chromosome mediated gene transfer
DEPD  Diethylpyrocarbonate
DMGT  DNA mediated gene transfer
DNA   Deoxyribonucleic acid
DNAase Deoxyribonuclease
dATP  Deoxyadenosine triphosphate
dCTP  Deoxycytidine triphosphate
dGTP  Deoxyguanidine triphosphate
dTTP  Deoxthymidine triphosphate
dITP  Deoxyinosine triphosphate
dsDNA Double-stranded DNA
ssDNA Single-stranded DNA
EDTA  Ethylenediaminetetra-acetic acid
FCS   Fetal Calf serum
mRNA  Messenger RNA
OD    Optical density
pfu   Plaque forming units
PolyA Polyadenylated
RPM   Revolutions per minute
RNA   Ribonucleic acid
RNAase Ribonuclease
SDS   Sodium dodecyl sulphate
TBR   Translocation breakpoint region
TCA   Trichloroacetic acid
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1 INTRODUCTION

1.1 Cancer.

Cancer is a ubiquitous genetically based disease. Statistical surveys of the UK suggest that as many as 1 in 3 people will suffer from cancer during their lifetime. Although this figure represents many forms of cancer, most deaths are caused by a small number of tumour types. For example, 26% of cancer cases in men are for lung cancer, 11% for skin cancers and 9% cancer of the prostate; whereas for women, 22% of cases are for cancer of the breast, 11% cancer of the skin and 10% cancer of the lung (OPCS, 1987). There is, however, a growing realisation that much of human cancer is linked with environmental affects and may thus be avoidable. Evidence for this comes mainly from i) the differences in cancer rates between migrants and those that remain in the country of origin, ii) variations in the time of onset of cancer in different countries, and iii) the actual identification of a number of factors contributing to the formation of cancers, such as tobacco smoke (Doll and Peto, 1981). However, perhaps the greatest insight into cancer aetiology has come from efforts to define its causes at the genetic level.

A genetic cause for cancer has been sought ever since the earliest speculation by Boveri (1929) that chromosomal abnormalities were linked with cancer. He observed that abnormal mitotic divisions leading to chromosomal loss in fertilised sea urchin eggs resulted in atypical tissue masses resembling the undifferentiated tissue masses found in tumours. Now, with the advent of recombinant DNA technology, alterations at both the chromosome and DNA level are now accessible to molecular investigation. The exploitation of such technologies has shown us, within the space of a few years, that the causes of cancer lie in alterations in the expression of genes which are intimately involved with the processes of cell growth and differentiation.
1.2 Molecular approaches to Cancer Investigation.

In vitro tissue culture techniques have revealed fundamental differences in both the lifespan and growth patterns of tumorous or transformed cells compared with normal cells (see table 1). Assays exploiting these differences have been used to identify genes involved in the cellular transition to the transformed phenotype.

Our current understanding of the processes involved in the development of cellular transformation has come mainly from the identification of mitigating genetic factors, the oncogenes, isolated from oncogenic retroviruses and tumour cells (reviewed by Varmus, 1984; Bishop, 1985;1987).

The retroviral oncogenes, which were first isolated from viral induced tumours in chickens and cats were the first genetic factors to be isolated. It was soon discovered that normal cellular counterparts of these retroviral genes, mutated versions of which could be isolated in DNA transfection assays, existed in the human genome and that altered versions appeared in some cancers. Over 50 such loci have now been identified in the human genome, using a number of criteria including: i) alteration of gene expression by retroviral integration, ii) coding sequences that are homologous to the transforming regions of oncogenic retroviruses, iii) sequences that have been detected in assays by their ability to induce malignant transformation in detector cell lines, and iv) sequences that appear to be altered or interrupted by tumour specific chromosomal rearrangements.

1.2.1 Retroviral Oncogenes.

The investigation of genes present in several retroviruses which are capable of transforming cells and inducing tumours in their hosts has had a great impact upon cancer research, giving researchers their first direct handle upon the DNA sequences directly involved in tumorigenesis.

It was as long ago as 1911 that Rous showed that an element not removed by filtration from an extract of a chicken sarcoma was capable of inducing tumours when injected back into healthy animals (Rous, 1911).
Table 1.1 Comparison of Normal and Transformed Cells

<table>
<thead>
<tr>
<th>Criteria</th>
<th>Normal</th>
<th>Transformed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lifespan</td>
<td>Limited</td>
<td>Indefinite</td>
</tr>
<tr>
<td>Monolayer Growth</td>
<td>Contact inhibited</td>
<td>Loss of contact inhibition</td>
</tr>
<tr>
<td>Growth in semi-solid medium</td>
<td>None</td>
<td>Yes</td>
</tr>
<tr>
<td>Xenografts</td>
<td>Non-tumorigenic</td>
<td>Tumorigenic</td>
</tr>
<tr>
<td>Growth factor dependence</td>
<td>Dependent</td>
<td>Partial or complete independence</td>
</tr>
</tbody>
</table>

Table 1.2 Retroviral Oncogenes

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Viral source</th>
<th>Virus</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>src</td>
<td>Chicken</td>
<td>RSV</td>
<td></td>
</tr>
<tr>
<td>fps(fes)</td>
<td>&quot;</td>
<td>FSV</td>
<td></td>
</tr>
<tr>
<td>yes</td>
<td>&quot;</td>
<td>Y73</td>
<td></td>
</tr>
<tr>
<td>roS</td>
<td>&quot;</td>
<td>UR2</td>
<td></td>
</tr>
<tr>
<td>S\ki</td>
<td>&quot;</td>
<td>SK770</td>
<td></td>
</tr>
<tr>
<td>erbA1(^1)</td>
<td>&quot;</td>
<td>AEV</td>
<td></td>
</tr>
<tr>
<td>erbB(^2)</td>
<td>&quot;</td>
<td>AEV</td>
<td></td>
</tr>
<tr>
<td>myc</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>myb(^3)</td>
<td>&quot;</td>
<td>AMV+E26</td>
<td></td>
</tr>
<tr>
<td>ets</td>
<td>&quot;</td>
<td>E26</td>
<td></td>
</tr>
<tr>
<td>mil(raf)(^4)</td>
<td>&quot;</td>
<td>MH2</td>
<td></td>
</tr>
<tr>
<td>crk</td>
<td>&quot;</td>
<td>CT10</td>
<td>Mayer et al, 1988</td>
</tr>
<tr>
<td>rel</td>
<td>Turkey</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mos</td>
<td>Mouse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fos</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>abl</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>raf(mil)(^5)</td>
<td>&quot;</td>
<td>ASV-17</td>
<td>Bos et al, 1988</td>
</tr>
<tr>
<td>jun</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ha-raS(^6)</td>
<td>Rat</td>
<td>HSV</td>
<td></td>
</tr>
<tr>
<td>Ki-raS(^7)</td>
<td>&quot;</td>
<td>KiMSV</td>
<td></td>
</tr>
<tr>
<td>fes(fps)</td>
<td>&quot;</td>
<td>Cat</td>
<td></td>
</tr>
<tr>
<td>fms</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>fgr</td>
<td>&quot;</td>
<td>HZ4FSV</td>
<td>Besmer et al, 1986</td>
</tr>
<tr>
<td>kit</td>
<td>&quot;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>sis</td>
<td>Monkey</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

All data taken from references given and from Hall, 1984.
These infectious particles, later identified as a virus, are now called the Rous sarcoma virus (RSV), and has been the subject of intense genetic and molecular research and much is known about the actions of its encoded products.

Analysis of the virus led to the identification of a single gene which was non-essential for viral maintenance, but was essential for transformation. Temperature sensitive mutants were isolated that could only transform cells at the permissive temperature, and the mutations were mapped to a single locus in the virus, the src gene (Toyashima and Vogt, 1969; Martin, 1970). We now know that the gene encodes a 599 amino acid protein, called pp60^src, which has tyrosine specific protein kinase activity. Despite an intensive research study, however, its normal cellular function is still obscure (Collet and Erikson, 1978; Collet et al, 1980; Schwärtz et al, 1983). To date, many more retroviral oncogenes have been isolated and characterised in the search for cancer causing agents (see table 2) and several have been used to map and isolate homologous genes in the human genome. They have therefore been essential towards the identification and understanding of genes involved in human tumorigenesis.

Why is it that when present in a retrovirus, genes apparently transduced from a normal cell should become transforming? Firstly, any gene that is carried by an actively transcribed retrovirus is expressed strongly due to the powerful LTR promoters, and is generally under no cell-specific control. This could be an important factor in escaping from the cellular restraints upon expression. Secondly, although the sequences are derived from their normal cellular homologues, they are often altered. Some are found to have simple base pair changes eg ras and neu, whilst others have lost portions, frequently their 5' amino terminal end, of their coding sequences (see section 1.3.2). Such alterations can result in an altered subcellular localisation of the onco-protein and alterations in the allosteric controls of its function or provide a cell with an unregulatable or ectopically expressed oncogene leading to transformation (Varmus, 1984; Bishop, 1987).

1.2.2 Viral Mutagenesis

The integration of a viral genome into host DNA can both bring genes in the surrounding chromatin under the control of its strong regulatory elements and also directly disrupt DNA sequences
as it integrates. This "insertional mutagenesis" by a virus has been shown to lead to cellular transformation when the cellular DNA sequences involved are proto-oncogenes (Bishop, 1987). This was first shown for the integration of the avian leukosis virus in viral induced chicken lymphomas which leads to the overexpression of the c-myc gene (Neel and Haywood, 1981; Haywood et al, 1981), and has been more recently highlighted in the isolation of the int family of oncogenes. This family of genes was isolated by studying the integration sites of the mouse mammary tumour virus (MMTV) in spontaneously arising mouse mammary tumours (Nusse and Varmus, 1982; Dickson et al, 1984). Several of these genes have been mapped and characterised (see Table 1.4) and in one case, that of the int-2 gene, the human homologue has been found to be amplified in a human breast neoplasia (Zhou et al, 1988).

1.2.3 DNA tumour viruses.

Unlike the retroviral oncogenes, the oncogenes encoded by the DNA tumour viruses such as polyomavirus, simian virus 40 (SV40) and adenovirus have no cellular homologues. They are capable of inducing cellular transformation and as such have been used as model systems for studying oncogenesis, although their precise functions remain unknown. Several are known to interact with host proto-oncogene products, for example the SV40 large T and adenovirus ElA proteins both interact with p53 (Lane and Crawford, 1979; Sarman et al, 1982) and more recently the adenovirus ElA protein has been shown to bind to the "anti-oncogene" (see 1.2.5) product of the retinoblastoma gene (Whyte et al, 1988).

1.2.4 The Focus Forming Assay and Cellular Oncogenes.

The focus forming assay was developed as a direct extension of the techniques of single gene transfer using naked DNA, developed in the late 1970's. It had been shown that fragments of the Herpes Simplex virus (HSV) thymidine kinase (Tk) gene could be used to complement Tk− mouse cells upon introduction of the HSV DNA using the calcium phosphate precipitation technique of Graham and van der Eb (1973) (Wigler et al, 1977; Maitland and McDougall, 1977). It was then shown that naked human genomic DNA could also be used to complement the same mutation and that the
complementation was due to the expression of transferred human thymidine kinase activity (Wigler et al, 1978).

Based upon these experiments, two groups demonstrated that specific sequences in particular human tumours could induce morphological transformation after DNA transfer (Shih et al, 1979; Cooper et al, 1980). Co-precipitation of tumour DNA with calcium phosphate and transfection into recipient cells resulted in repeatable, albeit low frequencies of morphologically transformed cells growing as foci on a monolayer of unaffected cells. These transformed cells were capable of anchorage independent growth in soft agar and were often tumorigenic in mouse tumour assays.

The first transforming sequence isolated using the focus forming assay was from the EJ bladder carcinoma cell line (Krontiris and Cooper, 1981; Shih et al, 1981). Multiple rounds of transfection, and the use of the human Alu repeated sequence to detect the transferred human DNA allowed the isolation of a single common genetic element responsible for the transformation. It was cloned and was found to be able to transform NIH3T3 cells at a much higher rate than the parental tumour DNA (Shih and Weinberg, 1982). Santos et al (1982) then showed that the transforming sequence that had been isolated was homologous to the viral oncogene from the Harvey sarcoma virus (HSV), the normal human homologue of which had already been cloned from the genome and shown to be non-transforming unless its transcription was driven by a powerful viral promoter (Chang et al, 1982a; 1982b). This paradox in transformation abilities of two apparently iso-genic sequences was not resolved until the full DNA sequences of the transforming genes were obtained. These were shown to differ by only one base-pair in their coding regions conferring a single amino-acid transition from glycine to valine in codon 12 (Capon et al, 1983). By implication, it was reasoned that this genetic lesion caused the in vivo malignancy.

Approximately 20% of tumours yield transforming sequences detectable in the focus forming assay. The majority are members of the ras family, consisting of Harvey-, Kirsten- and N-ras genes, although other classes of oncogene have been isolated. Most of the genes isolated carry alterations found specifically in the tumour material, but others have been isolated that are bi-products of the transfection process itself (see table 3).
TABLE 1.3 Oncogenes detected by DNA transfection.

<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Source</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Ha-ras-1</td>
<td>Various</td>
<td>Varmus, 1984</td>
</tr>
<tr>
<td>c-Ki-ras-2</td>
<td>Various</td>
<td>Varmus, 1984</td>
</tr>
<tr>
<td>N-ras</td>
<td></td>
<td></td>
</tr>
<tr>
<td>mcf-2</td>
<td>Human Mammary Carcinoma cell line MCF7</td>
<td>Noguchi et al,1987</td>
</tr>
<tr>
<td>B-lym</td>
<td>Intermediate B-cell lymphoma</td>
<td>Diamond et al,1983</td>
</tr>
<tr>
<td>T-lym</td>
<td>Intermediate T-cell lymphoma</td>
<td>Lane et al,1984</td>
</tr>
<tr>
<td>c-neu</td>
<td>Rat Neuroblastoma</td>
<td>Schecter et al,1984</td>
</tr>
<tr>
<td>met</td>
<td>Human osteosarcoma cell line</td>
<td>Cooper et al,1984</td>
</tr>
<tr>
<td>trk</td>
<td>Human Colon carcinoma</td>
<td>Martin-Zanca et al,1986</td>
</tr>
<tr>
<td>ks</td>
<td>Kaposi’s Sarcoma</td>
<td>Delli-Bovi et al, 1987a</td>
</tr>
<tr>
<td>ros1(mcf-3)</td>
<td>Human Mammary Carcinoma cell line MCF7</td>
<td>Birchmeier et al,1987</td>
</tr>
<tr>
<td>trk-2h</td>
<td>Human Breast Carcinoma cell line</td>
<td>Kozma et al,1988</td>
</tr>
<tr>
<td>trk-3mh</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ret</td>
<td>Human T-cell Lymphoma</td>
<td>Takahashi and Cooper,1987</td>
</tr>
<tr>
<td>ral</td>
<td>Multiple sources</td>
<td>Stanton and Cooper,1987</td>
</tr>
<tr>
<td>mas</td>
<td>human epidermoid carcinoma</td>
<td>Young et al; 1986</td>
</tr>
</tbody>
</table>

Notes

1 Result of a somatic rearrangement of a tyrosine kinase domain with the 5' sequences being donated by tropomyosin.
2 Arose by the rearrangement of the human ROS1 gene which deleted the extracellular domain leaving only the transmembrane and internal tyrosine kinase domain intact.
3 Both the trk-2h and the trk-3mh oncogenes are the product of recombination of the trk domain with other cellular sequences. See section 1.3.1 for more details.
4 Transfection artifact - transforming element has lost of 5' sequences leaving only 3' tyrosine kinase intact.
5 Has been isolated numerous times as an artifact of transfection. Sources include human adenocarcinoma, glioblastoma and rat hepatocellular carcinoma.
6 5' sequences donated by a ipr in both raf and met activation.(see King et al, 1988)
7 Now shown not to be an oncogene
The implications of the isolation of these recombinant fusion oncogenes and their potential uses for dissecting oncoprotein domains is discussed in more detail in section 1.3.3.

It is now known that the c-Ha-ras-1 gene can be activated by mutations in codons other than 12, including 13, 59, 61 and 63 (Varmus, 1984; Levinson, 1986). Codon mutagenesis studies, oligonucleotide analysis and direct sequencing has shown that such alterations are frequently found in human malignancies, particularly in the two evolutionarily related genes c-Ki-ras-2 and N-ras again indicating the importance of these mutations in the genesis of the tumours from which they were isolated. These genes are discussed in more detail in section 1.3.2.

The indicator cell lines used for the detection of transforming sequences have played a central role in the discovery of these cellular oncogenes. These cell lines retain many of the characteristics of normal fibroblasts (see Table 1.1) but do not senesce and can be grown indefinitely in vitro. The recipient cell line most often used for these assays is the mouse fibroblast cell line NIH3T3 which is abnormal when compared to primary cells, but it is precisely this degree of abnormality that makes it a suitable assay system (See 1.3.2). The focus forming assay has revealed many genes capable of inducing transformation in vitro and as an experimental procedure has been essential for oncogene research. Nevertheless, the predominant isolation of the ras gene family warrants a precautionary note to the use of these cells. In all certainty, this relates more to the repertoire of oncogenes detected by these cells rather than the preponderance of this class of oncogene in human cancer. Expansion of the assay to other gene transfer techniques may reveal a different sub-set of oncogenic sequences.

1.2.5 Tumour Specific Chromosome Alterations

Tumour cells frequently contain chromosomal rearrangements, most commonly translocations and deletions. In a few cases, there are consistent tumour specific alterations. A molecular analysis of such rearrangements have both demonstrated the involvement of cellular homologues of the myc and abl oncogenes, and identified novel putative oncogenes such as bcl-1 and bcl-2 (Tsujimoto et al, 1984a and 1984b).

B-cell Burkitt's lymphoma is characterised by the translocational activation of the c-myc gene at
8q24. A variety of different translocations lead to the abnormal expression of the gene by several different mechanisms (Rabbitts, 1986). These include alterations in the mRNA levels due to the mutation of RNA transcription pause sites (Bentley and Groudine, 1986a and 1986b) and alterations in the expression of alternatively encoded c-myc proteins (Hann et al, 1988). The complete mechanism of c-myc activation and function however, is still to be elucidated.

Chronic myelogenous leukemia (CML) is characterised by a common translocation between chromosomes 9 and 22 which juxtaposes the cellular homologue of the abl oncogene with a common region called bcr (breakpoint cluster region) (Heisterkamp et al, 1983). This results in the production of a fused transcript encoding a novel protein (Shtivelman et al, 1985).

Other tumours, notably sporadic cases of retinoblastoma and Wilm’s tumour, show constitutional deletion of specific chromosomal regions (Lele et al, 1969; Ricardi et al, 1978; van Heyningen et al, 1985). Both tumour types also show familial inheritance. There is growing evidence that both tumours result from mutations at a locus encoding a recessive gene or anti-oncogene. In the case of the inherited form, a germline mutation is thought to result in the tumour as a result of non-disjunction, mitotic recombination or somatic mutation (Knudson, 1985; Hansen and Cavenee, 1987). Sporadic cases are characterised by constitutional hemizygous deletion, which can homozygose by the same mechanisms as for the familial forms. Evidence to support the existence of such recessive loci comes mainly from three sources. Firstly, it has been known for some time that the fusion of normal and tumorigenic cells results in suppression of malignancy, suggesting that the normal cell can actively suppress the tumorigenicity (Harris et al, 1969; Craig and Sager, 1985). Malignancy reappears only on long-term culture and is associated with the loss of particular chromosomes, implying that specific genetic loci are involved (Stanbridge et al, 1981). Secondly, reduction to homozygosity of loci which are heterozygous in normal somatic tissue and which are syntenic with the putative suppressor gene, is frequently observed in tumours (Koufos et al, 1984; Orkin et al, 1984). The most direct source of evidence comes from the study of neoplasms in Drosophila melanogaster (Gateff, 1978). The best characterised mutation is the giant lethal-2 larvae. Flies that are homozygously mutated for this locus develop tumour-like growths in their larval stage. Reintroduction of the locus by P-element mediated transformation into
these homozygous mutant flies leads to normal development and suppression of the tumour formation
(Mechler et al, 1985).

In summary, evidence is steadily accumulating for an important role for recessive genes (which
have been termed anti-oncogenes) in normal and neoplastic growth (reviewed by Klein, 1987).

1.3 Oncogenes and Transformation

1.3.1 The c-Ha-ras-1 Oncogene.

Since its first isolation from the bladder carcinoma cell line (see section 1.2.2), the c-Ha-ras-1
gene and related genes have been amongst the most studied oncogenes. Much is known about the
biochemistry of their encoded proteins and about mutational alterations that confer tumorigenicity
(Levinson, 1986).

Gene Structure and Encoded Protein.

The c-Ha-ras-1 gene maps to the tip of the short arm of human chromosome 11 at band 11p15
(Huerre et al, 1983). Its coding region span approximately 4Kb of DNA and consists of 4 exons (Capon
et al, 1983). The 5' region of the gene contains an undermethylated CpG "island" characteristic of many
housekeeping genes (Bird, 1987), which has been shown to interact with the transcription factor SP1
(Ishii et al, 1986). Lying immediately 3' to the coding region is a stretch of tandemly repeated DNA
blocks termed the variable tandem repeat (VTR) (Capon et al, 1983). This region is highly polymorphic
in the population and certain rare length variants have been reported to be present in a dispropor-
tionately high number of cancer patients (Krontiris et al, 1985; Lidereau et al, 1986). This is a conten-
tious issue as others report no such association (Heighway et al, 1986; Thein et al, 1986; Gerhard et al,
1986; Mackay et al, in press).

The encoded protein has a molecular weight of 21Kd and is referred to as p21^{Ha-ras}. It is located
on the inner surface of the plasma membrane and is palmitylated at a cysteine residue at the C terminus
which acts as a membrane anchor and is essential for transformation (Willumsen et al, 1984). These
observations coupled with those that the ras proteins have a weak intrinsic GTPase activity and have
homology to the G-protein family of signal transduction proteins, has led to the suggestion that p21 functions as a transducing protein relaying growth stimulatory signals into the cell from cell surface receptors (Masters and Bourne, 1986). Recently it has been shown that the N-ras protein couples the bombesin and other growth factor receptors to the production of phosphoinositol compounds (Wakelam et al, 1986) linking the protein with established components of a cellular signal transduction system (Berridge, 1986).

Ras proteins purified after overexpression in E.coli have weak intrinsic GTPase activity which appears to be lowered in the oncogenic mutant proteins (Gibbs et al, 1984), although the relationship between this activity and transforming ability is not clear-cut. Recently, however, a protein present in mammalian cells called GAP (GTPase activating protein) has been identified which could resolve this matter. It has the ability to stimulate the GTPase activity of the wild-type proteins but has been found to have no affect on the mutant proteins (Trahey and McCormick, 1987). Whether GAP functions upstream to ras as a regulator or downstream as an effector is still to be clearly resolved (Sigal, 1988) although the evidence suggests that it interacts with the p21 effector domain (Cales et al, 1988; Adari et al, 1988). Thus, activation of the ras genes appears to result in an abnormally high level of p21-GTP in the cell, which results in a constantly "switched-on" signal.

Activation of ras genes.

The differences between the normal c-Ha-ras proto-oncogene and the activating transforming gene coding regions are usually in single base pair changes at one of a few critical positions, resulting in a single amino acid substitution in the encoded protein. These alterations have been identified in a wide range of human tumours including bladder carcinoma, urinary tract carcinoma, lung carcinoma and melanoma (Varmus, 1984). The codons most often affected are codons 12, 13 and 61. Seeburg et al (1984) constructed c-Ha-ras-1 genes bearing all possible mutations at codon 12 and all substitutions except proline gave rise to transforming ability. Mutational studies at codon 61 gave similar results (Der et al, 1986). In addition, two other sites, codons 59 and 63, have been identified as being transforming when altered by in vitro mutagenesis (Fasano et al, 1984). All six possible amino-acid changes due to single base-pair changes at codon 12 have been isolated as activating Ha-ras genes from human.
tumours (Hall, 1986).

The analysis of carcinogen induced tumours in the mouse and rat has shown that the same mutation is found frequently in independently arising tumours. For example, with the carcinogen DMBA (dimethylbenzanthracene) an A-T transition in codon 61 of the mouse Ha-ras gene is found in over 90% of the tumours (Quintanilla et al, 1986), whereas with NMU (nitrosomethylurea) predominantly G-A transitions in codon 12 of the rat Ha-ras gene occurred (Zarbl et al, 1985). Both systems used TPA (12-o-tetradecanoyl-phorbol-13-acetate) as the promoting agent.

More recently, Levinson's group have identified a mutation in the T24 bladder carcinoma Ha-ras gene that causes an increased transforming ability. They noticed that in co-transfer experiments with the myc gene into primary rat fibroblasts, a wild type Ha-ras gene carrying a mutant codon 12 was less transforming than the activated T24 gene. By constructing chimaeric genes, they were able to localise the modifying region to the intron between exons 3 and 4. A single A-G transition in the T24 gene apparently removes a splice site and leads to a more efficient production of ras coding mRNA. This single base-pair change increases the transforming ability 20 fold (Cohen and Levinson, 1988).

It is clear therefore that multiple mutations are occurring at this locus each of which may act alone or in concert with the other changes, either by contributing to tumorigenicity or by acting as predisposing factors.

Finally, comparison of the retroviral transduced Ha-ras oncogene with the structure of the genomic homologue led to the proposal that the activation of the Ha-ras gene can be brought about by the loss of an upstream untranslated exon (Exon -1) which is present in the normal gene but is absent in the viral genes (Cichutek and Duesberg, 1986).

It is clear that mutations that activate ras play an important role in tumorigenesis, but it is not clear what their precise role is in this process. Balmain and co-workers using the mouse skin as an animal model for mutagenesis (Balmain, 1986) have shown that mutations in ras occurred early in the initiation of a tumour (Balmain et al, 1984; Quintanilla et al, 1986; Brown et al, 1986). These studies showed both that DMBA induced benign papillomas contained Ha-ras mutations and that naked viral DNA could replace the initiating agent DMBA. This work shows clearly that ras mutations are
involved in the initiation stage of mouse skin papilloma formation. However, it has also been shown that ras plays a role in the maintenance of the transformed phenotype of a human tumorigenic cell line HT1080 (Paterson et al, 1988) and more recently that the Ki-ras gene is involved in the development of human colorectal tumours. Mutations of codons 12 and 13 coincide with the progression from small benign adenomas to dysplastic adenomas. Further changes then occur later to lead to the full development of adenocarcinomas (Fearon and Vogelstein, 1988). These apparently contradictory observations could reflect intrinsic differences between cell systems and species but more likely reflect the fact that there is no single pathway for tumour cell development.

The ras gene family.

Mammalian genomes contain other genes related to the c-Ha-ras gene. These are the N-ras and Ki-ras genes. Furthermore, comparisons with non-vertebrate species indicates a high degree of homology with genes in Drosophila, Dictyostelium, yeast and the sea-snail Aplasia, indicating that regions of the protein probably perform highly conserved functions (Marshall, 1986).

Whilst many ras genes are known to exist in a wide range of different organisms, none have been as well studied as those in yeast. S.cerevisiae contains two genes, RAS1 and RAS2, which both show extensive homology to the human ras genes. Remarkably, the residues which appear to be critical for the oncogenic activation of the mammalian genes are also conserved. This degree of conservation is also seen at the functional level. A truncated RAS1 (truncated by the removal of the C-terminal non-homologous section) containing a point mutation equivalent to the activating lesions of the mammalian genes, is capable of inducing morphological transformation of NIH3T3 cells (Defeo-Jones et al, 1985). It is also the case that human genes can complement the lethality of yeast doubly mutant in the RAS1 and RAS2 genes (Kataoka et al, 1985). It is obvious therefore that the proteins have some functional capabilities in common. The similarity of mutations in the yeast RAS genes to mutations in the cAMP transduction pathway led to the discovery that at least part of their function was as positive effectors of intracellular cAMP levels (Fasano, 1986). Whilst we know that direct control of cAMP production is not the primary functional role of the mammalian ras proteins, the yeast RAS genes are an ideal system for the further dissection of their activity. Yeast genetics enables the manipulation and dissection of
RAS activity not yet achievable in mammalian cells.

1.3.2 An integration of oncogenes.

Proto-oncogenes are normal cellular genes, so what activates them into becoming oncogenes? We have already seen that some mechanisms of oncogene activation were directly responsible for their first isolation, but it is clear that oncogene activation can come about by several mechanisms all of which lead to an abnormal expression of the encoded protein. Such mechanisms are known to include the acquisition of point mutations in the coding sequences, amplification leading to over expression, disruption of transcription patterns due to viral insertion or chromosome translocation or the inactivation of a constitutive suppressor.

Oncogene cooperativity and multistep progression to neoplasia.

It is clear that an isolated activated genomic oncogene alone is incapable of inducing malignant transformation of normal cells. Transfection of an activated c-Ha-ras-1 allele into primary fibroblasts gives no transformation despite the fact that the same DNA, when transfected into NIH3T3 cells results in transformation in an apparently dominant fashion. It follows therefore that activation of Ha-ras fulfils only one of the (several ?) necessary steps required for development of in vivo tumorigenicity.

It has been shown that groups of oncogenes such as Ha-ras and v-myc (Land et al, 1983), Ha-ras and E1a (Ruley et al, 1984) and v-Ha-ras and v-myc (Thomasson et al, 1985), which singly cannot transform primary cells, can lead to transformation when co-transfected into cells. This necessity for two or more oncogenic functions underlines the involvement of multiple steps in the transition from normal growth to the fully transformed state. It is clear therefore that cell lines such as NIH3T3 have already undergone one or more pre-requisite steps, including immortalisation, and hence the introduction of what might be more appropriately called co-oncogenic sequences can lead to the expression of the transformed phenotype in an apparently dominant fashion.
Roles of oncogenes in cellular physiology.

We now know a great deal about the normal role of proto-oncogene encoded proteins in cellular physiology, functions that have been assigned by both functional and structural homologies to other cloned or well studied proteins. A summary of the reported homologies and functions of a number of onco-proteins is in Table 1.4. From this it is clear that many are involved with growth regulatory pathways in the cell. Many oncogenes appear to lie on pathways that transduce positive growth regulatory signals from the cell surface to the nucleus. There are homologues of growth factors (sis, hst and int-2), growth factor receptors (fms, neu, erb-B, kit, trk and ret), tyrosine and serine/threonine kinases and nuclear proteins (myb, myc, jun, fos, ski and B-lym). It is now possible to construct a flow diagram of growth signal transduction in cells (see fig 1.1). This type of pictorial representation to unify the pathways and cellular roles of oncogenes, demonstrates the contribution that oncogene research has made to understanding cellular growth control.

Genes with homologies to genes known to be involved in the normal development of Drosophila (rel and int-1) have been identified also, although their roles in mammalian cells has yet to be elucidated.

Mechanisms of oncoprotein activation.

With such a wide variety of putative functions, how do perturbations in cellular growth occur, and are there common pathways into which the oncogenes all feed? The observation that certain genes are disrupted and that this disruption has caused the observed transformed cell has led to the identification of the oncogenes currently under study. Emphasis is now moving to study how the changes to oncoproteins directly affect the biochemistry of the protein's functions and their interactions with regulators and effectors.
<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>fms</td>
<td>Colony Stimulating Factor-1 receptor</td>
<td>Sherr et al, 1985</td>
</tr>
<tr>
<td>erbB</td>
<td>Epidermal Growth factor receptor internal domain</td>
<td>Downward et al, 1984</td>
</tr>
<tr>
<td>neu</td>
<td>Homology to erb-B. Receptor-like protein</td>
<td>Schechter et al, 198</td>
</tr>
<tr>
<td>mcf-3(ros1)</td>
<td>Receptor-like protein with loss of external domain, similar to trk, fms, erbB</td>
<td>Birchmeier et al, 198</td>
</tr>
<tr>
<td>kit</td>
<td>Receptor kinase</td>
<td>Qiu et al, 1988</td>
</tr>
<tr>
<td>trk, ret</td>
<td>Receptor kinases</td>
<td>Martin-Zanca et al, 1986; Takahashi and Cooper, 1987</td>
</tr>
<tr>
<td>mas</td>
<td>membrane protein</td>
<td>Young et al, 1986</td>
</tr>
<tr>
<td>bcl2</td>
<td>membrane protein</td>
<td>Tsujimoto et al, 1987</td>
</tr>
<tr>
<td>ras family</td>
<td>G-protein-related. Possible role in signal transduction</td>
<td></td>
</tr>
<tr>
<td>sis</td>
<td>B-chain of platelet derived growth factor</td>
<td>Styles et al, 1983</td>
</tr>
<tr>
<td>mos, raf</td>
<td>Serine/threonine protein kinases</td>
<td></td>
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Continued overleaf.
<table>
<thead>
<tr>
<th>Oncogene</th>
<th>Function</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>src, fps, tkl, yes, abl, fgr, lyn, lck, fes</td>
<td>Tyrosine specific protein kinases</td>
<td></td>
</tr>
<tr>
<td>crk</td>
<td>Homology to 180aa of Bovine brain Phospholipase C and non-catalytic region of src</td>
<td>Mayer et al, 1988</td>
</tr>
<tr>
<td>myc, fos, myb, ski, B-lym</td>
<td>Nuclear proteins: putative role in gene regulation</td>
<td></td>
</tr>
<tr>
<td>jun</td>
<td>Human homologue has homology to the transcription factor AP-1</td>
<td>Bohmann et al, 1987</td>
</tr>
<tr>
<td>int-2, hst, Ks</td>
<td>Members of the fibroblast growth factor family</td>
<td>Dickson et al, 1987; Taira et al, 1987; Delli-Bovi et al, 1987b</td>
</tr>
<tr>
<td>int-1</td>
<td>Homology to <em>Drosophila</em> segment polarity gene <em>wingless</em></td>
<td>Rijsewijk et al, 1987</td>
</tr>
<tr>
<td>rel</td>
<td>Homologous to the <em>Drosophila</em> Embryonic polarity gene <em>Dorsal</em></td>
<td>Steward, 1987</td>
</tr>
<tr>
<td>erb-A</td>
<td>Thyroid Hormone receptor</td>
<td>Sap et al, 1986; Weinberger et al, 1986</td>
</tr>
</tbody>
</table>
Flow diagram of positive growth stimulatory signal pathways across the cell membrane and into the nucleus of the cell. The figure shows some of the proposed general pathways of signal transduction such as phospho-tyrosine, phospho-serine, calcium ions (Ca²⁺), protein kinase C (PKC) and phosphoinositol (PI) and the proposed position of several oncoproteins in this pathway. See section 1.3.2. Diagram adapted from Hall, 1984 and Bishop, 1986.
There are several ways in which mutational alteration of proto-oncogenes might perturb normal growth including:

[1] Alterations which affect the biochemical characteristics of the protein such that its interactions with regulators or effectors is changed.

[2] Alterations in the level of expression which lead to an imbalance in cellular protein functions.

[3] Alterations in the protein structure eg loss of the NH$_2$ domains which lead to inappropriate or ectopic expression in a cell.

[4] Alterations which cause the expression in a cell type of a normally silent gene, with the consequence that its regulators are absent and hence its functions are unchecked.

The retroviral oncogenes $fgr$, $erb-B$, $abl$, $kit$ and $fms$ are all found as fusion proteins with viral $gag$ or other sequences. The cellular oncogenes $trk$ (and its derivatives), $bcr/abl$, $met$, $raf$ and $ret$ are also found in an active form as fusion partners. Several of the fusion oncogenes for which the fusion partners have been characterised are structural cytoskeletal proteins. The fusion partners for $met$, $trk$ and $v-fgr$ are laminin, tropomyosin and $\gamma$-actin respectively. The partners for the $trk$ derivatives are also proteins rich in basic amino acids suggesting a role in cell structure. Whether it is the generation of a fusion or the linking of two functions that is important remains to be seen.

The loss of an amino terminal domain appears to be common to the activation of several oncopenic sequences. The tyrosine specific protein kinases $kit$ (Qiu et al, 1988), $ret$ (Takahashi and Cooper, 1987), $v-erb-B$ (Downward et al, 1984), $v-abl$ (Wang et al, 1984), $fgr$ (Naharo et al, 1984) and the serine/threonine kinase $raf$ as well as the non-kinase nuclear protein $myb$ (Klempnauer et al, 1983) all have been isolated as active oncogenes and all have lost their usual 5' amino terminal domains. One explanation for this common method of activation is that the removal of a regulatory domain leaves a constitutive kinase activity. The protein kinase C family have such an amino terminal regulatory domain (Parker et al, 1986; Knopf et al, 1986).
Oncogenes and transgenics.

The development of transgenic mouse systems (Gordon and Ruddle, 1983) now allows the study of oncogene activity in vivo. It provides a new approach to the study of initiation and progression of tumours, to directly address the contributory roles of specific oncogenes towards tumour formation and may allow the dissection of multistep carcinogenesis. Transgenic oncogenes have been introduced in different forms, most notably under the control of various promoters. For example, the large T-antigen of SV40 has been introduced in several forms (see Hanahan, 1986 for review). When under the control of the rat insulin II promoter in transgenic mice it specifically induces pancreatic B-cell tumours (Hanahan, 1985) whereas brain tumours develop when under the control of the metallotheionein promoter (Brinster et al, 1984). The Hanahan insulin-TAg system is providing further insights into tumour development. When individual pre-neoplastic B-cells from transgenic mice carrying the insulin-SV40 construct are analysed, they are found to all be expressing the T antigen from a very early stage. However, only a small number of the cells will progress to form tumours. This number of cells which do progress has been shown to correlate with the number of cells capable of inducing blood vessel development in vitro culture systems. Firstly, this implies, as others have found, that the transgene alone is incapable of inducing transformation in vivo, and secondly that a further step towards tumorigenicity in this system is one of angiogenesis. Such systems offer the opportunity to also study the aspect of predisposition to tumourigenicity.

1.4 Gene Transfer Techniques

A number of systems have been used to facilitate the transfer of genetic material for donor to recipient cell, ranging from cell fusion to DNA mediated gene transfer (DMGT). Essentially, where these methods differ is in the amount and form of material transferred into the recipient cells. Cell fusion frequently results in the transfer of several chromosomes, whereas with DMGT contiguous stretches of DNA greater than 100Kb are rarely transferred. Each method has advantages and disadvantages, some of which are considered below, with particular reference to the study of the oncogenes, for both DNA- and chromosome-mediated gene transfer.
1.4.1 DMGT, its uses and limitations

As discussed earlier, DNA mediated gene transfer (DMGT) with focus formation as the selective criterion, has been essential for the identification and isolation of cellular oncogenes. (Section 1.2.3) The process of DMGT is invariably associated with DNA scrambling which greatly aided the isolation of specific single copy sequences as rarely are contiguous sequences of donor DNA greater than 50-100Kb in length transferred intact. This has several effects: Secondary and tertiary transfections from primary transfectants often have much higher frequencies of transformation than did the original tumour DNA. This is most likely due to the amplification of sequences transferred or the segregation of negatively controlling flanking DNA sequences. The scrambling also has the effect that in a series of primary transfectants, few sequences will be common to all transfectants, so making the identification of the transforming element easier.

Another result of the phenomenon of DNA scrambling has been the isolation of transfection-generated recombinant oncogenes such as \textit{irk-2} and \textit{raf} (see table 1.3). These have been isolated as transforming elements in focus forming assays from various tumours and have been found to be fusions between genes non-syntenic in the original tumour material. These events, not dissimilar to retroviral fusions, are useful as they identify proto-oncoprotein domains which might become activated for example in chromosome translocations. This generation of fusion oncoproteins has been exploited further by using the \textit{irk} domain in transfections to detect fusion partners that will transform cells (Kozma et al, 1988; Oskam et al, 1988). This approach has the potential for further exploitation.

As a gene transfer technique, DMGT has proved invaluable. However, with the growing realisation that many human genes are beyond the transfer capability of DMGT, the emphasis has turned to techniques of DNA such as chromosome mediated gene transfer (CMGT) that might transfer larger fragments of DNA.

1.4.2 Chromosome mediated gene transfer.

The introduction of chromosomal DNA into recipient cells was first demonstrated in 1973 by McBride and Ozer (McBride and Ozer, 1973) but until recently, it had remained a technique of limited
use, mainly due to the lack of selection systems and worries about the integrity of the transferred material.

McBride and Ozer transferred wild-type chinese hamster chromosomes into a mouse cell line defective for the purine salvage enzyme HPRT, selecting for the uptake of the chinese hamster HPRT locus using the selective HAT medium. Their transfer frequencies and those of other early workers were around 1 in $10^7$ cells, a figure very close to the spontaneous rate of reversion for some mutant alleles (Reviewed in Klobucher and Ruddle, 1981). This meant that each successful transfection had to be confirmed biochemically, which in the case of McBride and Ozer was done by electrophoretically separating the donor and recipient HPRT species.

These early transfection frequencies were improved upon by using techniques developed for the transfer of viral DNA into cells. The modifications found to be of most use were those of calcium phosphate precipitation and the technique of "shocking" the cells with DMSO (Miller and Ruddlle, 1978; Klobucher and Ruddle, 1981). These adaptations raised the transfection frequencies to about 3 in $10^5$ cells and form the basis of a reliable, reproducible and useful gene transfer system.

The transgenomes generated by CMGT range in size from 50Kb to a whole chromosome (Olsen et al, 1981; Murphy et al, 1985; Porteous et al, 1986) and rearrangements of the donor genetic material appear to be frequent although the transfer of syntenic markers is frequently observed (Porteous et al, 1986; Pritchard and Goodfellow, 1987). Whether these rearrangements are due to interactions in the precipitate, due to degradation in the cytoplasm or are transgenome stabilisation products remain to be seen. Upon initial transfection the transgenomes exist as independent units but these cells are unstable, stabilisation occurring upon the association of the donor chromosome with the host chromosomes (Klobucher and Ruddle, 1979).

Human transgenomes on a mouse background can be visualised in a number of ways. Only the largest transgenomes can be visualised by conventional histochemical staining techniques, but for the smaller transgenomes an estimate of transgenome size and copy number can be obtained by in situ hybridisation with fluorescence detection or autoradiography (Mitchell et al, 1986; Gosden and Porteous, 1987). At the molecular level, several methods have been used to analyse the transfer of DNA
sequences. Unique-sequence X chromosomal DNA probes (Olsen et al, 1981) and expressed X sequences (McBride et al, 1982) made by the removal of repetitive and non-expressed DNA elements by \textit{in vitro} pre-annealing of DNA, were used to analyse HPRT-selected X chromosome transfectants.

The use of cloned human \textit{alu} and L1 repeats has been demonstrated (Minden et al, 1984; Porteous et al, 1986; Pritchard and Goodfellow, 1987) to be a more generally applicable approach. Due to varying sizes of repeat and the loss of restriction sites around individual repeats a ladder of specific restriction fragment length variations is generated upon restriction enzyme digestion and hybridisation (Porteous et al, 1986). As the human L1 repeat is estimated to be present once every 150Kb on average (Shafit-Zagardo et al, 1982), the number of bands present not only gives an indication of faithful transfer but can be used as an estimate of transgenome size.

Until recently, one of the major limitations of CMGT was the lack of suitable selectable markers present in the human genome. Novel methods to overcome these problems have now been developed:

1. The integration of a marker dominantly selectable in mammalian cells, that of neomycin resistance (Southern and Berg, 1982) by both homologous recombination (Smithies et al, 1985), with the use of retroviral vectors (Weiss et al, 1986) and by conventional DMGT means that new selectable markers can be integrated into the genome. Isolation of clones carrying the insert in the required chromosomal location can then be used as donors for gene transfer techniques.

2. If the neomycin resistance encoding plasmid DNA is mixed with mitotic donor chromosomes and a co-precipitate is formed, both plasmid and chromatin become physically linked and will co-integrate. Therefore only the minority of cells carrying the resistance marker will survive selection. This facilitates the co-selection of rare, chromatin encoded functions (Pritchard and Goodfellow, 1986; Porteous et al, 1986).

3. The use of activated cellular oncogenes as selectable markers for transfer into transformation competent cell line such as NIH3T3 or C127 means that a wide range of intrinsic loci dispersed throughout the genome are now useable (Morten et al, 1987). This selection is not limited to cellular oncogenes but can also be used with integrated transforming viruses such as SV40 (see Table 1.6 and Porteous, pers comm).
Since its first use in 1973, CMGT has had limited uses, but it is now finding a new lease of life as a tool for the molecular dissection of human diseases such as cystic fibrosis (Scambler et al, 1986; Porteous and van Heyningen, 1986) and Wilm's tumour (Porteous et al, 1987; Bickmore et al, 1988). CMGT is now set to play a role in more global human molecular mapping of the genome (Porteous, 1987).

1.4.3 The focus forming assay and CMGT.

We have seen that DMGT in conjunction with the focus forming assay has played an essential role in the isolation and understanding of the cellular oncogenes (see sections 1.2.4, 1.3.2) but as long ago as 1974 the transfer of transforming elements had been demonstrated using naked mitotic chromosomes (Shani et al, 1974), although transforming elements from these early transfers were not characterised.

The work of Shani et al, Cassingena et al and Spandidos and Siminovich (see table 1.5) all suggested that genetic elements for anchorage independent growth and tumorigenicity could be transferred to recipient cells using CMGT. However, the analysis of the transgenomes generated was hampered by the fact that these transfers were between similar or identical species (Shih et al, 1979) and DNA analysis was not available.

The successful transfer of malignancy using chromosomes from the bladder carcinoma cell line is the most fully investigated oncogene selected transfer to date (Porteous et al, 1986a; Porteous et al, 1986b; Morten et al, 1987). This is described in detail in section 1.5.

1.5 c-Ha-ras-1 selected CMGT

The transfer of mitotic chromosomes from the EJ bladder carcinoma cell line EJ-18-8D, followed by selection for cells receiving a copy of the c-Ha-ras-1 oncogene, has been described (Porteous et al, 1986a; 1986b) (see fig 1.2).
<table>
<thead>
<tr>
<th>Transferred locus</th>
<th>Donor</th>
<th>Recipient</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>SV40</td>
<td>Chinese hamster cells</td>
<td>Monkey cells</td>
<td>Shani et al, 1974</td>
</tr>
<tr>
<td>Unknown-transfer of anchorage independence and tumorigenicity</td>
<td>Chinese hamster ovary cells</td>
<td>Primary hamster lung fibroblasts</td>
<td>Spandidos and Siminovich, 1977 and 1978</td>
</tr>
<tr>
<td>SV40, Ad5 and spontaneous mouse and human tumours</td>
<td>Various mouse and human cell lines</td>
<td>Normal diploid human cells</td>
<td>Cassingena et al, 1978</td>
</tr>
<tr>
<td>Unknown</td>
<td>Chemically transformed mouse cells</td>
<td>NIH3T3 cells</td>
<td>Shih et al, 1979</td>
</tr>
<tr>
<td>N-ras?</td>
<td>Human CML cells</td>
<td>NIH3T3 cells</td>
<td>Minden et al, 1984</td>
</tr>
<tr>
<td>Ha-ras-1</td>
<td>Human bladder carcinoma cell line EJ-18-8D</td>
<td>Mouse C127 cells</td>
<td>Porteous et al, 1986</td>
</tr>
<tr>
<td>met</td>
<td>Chemically transformed human cell line MNNG-HOS</td>
<td>NIH3T3 cells</td>
<td>Scambler et al, 1986</td>
</tr>
</tbody>
</table>
Figure 1.2

Oncogene selected chromosome mediated gene transfer. The figure represents the general chromosome transfer protocol and the selection strategies used by Porteous et al. (1986a) to generate the series of transfectants discussed in section 1.5. Figure taken from Morten et al., 1987.
These experiments generated a set of transfectant mouse/human hybrid cell lines carrying varying lengths of human DNA linked to the oncogene. A tumorigenic analysis of these transfectants revealed variations in both the *in vivo* and *in vitro* phenotypes (Porteous et al, 1986b). This analysis has been continued in the present study and one particular cell line, E65.5, has been studied most closely at both the molecular and tumorigenic level. Below I shall describe the transfectants in general and then proceed to describe E65.5 in more detail. All the observations in section 1.5 are taken from Porteous et al, 1986a, 1986b and Morten et al, 1987.

1.5.1 The Transfectants

In contrast to both DNA- and plasmid mediated gene transfer, which give rise to foci between 1 and 3 weeks post-transfection, the foci generated from chromosome transfection can take up to 4-5 weeks to appear (Porteous et al, 1986). Although the reason for this is unknown, it probably reflects the time taken for transgenome stabilisation and oncogene expression.

The transfectants generated can be distinguished by characteristic morphological and phenotypic differences which are stable on sub-culturing, extended passage *in vitro* and passage through mice. The cells vary considerably in their adherence to the tissue culture vessel, ranging from ones attached only loosely which can be easily detached by gentle washing with PBS to very well attached cells which require trypsin/EDTA for detachment. The morphologies of the cell lines vary in parallel, ranging from highly refractile dendritic cells to flat 'pavement-like' cells that resemble the parent C127 cells. As can be seen from figure 1.3, transfectant E65.7 grows to a low cell density and forms small colonies in soft agar. Transfectant E65.6 is more dendritic than E65.7 and grows to a higher cell density. It also forms large soft agar colonies which are, however, never as dense as those seen with the transfectant E65.5 which grows to a very high cell density in culture.

The ability to form tumours in neonatally thymectomised mice (Hay et al, 1985; Morten et al, 1984) reveals yet another facet of the variation present (See Fig 1.4) All the transfectants generate tumours, but with considerable variation in tumour incidence and take rate, especially upon co-innuculation of a decreasing numbers of transfectant cells with C127 cells.
Figure 1.3

Variation in phenotypes in HRAS1-selected CMGT. Three individual transformants (E65.5 a,b,c, E65.6 d,e,f and E65.7 g,h,i) are shown growing in monolayer culture (a,d,g), tumour histology (b,e,h) and growing in soft agar (c,f,i). Figure taken from Morten et al, 1987. See section 1.5.1.
Both as a single innocula down to the level of $10^3$ cells and as low as 10 cells when co-
innoculated with C127 cells, the transfectant E65.5 will form tumours. This is over 100x higher than
any other transfectant. While the transfectant E65.7 grows only poorly in soft agar and forms tumours
very weakly, 10% of mice bearing E65.7 tumours show invasion of the mouse peritoneal cavity. In the
majority of other transformants we see little if any invasive spread. This low level of invasion is in con-
trast to many reported cases of the induction of invasive and metastatic phenotypes with re-introduced
cloned ras genes in NIH3T3 (Bradley et al, 1986; Vousden et al, 1986; Thorbjergsson et al, 1985).

At the physical level, the amounts of human DNA present in the transfectants has been studied
using several methods; in situ hybridisation with fluorescently labelled anti-AAF monoclonal antibodies
for detection of AAF- (N-acetoxy-2-acetylaminoflourine) modified human DNA probe (Mitchell et al,
1986) has shown that the transgenomes are present as discrete blocks of DNA inserted into mouse chro-
mosomes. A more sensitive double-labelling technique using biotinylated human DNA in combination
with a tritiated Ha-ras probe shows that each block of human DNA contains at least one copy of the
Ha-ras gene (Gosden and Porteous, 1987). The transfectant E65.5 contains 4 blocks of human DNA
which can only be visualised with gold-enhancement. This precludes the use of a tritiated ras probe and
so the oncogene copy number remains uncertain.

At a molecular level, the use of the L1 repeat probe to study the integrity of the DNA transferred
has revealed that several transfectants have essentially subsets of the chromosome 11 fingerprints
(transfectants E67.4 and E67.1) However, others appear to have rearranged or amplified bands present,
indicating DNA rearrangements (transfectants E65.6 and E65.5).

With two notable exceptions, restriction mapping around the Ha-ras locus suggests that this
region has been transferred intact. The transfectants E65.5 and E65.7 had both lost regions around the
locus. As can be seen from figure 1.5, E65.5 has lost all restriction sites 3' to the Ha-ras gene indicat-
ing a translocational-like rearrangement, whereas both 3' and 5' sites have been lost in E65.7 resem-
bling a transpositional-like rearrangement. It is interesting to note that the two transfectants which have
rearrangements at the Ha-ras locus are at the two extremes with respect to tumorigenicity.
Figure 1.4

The effects of both absolute cell inoculum and of co-inoculation with untransformed C127 cells upon the in vivo tumorigenicity of independent Ha-ras selected CMGT's as described by Porteous et al (1986b). Figure taken from Morten et al, 1987.

<table>
<thead>
<tr>
<th>CELL INOCULUM PER SITE</th>
<th>Tumour Incidence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C127</td>
</tr>
<tr>
<td>C127 HRAS1-CMGT</td>
<td></td>
</tr>
<tr>
<td>0 5x10^5</td>
<td>4/4</td>
</tr>
<tr>
<td>0 10^6</td>
<td>2/2</td>
</tr>
<tr>
<td>0 5x10^4</td>
<td>0/2</td>
</tr>
<tr>
<td>0 10^4</td>
<td>0/4</td>
</tr>
<tr>
<td>0 10^3</td>
<td>0/4</td>
</tr>
<tr>
<td>10^7 10^4</td>
<td>1/2</td>
</tr>
<tr>
<td>10^7 10^3</td>
<td>1/4</td>
</tr>
<tr>
<td>10^7 10^2</td>
<td>0/4</td>
</tr>
<tr>
<td>10^7 10^1</td>
<td>0/8</td>
</tr>
<tr>
<td>10^7 0</td>
<td>0/26</td>
</tr>
</tbody>
</table>

N.T. Not tested.

Figure 1.5

Rearrangements at the HRAS1 locus in CMGT. Genomic restriction maps of the Ha-ras locus of EJ-18-8D (indicating four exons and the VTR region), E65.6 (no changes), E65.5 (all sites 3' to the SacI site lost) and E65.7 (all sites 5' to the TaqI and 5' to the BamHI sites lost). B=BamHI, S= SacI, H=HindIII, K=KpnI, E=EcoRI, T=TaqI, Bg=BglII and X=XhoII. Figure taken from Morten et al, 1987.
1.6 Molecular mechanisms of chromosomal rearrangements.

Chromosomal changes in tumour cells are very common, but the molecular basis of such changes is still to a great extent unknown. The most widely understood mechanism of rearrangement is found in the human and mouse T- and B-cell neoplasias (reviewed by Showes and Croce, 1987).

The somatic rearrangement of the variable regions of the immunoglobulin and T-cell antigen receptors involves the recombination of specific chromosomal regions (V, D and J) into expressed units. This recombination is directed by a series of conserved signals which flank each of the elements and consist of a palindromic heptamer and a nonamer separated by a spacer of 12 or 23 base pairs (reviewed by Tonegawa, 1983). These rearrangements are performed by a VDJ recombinase (Yancopoulos et al, 1986). Aberrant activity of the recombinase, where a genuine signal is linked to an erroneous signal, has been found in a small number of B- and T-cell neoplasias (Haluska et al, 1986; Finger et al, 1986; Boehm et al, 1988a) and it has been suggested that this mechanism may have a more general role to play in other translocations (Showes and Croce, 1987).

A mechanism of chromosomal rearrangements not involving immunoglobulin recombinase activity is that which involves recombination between two alu repetitive DNA elements. These have been found at the breakpoints of the 9:22 Philadelphia chromosome translocation (Heisterkamp et al, 1985; Rogers 1985; Heisterkamp and Groffen 1985; de Klein et al, 1986), deletions at the LDL receptor locus (Lehrman et al, 1985) and deletions in the human B-globin gene cluster (Vanin et al, 1983).

Many translocations appear not to be mediated by either of the aforementioned mechanisms, and in these cases analysis has revealed conserved motifs or small regions of homology at or near to the breakpoint. Examples of this include the c-myc locus in mouse plasmacytomas (Picolli et al, 1984) and an X:21 translocation associated with muscular dystrophy in a female (Bodrug et al, 1987). Steinmetz et al (1986) identified a repeated motif at a recombinational hotspot in the mouse major histocompatibility complex which is highly homologous to the minisatellite core sequence of Jeffreys et al (1985).

Even when a recombination event is reciprocal, it is frequently not conservative in nature i.e. base-pairs are added or lost at the site of cross-over. For example, the muscular dystrophy-associated X:21 translocation described by Bodrug et al (1987) which appears to have been driven by a single 4
base pairs of homology (CGGC) results in the loss of 71-72bp from the X chromosome and 16-23bp from chromosome 21.
1.7 Approaches to the study of E65.5.

The extreme degree of tumorigenicity of the transfectant cell line E65.5 and the presence of a rearrangement within 2Kb of the Ha-ras gene raises the question as to whether or not the two are causally linked. The major aims of the present study have been to examine this possibility and to gain a molecular insight into the chromosomal rearrangement that has occurred at the E65.5 HRAS1 locus. To fulfil this, a study of the transfectant E65.5 has been undertaken at both the molecular and cellular levels.

In chapter 3, I extend the expression analysis of Porteous et al (1986b) to an analysis of HRAS1 protein levels to delineate any relationship between expression and tumorigenic potential.

An analysis of both DNA- and chromosome-mediated secondary gene transfectants isolated using the focus forming assay has been used to in an attempt to relate transforming ability to physical structure around the HRAS1 locus in E65.5. This work is presented in chapter 4.

In an attempt to understand the possible mechanism of rearrangement, the E65.5 HRAS1 locus has been cloned. This has allowed me to determine whether the Ha-ras coding sequences are directly involved in the rearrangement. The construction of a purpose built library in the vector EMBL3, isolation of the rearranged Ha-ras clone and localisation of the breakpoint region is presented in chapter 5. A characterisation of and sequence analysis of the breakpoint is presented in Chapter 6.

The normal unrearranged progenitor to the DNA translocated next to the HRAS1 locus in E65.5 has been cloned and an initial characterisation of it is presented in chapter 7.

The transfectant E65.5 has been analysed at a more global level and in relation to the family of chromosome transfectants by the isolation of random lambda clones and by repeat mapping the human DNA content.
CHAPTER 2
2 MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES.

2.1.1 Cell Line.

C127 is an immortal but morphologically non-transformed mouse cell line derived from a mammary tumour of an RIII mouse (described in Lowy et al, 1978). EJ-18-8D is a clonal derivative of the human EJ Bladder carcinoma cell line. It is essentially diploid and is tumorigenic in nude mice (Hastings and Franks, 1983). All transfectant cell lines including E65.5 were generated by the introduction of CaPO₄ precipitated mitotic chromosomes from EJ-18-8D into the mouse C127 line as described in Porteous et al, 1986a.

2.1.2 General maintenance.

All cells were grown attached to plastic tissue culture vessels in Dulbecco's modified minimal essential medium (DMEM) supplemented with 5% fetal calf serum at 37°C under 10% carbon dioxide. Cells were routinely fed or subcultured twice-weekly. Subculturing was done by splitting the cells between 1:5 and 1:40 into fresh medium. Cells to be subcultured and harvested were first washed with Dulbecco's phosphate buffered saline (PBS) and detached from the culture vessels. EJ-18-8D, EJ-18-3E and C127 cells were detached with versene containing 5% trypsin; E67.4 was detached with versene alone and the transfectant cell lines (including secondary DNA, plasmid and lambda clone transfectants) were all detached with PBS containing 10% versene. Harvested cells for DNA preparation were washed in PBS, pelleted and stored frozen at -70°C. Frozen stocks were made by resuspending harvested cells in 90% FCS/10% dimethylsulphoxide (DMSO) at not less than 10⁶ cells /ml and freezing slowly overnight in vials at -70°C in a polystyrene box followed the next day by transfer to liquid nitro-
gen. Cell lines were regenerated from frozen storage by resuspending rapidly thawed cells in 10mls of medium and plating in fresh tissue culture vessels in DMEM +5% FCS.

2.1.3 Calcium Phosphate/DNA mediated gene transfer.

DNA transfection was carried out using calcium phosphate precipitation and incorporating a glycerol shock to increase the transfection frequency (Graham and Van der Eb, 1973; Parker and Stark, 1978).

5x10^5 recipient C127 cells were plated per 90mm dish 16-20 hours prior to the transfection.

Precipitate per dish: Aliquots of DNA were made to a volume of 350μl with sterile water and mixed thoroughly with 50μl of sterile 50mM calcium phosphate. This was added dropwise to 0.4mls of 2xHEBS with a gentle stream of air bubbles passing through it to ensure complete mixing and even precipitate formation. The precipitate was left to stand whilst the cells were prepared. Cells were drained of medium, washed once with PBS and the precipitate was added and spread evenly over the dish. This was left to stand for 20 minutes at room temperature after which 8mls of medium was added and the dish incubated at 37°C for 4-6 hours under 10% CO₂. The medium/precipitate was removed and 2.5mls of 15% glycerol in HEPES buffered saline was added for exactly 2 minutes then aspirated and washed off completely. 10mls of fresh medium was added and the cells incubated overnight as normal. The following morning cells were detached with trypsin/versene and reseeded evenly in two T175cm² flasks. The cells were maintained by twice weekly feeds thereafter.

2xHEBS is 280mM NaCl, 50mM HEPES, 1.5mM Na₂HPO₄, 10mM KCl pH7.1.

HEPES buffered saline +15% glycerol is 140mM NaCl, 25mM, 0.75mM Na₂PO₄, pH7.15. Make to 15% glycerol and autoclave.

HEPES is 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

2.1.4 Colony isolation by ring-cloning.

The tops were removed from tissue culture vessels and a sterile plastic cloning ring, sealed with grease was placed over a suitable colony. Cells were detached with a drop of versene for 30 seconds, washed-off with medium and seeded into microwells.
2.1.5 Soft agar cloning.

$10^3$ cells were innoculated into 2mls of 0.25% low gelling temperature agarose containing DMEM + 5% FCS. After gentle mixing these were poured onto a 0.5% agarose/medium base and allowed to harden for 5 minutes at 4°C. Colonies were fed every 5 days by the addition of a few drops of FCS and were scored at 9 or 14 days.

2.2 CELLULAR DNA AND RNA ISOLATION.

2.2.1 Extraction of Genomic DNA from cultured cells.

High molecular DNA was prepared by standard protocols as modified by Porteous (1985). Cell pellets were resuspended in a small volume of PBS and lysed in a volume of 0.5% SDS, 100mM NaCl, 100mM TrisCl, 100mM EDTA, pH8.0, to give approximately $10^7$ cells per ml. RNA and protein was digested using first RNase A at a concentration of 0.1mg/ml for 1 hour followed by Pronase at 0.25mg/ml for 4 hours, both at 37°C. The lysates were extracted gently for 5 minutes with an equal volume of phenol. The phases were seperated by centrifugation at 2500rpm for 10 minutes and the aqueous phase was sequentially re-extracted with equal volumes of 25:24:1 phenol/chloroform/iso-amyl alcohol and 24:1 chloroform/iso-amyl alcohol. A half volume of 7M ammonium acetate and 2 volumes of absolute ethanol were used to precipitate the DNA which was spooled-out with a sterile glass rod, air dried, rinsed in 70% ethanol, and finally dissolved overnight at 4°C in TE.

2.2.2 Extraction of total RNA from cultured cells.

Total RNA was prepared as described by Meehan et al (1985). $10^8$ cells were harvested, washed thoroughly with PBS, pelleted and resuspended in 10mls of 8M guanidine HCl pH7.5. They were then homogenised in a hand held sterile B homogeniser on ice. The debris was removed by centrifugation at 8000rpm for 5 minutes at 4°C and the RNA precipitated from the supernatant by the addition of a half volume of cold absolute ethanol at 20°C for 45 minutes. The RNA was pelleted by centrifugation at 10,000rpm at 4°C for 5 minutes, resuspended in 6mls of guanidinium, re-homogenised, re-precipitated and re-pelleted as before. The RNA pellet was then resuspended in 4mls of DEPC water, homogenised
in a clean tube to remove traces of guanidinium and precipitated with a half volume of 7.5M ammonium acetate and 2 volumes of cold absolute ethanol at -70°C for one hour. After pelleting as before and resuspending in DEPC water, the RNA was finally precipitated with one tenth volume of 2M sodium acetate pH5 and 2.5 volumes of cold absolute ethanol at -70°C for one hour. The RNA was resuspended in DEPC water and stored frozen at -70°C. The concentration was estimated from the absorption at OD_{260nm} (see 2.2.4).

DEPC water is prepared by mixing thoroughly 0.5ml of DEPC (diethyl pyrocarbonate) with 500mls of distilled water. The DEPC is then destroyed upon autoclaving.

2.2.3 Isolation of Poly A⁺ RNA.

Polyadenylated RNA was purified by passing total RNA over an oligo-dT cellulose matrix column as described in Davies et al (1986). Non-adenylated species were washed from the column and the poly-A RNA was eluted with low salt.

A sterile, glass-wool plugged Pasteur pipette was loaded with approximately 0.5mls of oligo-dT slurry suspended in buffer I and rinsed with 1ml of 0.1M NaOH containing 5mM EDTA, and then with DEPC water until the pH of the effluent was less than 8.0. The column was equilibrated with 5mls of buffer II. 5mg of total RNA was made to a volume of 500μl with DEPC water, heated to 65°C for 5 minutes and 500μl of buffer II was added. These were mixed thoroughly, cooled to room temperature and loaded onto the column. The eluate was re-heated to 65°C and, as before, cooled and reapplied to the column.

The non-polyA species of RNA were removed from the column by washing with 5mls of buffer II and then 3mls of elution buffer III was added and 0.2ml fractions were collected. The OD's of the fractions were read at 260nm and fractions containing RNA were pooled and precipitated with one tenth volume of 3M sodium acetate and 2.5 volumes of absolute ethanol. The polyA RNA was stored frozen at -70°C.

Buffer I is 20mM Tris pH7.4, 0.1M NaCl and 1mM EDTA; Autoclaved and made to 0.1% SDS.

Buffer II is 40mM Tris pH7.4, 1M NaCl and 1mM EDTA; Mixed, autoclaved and made to 0.1% SDS.
Buffer III is 10mM Tris pH 7.4 and 1mM EDTA; Autoclaved and made to 0.05% SDS.

DEPC water was used to make all solutions. SDS was heated at 68°C for 2 hours to destroy RNA‘ase activity.

2.2.4 Determination of nucleic acid concentration by optical density measurement.

The concentration of DNA and RNA was estimated by reading the $OD_{260nm}$. An OD of 1.0 is equivalent to 50ugm/ml DNA and 40ugm/ml RNA and ssDNA.

To estimate the concentration of oligonucleotide solutions the molar extinction coefficient for the oligo must first be determined by summing the contribution made by each individual nucleotide. This is done using the following values: A 15,200, C 7050, G 12,020 and T 8400. The molar extinction coefficient = $OD_{260nm}$ equivalent to a 1M solution.

2.3 BACTERIAL CULTURE AND MANIPULATION.

2.3.1 Bacterial strains used.

All bacteria used were of strain K12.

Q359 $hsdR^-_k$, $hsdM^+_k$, supF,Φ80,P2 (Karn et al 1980).

Q358 as for Q359 but does not carry a P2 prophage (Karn et al 1980).

These strains were used for the production of EMBL3 libraries. The P2 prophage is the basis of the selection against non-recombinant EMBL3 clones.

1255 is a recombination deficient strain used for the isolation and maintenance of unstable phage clones (Arthur Mitchell, pers comm).

JM83 araΔ(lac-proAB), rPL(=srA),Φ80,lacZΔM5 (Vierra and Messing, 1982).

JM83 was the host for all manipulations with the plasmid vector pTZ.

NM522 $hsd$ ID 5,Δ(lac-proAB0, supE, hi[F',proAB,lacIqZΔM15]) (Gough and Murray, 1983).

NM522 was the host used for the production of single-stranded DNA from recombinant pTZ plasmids.
DH1 F, endA1, hsdR17(rk-,mk-), supE44, his-1, recA1, gyrA96, relA1 (Hanahan, 1985).

DH1 was used as a host to grow the plasmid pEJ.

2.3.2 Media and additives.

All media was sterilised by autoclaving.

**CY Broth.**

Per litre: 10 gm casamino acids, 5gm yeast extract, 3gm sodium chloride, 2gm potassium chloride. pH 7.2.

**H Broth.**

Per litre: 10gm tryptone and 8gm sodium chloride. pH 7.2.

**L Broth.**

Per litre: 2.46gm magnesium sulphate, 10gm tryptone, 5gm yeast extract, 5gm sodium chloride, 1gm glucose. pH 7.2.

**2xTY Broth.**

Per litre: 16gm disodium hydrogen orthophosphate, 10gm yeast extract, 5gm sodium chloride. pH 7.0.

"Terrific Broth ". (BRL Focus 1987.)

Per 900ml: 12gm tryptone, 24gm yeast extract, 4.0mls glycerol. Add 100mls of 0.1M KH₂PO₄/0.72M K₂HPO₄ after sterilising both solutions.

**SOB and SOC Broth.**

Basic Media Per Litre: 20gm tryptone, 5gm yeast extract, 0.59gm sodium chloride, 1.86gm potassium chloride.

SOB: Just prior to use make media 10mM magnesium chloride and 10mM magnesium sulphate from filter sterilised stock 1M solutions of each.
SOC: SOC medium is SOB plus 20mM glucose. (From a Filter sterile 2M Stock.)

Agar and Agarose.

All solid media were made by the addition of agar or agarose in the following amounts: Top agar/agarose 6gm/litre; Bottom agar 12gm/litre.

Glucose/Minimal agar plates.

1 Litre: 15gms of agar in 900ml water. Autoclave and whilst still liquid add 100mls 10x M9 sals, 1ml 1M magnesium sulphate, 1ml 0.1M calcium chloride, 1ml 1M thiamine HCL and 10mls 20% glucose. All these should be sterile solutions.

10xM9 salts is: Per litre 60 gm NaHPO₄, 30gm KH₂PO₄, 10gm NH₄Cl 5gm NaCl. Sterilise by autoclaving.

Media Additives.

Ampicillin was used a a working concentration of 40µg/ml to select for bacteria carrying resistance to the antibiotic. BCIG (X-Gal, 5-Bromo-4-chloro-3-indoyl-galactopyranoside) was used at a working concentration of 20µg/ml and was added to the agar at 56°C just prior to pouring. X-gal acts a substrate for B-galactosidase. It was used to screen for the lack of B-galactosidase activity in bacteria carrying recombinant plasmids with inserts in the lacZ gene.

Bacteriophage buffer SM.

Per litre: 5.8gm sodium chloride, 2gm magnesium sulphate (heptahydrate), 50mls 1M TrisCl (pH7.5) and 5mls 2% gelatin. Sterilised by autoclaving.

2.3.3 Preparation and transformation of frozen competent JM83 bacteria.

Frozen competent cells were made by the method of Simanis (described in Hanahan, 1985). Several large freshly plated JM83 colonies on a SOB plate were innoculated into 20mls of SOB medium, shaken a 37°C to an OD₆₀₀nm =0.05-0.1 (2.5-5x10⁷ viable cells /ml) and after standing on ice for 15 minutes were collected by centrifugation at 2500rpm for 10 minutes at 4°C in a 50ml polypropylene
centrifuge tube. The pellet was resuspended in 7mls of RF1, kept on ice for 15 minutes and respun as before. The cells were resuspended in 1.8mls of RF2, held on ice for 15 minutes and then 200μl aliquots were dispensed into pre-cooled Eppendorf tubes. These were then flash frozen in liquid nitrogen and stored a -70°C until use. Each 200μl aliquot was used for a single transformation. The cells were thawed in air and placed on ice. DNA was added (0.2μgm) and after an incubation on ice of 30 minutes the cells were heat shocked a 42°C for 90 seconds and recooled on ice. 800μl of SOC medium was added and the cells were shaken at 37°C for 1 hour followed by plating on the appropriate agar.

RF1 is 100mM rubidium chloride, 50mM manganese chloride (tetra-hydrate), 30mM potassium acetate, 10mM calcium chloride (dihydrate) and 15% w/v glycerol, pH adjusted to 5.8 with 0.2M acetic acid. It was sterilised by filtration through a 0.2μm membrane.

RF2 is 10mM MOPS, 10mM rubidium chloride, 75mM calcium chloride(dihydrate) and 15% w/v glycerol, pH adjusted to 6.8 with sodium hydroxide and filter sterilised through a 0.2μm membrane.

Both RF1 and RF2 are made up fresh just before use and the highest quality ingredients possible are used.

MOPS is 3-[N-Morpholino]propanesulphonic acid.

2.3.4 Preparation of transformation competent NM522 cells.

Cells were prepared as described in Davies et al (1986).

Several colonies of NM522 cells were taken from a minimal agar plate and innoculated into 20mls of 2xTY medium in a sterile culture flask. Cells were grown at 37°C with shaking until their OD<sub>600nm</sub> = 0.3. The cells were collected by centrifugation at 2500rpm for 5 minutes, resuspended in 20mls of serile 50mM calcium chloride, left on ice for 20 minutes and then collected by centrifugation as before. The cells were then resuspended in 2.5mls of ice-cold 50mM calcium chloride and left on ice 30 minutes before use. Cells prepared in this way were stored at 4°C and used within 2 days.

2.3.5 Transformation of competent NM522 cells with recombinant pTZ plasmids.

Plasmid DNA was added to 200μl of freshly prepared competent NM522 cells and left on ice for 20
minutes. The cells were heat-shocked at 42°C for 90 seconds, cooled on ice, then 800μl of 2xTY medium + 20mM glucose was added and the cells grown with shaking at 37°C for 1 hour. Aliquots were plated onto L-agar + ampicillin and incubated overnight.

2.3.6 Preparation of helper phage M13K07.

Prepared as recommended by Pharmacia. 20-30 closely spaced freshly plated M13K07 plaques grown on NM522 cells were scraped into 50mls of 2xTY + 70μg/ml Kanamycin and grown for 10-14hrs at 37°C shaking with good aeration. The cells were removed by centrifugation at 9000rpm for 10 minutes at 4°C and the supernatant was aliquoted into sterile containers to serve as a helper phage stock. Typical titers of approx 5x10^10 pfu's were obtained and were stable at 4°C.

2.3.7 Preparation of bacteria for bacteriophage infection.

A single colony of cells was innoculated into 40mls of L-broth + 0.4% maltose and grown overnight shaking at 37°C. Cells were collected by centrifugation at 2500rpm and resuspended in 20mls of 10mM magnesium sulphate.

Cells prepared in this way were stored at 4°C for 4 days without a significant decrease in viability. The use of maltose in the medium increases the infectability of the cells by increasing the copy number of the receptors used by the phage for entry into the cell.

2.4 VECTORS FOR CLONING IN E.coli.

2.4.1 Plasmid and Bacteriophage Lambda vectors.

pTZ is a 2.9Kb derivative plasmid of the pUC series (Vierra and Messing, 1982) with an insertion of the F1 origin of replication which allows the production of single-stranded versions of the plasmid (Mead et al, 1986). It was used as a general cloning vector and also to produce single-stranded DNA for sequencing. The vector used in all experiments was pTZ18R.

All genomic cloning was carried out in the vector EMBL3 (Frischauf et al, 1983). This is a lambda replacement vector that carrying two short polylinker sequences which release the two vector 'arms' for
cloning use when cut with suitable enzyme. EMBL3 has the advantage that recombinant phage ie those
carrying a genomic insert in place of the lambda 'stuffer' fragment that occupied the space between the
two vector arms, can be genetically selected for using a host E.coli carrying a P2 prophage, such as
Q359 (see 2.3.1). This is called the spi selection system (see main text).

2.4.2 Large-scale isolation of Plasmid DNA.

Plasmid DNA was isolated by the alkaline lysis method of Birnboim and Doly as described in Maniatis

1 Litre of overnight bacterial culture was collected by centrifugation at 6000rpm for 10 minutes at 4°C
in four 250ml bottles. Each pellet was drained, resuspended in 5 mls of solution I and lysozyme added
to 5mg/ml. This was left at room temperature for 15 minutes after which 10mls of solution II was
added, followed by gentle mixing by inversion and standing on ice for 10 minutes. 7.5mls of solution
III was added and mixed by rapid inversion and after standing on ice for 15 minutes the resultant pre-
cipitate was removed by centrifugation at 8000rpm for 20 minutes at 0°C. The supernatant was col-
lected through muslin and the DNA precipitated by the addition of 13.5mls of isopropanol. After 20
minutes at room temperature the DNA was pelleted at 10,000rpm for 30 minutes at 4°C. The plasmid
DNA pellet was resuspended in 3mls of TE and treated with RNAase A at 10ug/ml for 20 minutes at
37°C followed by extraction with equal volumes of phenol and 24:1 chloroform/isoamyl alcohol. The
DNA was precipitated with one tenth volume of 3M sodium acetate and 2.5 volumes of ethanol and
resuspended in TE ready to purify on a caesium chloride gradient.

Solution I is 50mM Tris-HCl (pH8), 10mM EDTA (pH8) and 25% Glucose (w/v).

Solution II is 0.2M NaOH, 1% SDS made fresh just before use.

Solution III is 3M Potassium acetate pH 4.8 with acetic acid.

2.4.3 Small-scale plasmid preparations.

Small-scale plasmid preparations were carried-out by two methods depending on the plasmid concerned.

When the plasmid insert was large the alkaline-SDS method gave a better yield of DNA.
Alkaline-SDS method.

The solutions used were as for the large scale plasmid prep method (2.4.2). 1.5mls of overnight culture was collected in an Eppendorf tube by centrifugation, drained and carefully resuspended in 200μl of solution I. A few flakes of lysozyme were added and the cells left for 5 minutes at room temperature. 400μl of solution II was added followed by gentle mixing by inversion and left on ice for 10 minutes. 200μl of cold solution III was added, left on ice for 10 minutes and then the precipitate was removed by centrifugation for 10 minutes in a microfuge at room temperature. Plasmid DNA was precipitated from the supernatant with 480μl of isopropanol, frozen for 10 minutes at the top of a liquid nitrogen tank, thawed and collected by centrifugation for 15 minutes. The pellet was washed in 70% ethanol and resuspended in 50μl of TE. 5μl of the DNA was checked on an agarose gel.

TELT method.

As described by Willimzig, 1985)

1ml of overnight culture was collected, drained and resuspended in 100μl of TELT buffer. A few crystals of lysozyme were added and left 5 minutes at room temperature. The cells were heated in a boiling water bath for exactly 60 seconds, cooled on ice for 5 minutes and the debris removed by centrifugation in a microfuge for 8 minutes. Plasmid DNA was precipitated with 66μl of isopropanol on ice for 20 minutes. After collecting by centrifugation and washing with 70% ethanol, the pellet was resuspended in 30μl of TE and 5μl was checked on an agarose gel.

TELT buffer is : 0.4% Triton X-100, 62.5mM EDTA, 2M LiCl, 50mM Tris.Cl. pH7.5.

2.4.4 Purification of plasmid DNA by CsCl density centrifugation.

Plasmid DNA was purified on caesium chloride gradients according to the method of Maniatis et al (1982). Plasmid DNA from a 100ml terrific broth preparation was dissolved in TE to a total volume of 3.21mls. To this was added 3.55gm caesium chloride and 0.34ml of 10mg/ml ethidium bromide. The resultant mixture was spun in a vertical TV865 rotor at 40,000rpm for 20 hours at 20°C. The plasmid DNA band was visualised by exposure to UV light and was collected with a needle and syringe. Ethi-
diium bromide was removed from the DNA solution by extraction with equal volumes of water saturated butanol until no ethidium bromide remained in the organic phase, whereupon the plasmid DNA was precipitated by the addition of 1/10th volume 3M sodium acetate and 2 volumes of absolute ethanol on ice for 10 minutes which leaves the caesium chloride in solution. The DNA was collected by centrifugation, resuspended and reprecipitated.

2.4.5 Large-scale bacteriophage lambda DNA preparation.

Bacteriophage DNA was prepared by an adaptation of the method described in Maniatis et al (1982). The phage from a large plate lysate (20cm x 20cm) were collected in 20mls of phage buffer and shaken with chloroform for 15 minutes. The chloroform was removed by centrifugation at 3000rpm for 5 minutes and DNAase I and RNAase A were added to 1μgm per ml. Solid NaCl to 1M was added to the phage solution and after 1 hour on ice the precipitated debris and proteins were removed by centrifugation at 8000rpm for 10 minutes at 0°C. Bacteriophage particles were collected by centrifugation at 25,000rpm for 2 hours at 4°C and were resuspended in 5mls of phage buffer overnight at 4°C. DNA was prepared from the collected particles by lysing at 65°C in the presence of 20mM EDTA, 50μgm/ml proteinase K and 0.5% SDS for 30 minutes. The crude DNA was extracted with equal volumes of phenol and chloroform and dialysed overnight to remove any additional impurities. Finally the DNA was precipitated with the addition of a tenth volume of NaOAC and two volumes of ethanol, collected by centrifugation and resuspended in TE.

2.4.7 Small-scale Bacteriophage lambda DNA preparation.

Small scale phage preparations performed by an altered version of the large scale method described above. A small plate lysate (90mm dish) was collected in 5mls of bacteriophage buffer and directly centrifuged at 25,000rpm as above. The particles were lysed as above and the DNA was not dialysed. This method usually yielded DNA suitable for random priming or nick translation but was unsuitable for restriction digestion.
2.5 MANIPULATION OF DNA WITH ENZYMES.

2.5.1 Restriction endonuclease cleavage of DNA.

Restriction enzyme digestion of DNA was carried out under the conditions recommended by the manufacturers. Genomic DNA digests were generally carried out using 2-4 units of enzyme for every 1µg of DNA. In a few cases enzymes required a special buffer, however most enzymes cut satisfactorily using the High-Medium-Low salt system.

Restriction enzyme buffers.

10xLow Salt 100mM Tris.Cl pH7.4, 100mM MgSO$_4$, 10mM DTT
10xMid Salt 100mM Tris.Cl pH7.4, 100mM MgSO$_4$, 50mM NaCl, 10mM DTT.
10xHigh Salt 500mM Tris.Cl pH7.4, 100mM MgSO$_4$, 1000mM NaCl, 10mM DTT.
10xAcll Buffer 60mM NaCl, 60mM Tris.Cl pH7.5, 60mM MgCl$_2$, 60mM 2-mercaptoethanol.
10xSma1 Buffer 200mM KCl, 60mM Tris.Cl pH8, 60mM MgCl$_2$, 60mM 2-mercaptoethanol.
10xSphl Buffer 1500mM NaCl, 100mM Tris.Cl pH7.4, 100mM MgCl$_2$, 100mM 2-mercaptopoethanol.

Bovine serum albumin was added to reactions at a concentration of 100µg/ml.

2.5.2 Ligation of DNA fragments.

2.6 DNA AND RNA ELECTROPHORESIS AND IMMOBILISATION ON MEMBRANES.

2.6.1 Electrophoresis of DNA through agarose.

Restricted DNA fragments were size separated in agarose gels by electrophoresis. Cut DNA samples were made to 1x stop/loading mix, loaded into the wells of an agarose gel and run at a suitable voltage.

1x TAE is 0.04M Tris-acetate 0.002M EDTA pH7.2.
1x TBE is 0.089M Tris-Borate, 0.089M Boric acid 0.002M EDTA.
Stop mix is 0.2M EDTA (pH8.1), 15% ficoll and orange 'g' to suitable colour.

2.6.2 Southern transfer of DNA.

DNA was transferred to membranes by the method of Southern (1975). After ethidium staining and photography, gels to be transferred were denatured for 30 minutes in 1.5M NaCl/0.5M NaOH and then neutralised with 2M NaCl/1M Tris.Cl pH5.5 for 30 minutes. The DNA was then capillary blotted with 20xSSC overnight onto a membrane of nitrocellulose or nylon. Nitrocellulose was pre-wetted in 2xSSC whereas Hybond-N nylon membranes were applied to the gel dry as they wet instantly on contact with the gel. One layer of pre-wetted (2xSSC) 3mm filter paper was placed onto the membrane and layered on top of this, paper towels a height of 6cm. A glass plate and a 1Kg weight were then placed on top of the towel. After transfer, the membrane was washed in 2XSSC to remove any attached agar fragments and allowed to air dry. The DNA was covalently bound to the membrane according to the manufacturers instructions. Nitrocellulose membranes were baked at 80°C for 90 minutes in vacuo and Nylon filters (Hybond-N, Amersham) were exposed to UV light on a UV transducer for 3.5 minutes.

20xSSC is 3M NaCl/0.3M Na3Citrate pH7.0.

2.6.3 Preparative agarose electrophoresis of DNA.

DNA was recovered from agarose gels after electrophoresis by the method of Vogelstein (1987). Inserts prepared in this way were suitable for both cloning and radiolabelling.

DNA fragments were electrophoresed at through a 0.9% agarose gel containing 1xTAE at 20V overnight. The DNA was stained with ethidium bromide, visualised with UV light and the required fragments were cut-out from the gel. These were placed into a Spin-X tube (Costar) and spun for 15 minutes in a microfuge. The tube was then frozen at -20°C for 1 hour and respun for 15 minutes. DNA was precipitated with 1/10th volume 3M sodium acetate and two volumes of absolute ethanol and collected by centrifugation after standing at -20°C for 1 hour.

2.6.4 Electrophoresis of RNA.
All RNA samples were run on 1.5% agarose gels containing 10mM NaPO_4 pH 6.5 and 6.6% formaldehyde. After adding 1.5 volumes of sample buffer to each 10μgm sample, the samples were heated to 50°C for 10 minutes and after adding loading mix containing 8% bromophenol blue, loaded onto a gel. Gels were run with recirculated 10mM NaPO_4 buffer to avoid any damaging pH changes.

1M Phosphate Buffer pH 6.5 was made by mixing separately autoclaved 1M solutions of Na_2HPO_4 and NaH_2PO_4 in the ratio of 26.5:73.5.

Sample buffer (1.5x) is 15mM phosphate, 27% formaldehyde and 75% formamide.

2.6.5 Northern blotting of RNA.

After electrophoresis was complete, gels were soaked in 10XSSC for ten minutes before transfer. The RNA was capillary blotted overnight as for Southern's but using 10xSSC as the transfer buffer. After transfer, the RNA was bound to the filter by UV or baking depending on the filter being used.

2.7 RADIO-LABELLING OF DNA.

2.7.1 Nick translation.

DNA was nick-translated by the methods of Rigby et al (1977). The method involves the controlled introduction of nicks into a dsDNA probe with DNAse I, followed by the repair of the nick using DNA polymerase I. The action of polymerase I is to move the nick along the template by addition of new nucleotide to the 3' end of the nick and the removal of nucleotides at the 5' end. Thus radiolabelled DNA probes can be generated by the incorporation of ^32P labelled dNTP's into the newly synthesised DNA.

200ngm of DNA was mixed with 2.5μl 10x Buffer and 0.5nmole each of dGTP, dCTP and dATP. To this was added 2.5μl [α^32P]dTTP (25μCi) and 0.25/(*ml of DNAase I (0.1μgm/ml). The volume was made to 24μl with distilled water. 1μl of E.coli DNA polymerase I was added and the reaction incubated at 15°C for 1 hour after which the incorporation was measured by TCA precipitation. The reaction was stopped by the addition of 1/10th volume of 0.2M EDTA pH 8.0.
Determination of radiolabel incorporation.

A small aliquot of labelling reaction was spotted onto the centre of a glass-fibre filter and the counts read by Cerenkov counting. The filter was then washed 3 times with 10% TCA under suction and recounted. The % of incorporation was determined.

2.7.2 Random Priming.

DNA probes were random primed by the method developed by Feinberg and Vogelstein (1983 and 1984). Random hexanucleotides were allowed to anneal to denatured probe DNA which then served as primers for DNA synthesis. Klenow polymerase was used to extend the complementary strand in the presence of $^{32}$P labelled dNTP's. Subsequent denaturation produces a radiolabelled DNA probe ready for hybridisation.

100ngm of heat denatured DNA (90°C for 5 minutes then into ice) was added to 0.5nMole each of dATP, dCTP and dGTP (1ul of 0.5mM). 2μl of reaction mix (as for nick translations), 3μl [α-$^{32}$P]dTTP (30μCi) and distilled water to 19μl were added. After the addition of 1μl of Klenow polymerase and mixing, the reaction was allowed to proceed at 37°C for 45 minutes. The incorporation of radionucleotides was checked by TCA precipitation as above and the reaction stopped by the addition of 1/10th volume of 0.2M EDTA pH8.0.

2.7.3 End-labelling.

The 5' OH group of an oligonucleotide probe was labelled with $^{32}$TTP by the action of the enzyme T4 polynucleotide kinase (T4 PNK).

0.2μgm of oligo was added to 2ul of 10x kinase buffer, 5μl $^{32}$P -TTP (50μCi) and 1μl T4 PNK in a total volume of 20μl. This was incubated for 1 hour at 37°C and incorporated counts checked by TCA precipitation. Short oligo's are at the limit of TCA precipitability and consequently only a small % of incorporation represents a high efficiency of end-labelling.

10x PNK buffer is 500mM Tris.Cl pH7.4,100mM MgCl$_2$,50mM DTT.

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2.9 NUCLEIC ACID HYBRIDISATION AND AUTORADIOGRAPHY.

2.9.1 Uses of Hybridisation

Homologous and closely related DNA sequences were detected by hybridisation of radiolabelled probes (oligonucleotides, plasmids and lambda clones) to nucleic acid immobilised on membranes.

2.9.2 Hybridisation Protocols.

Normal hybridisation conditions.

The filter to be probed was placed and sealed inside a plastic bag containing a volume of suitable hybridisation mix (5mls per 100cm\(^2\) of filter) containing 100\(\mu\)g/ml denatured sonicated salmon sperm. The bag was incubated at 68°C for 1 hour after which the heat-denatured radiolabelled probe was added, the bag resealed and reincubated overnight at 68°C. After hybridisation, the filter was removed into 2xSSC hybridisation wash and washed with increasing stringency to 0.5xSSC or 0.1xSSC at 68°C.

Hybridisation with oligonucleotides.

End-labelled oligonucleotide probes require special hybridisation conditions reflecting their base composition as determined by the equation \(T_m = 4(G+C) + \frac{2}{A+T}\). This temperature was used for both hybridisation and washing of the filters.

Hybridisation mixes.

Standard Mix for Nitrocellulose was 5xSSC,5xDenhardts,0.1% sodium pyrophosphate(PPi), 0.1% sodium dodecylsulphate( SDS).

Standard Mix for Nylon Hybond-N was 5xSSC,5xDenhardts,0.1% PPi,0.5% SDS.

pEJ Hybridisation Mix was 4xSSC,0.1% PPi,0.2% SDS,50ugm/ml Heparin.

Oligonucleotide Hybridisation Mix is

100xDenhardts was 2% bovine serum albumin, 2% PolyvinylPyrrolidine, 2% Ficoll 400.
2.9.3 Removal of repeat DNA by pre-annealing

Probes that contained repetitive DNA elements were used to detect their single copy homologues on Southern and Northern blots by using the pre-annealing technique of Sealey et al (1985). This technique allows repetitive DNA elements to be reannealed to their homologues in unlabelled genomic DNA, leaving only single copy sequences denatured and able to take part in the hybridisation reaction.

100ngm of labelled DNA was denatured with 50μl 5mg/ml sonicated total human DNA and allowed to reassociate at 65°C for 30 minutes before adding to the hybridisation mix.

2.9.4 Autoradiography.

After washing to the required stringency the filters were padded dry on 3mm paper and wrapped in cling-film before being exposed to film. To increase the degree of exposure, the films were pre-flashed with a photographic flash unit before exposure to the filters. Autoradiographs were carried out in cassettes containing intensifying screens. Filters were usually exposed overnight at -70°C for the first instance and then re-exposed for a longer period if necessary.

Kodak XAR and Fuji Medical X-ray film was used, and all autoradiographs were developed using a Fuji XRII Xray film developer.

2.10 GENOMIC LIBRARY CONSTRUCTION.

2.10.1 Size-fractionation of restricted DNA fragments by sucrose gradient centrifugation.

200μgm of Genomic DNA was digested to completion with restriction enzyme BamHI, the resultant fragments extracted with an equal volume of 25:24:1 phenol/chloroform iso-amyl alcohol and precipitated with two volumes of absolute ethanol. The DNA was resuspended in 200μl of TE, heated to 68°C for 5 minutes, cooled to 20°C and loaded onto a sucrose gradient made from layered 3.3mls volume of 40%, 30%, 20% and 10% sucrose solutions (in 1M NaCl, 20mM Tris.Cl pH8.0 and 5mM EDTA) in a 17ml polyallomer tube. This was spun for 24 hours at 26,000rpm on a Sorvall AH-627 rotor at 20°C.

250μl samples were collected and 10μl of each was run out on a 0.6% agarose gel. The DNA was transferred overnight and hybridised overnight at 68°C with a radiolabelled probe. The filter was washed.
to a stringency of 0.5xSSC and exposed to film at -70°C. Positive fractions were pooled and dialysed in a 12.5mm dialysis bag against TE at 4°C for 24 hours. The resultant fragments were concentrated by butanol, extraction, precipitated with ethanol and resuspended in 20μl of TE.

2.10.2 Construction of size-selected libraries in the vector EMBL3.

To ensure efficient ligation, the size-selected DNA was concentrated to approx 0.5mg/ml and checked to ensure that we had not inadvertently concentrated any ligation-inhibiting contaminants by doing a trial self-ligation and a ligation of HindIII cut lambda DNA in the presence of the size-selected DNA.

Approximately 0.5μgm of the size-selected DNA was ligated to 1μgm of EMBL3 arms (Stratagene) under the following conditions: 50mM Tris.Cl pH8, 7mM MgCl₂, 1mM DTT, 1mM rATP and 3 Weiss units T4 DNA Ligase (Biolabs). After a trial packaging reaction, the library was packaged with two Giga-Pack Gold reactions (Stratagene), infected into E.coli Q359 and plated in CY-Agarose (0.6%) onto 22cm X 22cm plates containing CY-Agar (1%). The plates were then allowed to solidify and incubated overnight at 37°C.

2.11 IDENTIFICATION OF RECOMBINANT PHAGE AND PLASMIDS BY HYBRIDISATION.

2.11.1 Plating of recombinant phage in agarose.

Agarose was used as the top layer in place of agar when screening phage as it doesn’t tear or peel-off when taking lifts with nitrocellulose filters.

Per 90mm² dish : 200ul of plating bacteria (see 2.3.6) were inoculated with the necessary number of phage, mixed gently and allowed to stand for 10 minutes at room temperature in order to synchronise the infection and get even-sized plaques. 3 mls of top agarose (cooled to 50°C) was added, gently mixed and poured onto the bottom agar surface. The plate was left to harden for 20 minutes and incubated inverted overnight at 37°C.

For the larger 220x220mm plates, 0.8ml of plating bacteria were used and mixed in 30mls of top agarose.
2.11.2 Screening by plaque hybridisation.

Recombinant phage can be identified in a lawn of phage by taking a replica of the lawn onto nitrocellulose and screening it with a suitable probe as described by Benton and Davis (1977). This takes advantage of the presence of unpackaged DNA present in the lysed bacterial debris in a phage plaque on a lawn. After screening in this way it is possible to identify clones of interest and pick the corresponding infective phage particles still present in the plaque on the lawn.

Plates containing fresh plaques were hardened at 4°C for 1 hour before taking lifts with nitrocellulose in order to harden the agar. The sheets were placed onto the plate, the position marked asymmetrically with ink and left for 60 seconds to adsorb the phage DNA. The sheet was then peeled off the lawn and placed phage side up onto a sheet of 17mm filter paper soaked in denaturant. After 1 minute the filter was placed onto neutraliser for 5 minutes, washed in 2xSSC to remove any bacterial debris and air dried. The filters were baked for 90 minutes at 80°C in a vacuum oven and hybridised with the appropriate probe.

When multiple lifts were taken from the same plate the first lift was left on the agar for 30 seconds, the second for 60 seconds and the third for 2 minutes (etc).

Denaturant is 1.5M NaCl 0.5M NaOH.

Neutraliser is 1.5M NaCl, 0.5M TrisCl,pH5.5.

Positive plaques were identified by aligning the autoradiograph with the appropriate filters and marking the ink mark location onto the film. The film was then alligned to the plate and hybridisation positive plaques identified. These were picked into 1ml of bacteriophage buffer, few drop of chloroform added and stored at 4°C. Positively identified phage were rescreened until a clonal population was obtained.

DNA was then made using one of the methods in section 2.4.

2.12 SEQUENCING.

2.12.1 Preparation of single-stranded template DNA.

Infection of cells carrying plasmids containing the F1 origin of replication results in the production of
single-stranded viroid copies of the plasmid. The plasmid pTZ (Pharmacia) is such a vector and was used to produce single-stranded DNA from cloned genomic fragments. The protocol of DNA purification is an adaptation of the Pharmacia protocol (Dr Robert Hill, pers comm.).

80μl of an overnight culture of NM522 cells carrying the recombinant pTZ plasmid grown in 2xTY+0.001% thiamine+150μg/ml ampicillin was inoculated into 5mls of the same medium, and grown for approx 4hrs until the OD₆⁵₀ₐ₉=0.8. 2mls of this culture was infected at a multiplicity of infection of 10 with helper phage M13K07 and shaken vigourously for 1 hour at 37°C in a 50ml polypropylene tube. After 1 hour, 400μl of infected cells were added to 10mls of 2xTY+0.001% thiamine + 150μg/ml ampicillin + 70μg/ml kanamycin and grown for 14-18hrs at 37°C with very good aeration. The cells were repeatedly removed by centrifugation until no pellet was observed, and the phage were precipitated from 8mls of supernatant with 2mls of 2.5M NaCl/20% Peg6000. After standing for 15 minutes at room temperature the phage particles were collected by centrifugation at 10,000rpm for 5 minutes. The pellet was resuspended in 200μl of TE and phenol extracted with ultra-pure phenol (BRL) to release the phage DNA and then chloroform extracted. The DNA was precipitated, visualised on an agarose gel, transferred to a filter and hybridised with the primer oligonucleotide.

2.12.2 Synthesis of oligonucleotide primer.

Oligonucleotides were all synthesised on an Applied Biosystems 851A DNA synthesiser by Mr John Inglis. The oligo's were formed by B-phosphoramadite reaction.

2.12.3 Preparation of polyacrylamide sequencing gels.

Sequencing gels were run in 400x200x4mm glass plates. These were cleaned thoroughly before use by washing in detergent and water and then rinsing in ethanol and allowed to air-dry. To make pouring the gel easier and to ensure adhesion of the gel to one plate only (upon seperation for autoradiography), the notched plate was treated with 3mls of 2% dimethyl-dichlorosilicone in 1,1,1-trichloromethane. This was applied to the plate, allowed to dry for 10 minutes and then any excess was removed by wiping clean with ethanol. The unnotched plate was treated to prevent the gel curling upon drying by treating it with a silane solution. 30μl of silane (-methylacryloyxpropyl-trimethoxysilane) was added to 10mls of
ethanol. This was mixed with 30µl of acetic acid in 300µl of water in a fume hood and rubbed into the unnotched plate. This was left to dry for 3 minutes and wiped clean with ethanol. Spacers were placed between the plates (treated faces facing inwards) and the edges completely sealed with tape leaving the top of the plates open.

The gel was prepared by mixing 21gm ultrapure urea, 7.5mls stock acrylamide, 5mls 10x TBE and water to 50mls. This was warmed to dissolve the urea and 300µl of fresh 10% ammonium persulphate and 30µl of TEMED were added. (TEMED is NNN'N'-tetramethylethylenediamine). This was mixed thoroughly and the gel poured into the open end of the casting plates using a pipette. The comb was inserted and the gel left to set for 1 hour.

Stock acrylamide is a 40% solution made as follows: 38gm acrylamide, 2gm bis-acrylamide, distilled water to 100mls. Add 5gm Amberlite MB1 and stir gently for 30 minutes. Then remove the resin by filtering through a sintered glass filter and finally filter through a 0.45µm filter. The stock was stored in the dark at 4°C.

2.12.4 Sequencing by Dideoxy chain termination.

All sequencing reactions were carried out using dideoxy chain termination (Sanger et al, 1977). Two commercially available kits were employed; the Amersham M13 sequencing kit and the Sequenase kit (USB Corporation). Sequencing with the Amerham kit involved chain elongation/termination and then an elongation step to remove any unterminated chains. In contrast, the first step in sequencing with the Sequenase kit involves elongation/labelling and the second step is the termination reaction. The two kits use different enzymes and radio-labelled nucleotides. The Amersham kit uses the large fragment of E.coli DNA polymerase I (Klenow Fragment) whereas the Sequenase kit uses a modified bacteriophage T7 DNA polymerase (Tabor and Richardson, 1987).

Where stretches being sequenced were prone to secondary structure, the dGTP analogue dITP was used in parallel to resolve compression zones in the sequence.

The primer oligonucleotide was allowed to anneal to the template DNA in a mole ratio of 1:1. For the 4 reactions (A,C,T,G(or l)), 1-2µgm of template was annealed to approx 0.5pmol of primer in a volume
of 10μl with the appropriate buffer. This was done by heating the mixture to 65°C for 2 minutes and then allowing it to cool gradually to room temperature in a beaker of 65°C water at room temperature.

Sequencing with Sequenase.

Annealing was carried out in 20mM Tris.HCl pH 7.5, 10mM MgCl₂, 25mM NaCl. To this was added 1μl 0.1M DTT, 2μl Labelling mix, 0.5μl [³⁵S]-dATP(10μCi,1400Ci/mmol) and 2μl 1/8th dilution of Sequenase. This was incubated at room temperature for 5-10 minutes after complete mixing.

Labelling mix is 1.5μM dCTP, 1.5μM dTTP, 1.5μM dGTP (or 3μM dTTP).

Four reaction tubes labelled A,C,G,T containing 2.5 μl of their respective ddNTP termination mixes were pre-incubated at 37°C for 1 minute and 3.5μl of the labelling reaction was added to each, mixed thoroughly and incubated at 37°C for a further 5 minutes. The reactions were stopped by adding 4μl of stop solution and the samples were stored on ice until ready for loading onto the gel.

A mix 80μM dCTP,80μM dGTP,80μM dTTP,8μM ddATP,50μM NaCl.
C Mix 80μM dCTP,80μM dGTP,80μM dTTP,8μM ddGTP,50μM NaCl.
G Mix 80μM dCTP,80μM dGTP,80μM dTTP,8μM ddGTP,50μM NaCl.
T Mix 80μM dCTP,80μM dGTP,80μM dTTP,8μM ddGTP,50μM NaCl.

Stop mix contained 95% formamide, 20mM EDTA, 0.05% Bromophenol Blue and 0.05% Xylene cyanol FF.

Sequencing using the Amersham Kit.

The primer was annealed as above in 10μl containing 1.5μl Klenow buffer. To this was added 1μl [³²P]-dATP (10μCi,>400Ci/mmol) and 1 unit of Klenow polymerase. 2.5μl of template/primer/label mix was added to 2μl of respective termination mixes(A,C,G,T) and incubated at room temperature for 15 minutes after which 2μl of chase buffer was added. This was incubated for a further 15 minutes and the reactions stopped by the addition of 4μl of formamide stop mix.

A Mix 3.5μM dATP,75μM ddATP,100μM dGTP,100μM dCTP.
C Mix 3.5μM dCTP, 10μM ddCTP, 100μM dGTP, 100μM dATP.

G Mix 3.5μM dGTP, 25μM ddGTP, 100μM dCTP, 100μM dATP.

T Mix 250μM ddTTP, 100μM dATP, 100μM dCTP, 100μM dGTP.

Klenow Buffer is 100mM Tris pH8.5, 50mM MgCl₂.

Chase Buffer is 0.5mM dATP, 0.5mM dCTP, 0.5mM dGTP, 0.5mM dTTP.

Formamide stop mix is as for Sequenase.

2.12.5 Double stranded sequencing of plasmid DNA.

Double stranded DNA sequencing was carried out on plasmid denatured as described by Chen and Seeburg (1985). 2μgm of purified plasmid DNA was denatured by resuspending dried down DNA in 40μl of denaturant buffer (0.2M NaOH). This was allowed to sit for 5 minutes at room temperature, neutralised by the addition of 4μl of 2M ammonium acetate (pH 4.5) and precipitated with absolute alcohol. The pellet was washed in 70% ethanol and the pellet resuspended in sequenase buffer (see above). To this was added 2.5pmol of oligonucleotide primer, 30μCi [³²-P]-dATP and DTT to 10mM. This was allowed to sit at 37°C for 30 minutes after which sequenase enzyme was added. This reaction mix was alliquotted into four reaction tubes containing sequenase terminase buffers. These were incubated for 10 minutes and then stopped using formamide stop mix.

2.12.6 Running, fixing and autoradiography of sequencing gels.

Samples were heated to 95°C for 5 minutes immediately prior to loading on the gel. They were loaded into freshly washed wells using a fine-tipped micro-pipette. The gels were run in 1xTBE at a constant current of 25mA until the bromophenol blue dye was at the bottom of the gel.

After running, the clamps were removed and the plates separated. The gel (attached to the unnotched plate) was fixed in 2 litres of 10% acetic acid/10% methanol for 20 minutes and then washed under flowing water for 10 to remove the urea. The gel was then dried in an oven at 90°C for 1 hour and exposed to unflashed film overnight at room temperature.
2.12.7 Sequence homology comparison with nucleic acid data bases.

Sequence comparisons were carried out using the Wordsearch program of the University of Wisconsin Genetics Computer group's software package (Deveraux et al, 1984). The wordsearch program is based on the algorithm of Wilbur and Lipman and detects sequences of similar sequence by looking for regions with an unusual number of short perfect matches. The Genbank data-base was searched for homologies and the results interpreted using the Segments program.

2.13 PROTEIN ISOLATION AND IMMUNOPRECIPITATION.

Proteins were labelled, harvested and immunoprecipitations carried out as described by Quintanilla et al, 1986 using the antibody YA6-172 (kindly donated by Dr Alan Balmain) as described in Furth et al, 1982.

2.13.1 In vivo cell labelling.

Proteins were labelled in vivo with L-[\textsuperscript{35}S]-Methionine(Amersham) by the addition of 1.5mls of labelling medium(MEM w/Earles salts, w/o L-methionine, w/o L-glutamine(GIBCO) and glutamine 200mM, 5% dialysed FCS and 5% normal DMEM medium +5% FCS containing 200μCi L-[\textsuperscript{35}S]-methionine per ml) to 5x10\textsuperscript{5} cells at confluence in 9.6cm\textsuperscript{2} microwells overnight at 37°C. The following morning, cells were washed thoroughly in chilled PBS and harvested with a rubber policeman in 1.25mls of lysis buffer.(1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.1M NaCl, 50mM Tris.Cl pH7.4, 5mM MgCl\textsubscript{2}, 3mM PMSF)

Lysates were homogenised with a hand held homogeniser, centrifuged at 35,000rpm for 30 minutes at 4°C and the supernatants collected and stored frozen at -70°C. Incorporation of label into protein was determined by measuring the cpm precipitable by 10% TCA.

2.13.2 Immunoprecipitation.

0.5gm Protein-A-Sepharose(Sigma) was coated with rabbit anti-rat Ig(Whole molecule-Sigma specified protein content 34mg/ml) and stored at 4°C in PBS containing 0.05% sodium azide. 100μl of this Ig-PAS was used to pre-clear 1-5x10\textsuperscript{5} cpm(TCA precipitable) of labelled extract by incubation for 2 hours
on a rotator at 4°C. The Ig-PAS was removed by centrifugation and the extracts were incubated for 2 hours as before in the presence of antibody YA6-172 (10μl of a 1/50 dilution of stock 1mg/ml). 30μl of Ig-PAS was added to this to precipitate the antibody-p21 complex which was collected by centrifugation, washed three times with lysis buffer and resuspended in 50μl loading buffer.

2.13.3 SDS-Polyacrylamide gel electrophoresis and fluorography.

Proteins were separated on a 12.5% separating polyacrylamide gel in 15cm-long glass plates by the method of Laemmli (1982). A 3% stacking gel was poured onto the separating gel to increase protein resolution. Samples were heated to 100°C for 5 minutes and loaded onto the gel which was run at a constant current of 8mA overnight. When the dye-front was at the bottom of the gel the plates were separated and the gel removed into 7% acetic acid for fixing. After 20 minutes in fix, the gel was transferred to "Amplify" for 20 minutes, dried under a vacuum at 80°C for 2 hours and exposed to pre-flashed film at -70°C with intensifier screens.

4X running gel buffer is 1.5M Tris-Cl 0.4% SDS pH8.8.

2X stacking gel buffer is 0.25M Tris-HCl 0.2% SDS, pH6.8.

10X running buffer is made by mixing 30.3gm Tris base, 145gm glycine and 100mls 10% SDS. The pH was adjusted to 8.3.
CHAPTER 3
Chapter 3 HRAS1 expression in HRAS1-selected CMGT.

The introduction of mitotic chromosomes from an EJ bladder carcinoma cell line into mouse C127 cells generated a series of transformed cell lines with varying in vivo tumorigenic potentials (Porteous et al, 1986b). All expressed the HRAS1 oncogene but there was no apparent correlation between HRAS1 RNA levels and tumorigenic potential (Porteous et al, 1986b). In order to further investigate the relationship between expression and tumorigenicity I have extended the analysis to the in vitro study of HRAS1 protein levels in the transfectant cell lines and several other cell lines carrying the HRAS1 oncogene. These include EJ-18-3E, a non-tumorigenic subclone of the EJ-bladder carcinoma cell line, EJNAC, an in vivo-transformation selected fusion hybrid derived from the bladder carcinoma cell line, and 178C1, a secondary chromosome transfectant derived from E65.5 (see chapter 4).

3.1 HRAS1 mRNA expression.

To establish the levels of HRAS1 messenger RNA, total RNA was made from cells growing rapidly in vitro. As can be seen from figure 3.1, the levels of hybridising HRAS1 RNA, when normalised against actin, show little variation between cell lines.

The tumorigenic human progenitor cell line EJ-18-8D and the non-tumorigenic variant EJ-18-3E show comparable levels of message. EJNAC and each of the chromosome transfectants, including E65.5, show message levels similar to the EJ cell lines. C127 shows no cross hybridisation to the HRAS1 probe at this stringency.

No change in message size or additional messages were detected in any of the transformants including E65.5.
Figure 3.1 Expression of HRAS1 messenger RNA in chromosome transfection.

Total RNA was prepared from individual cell lines and electrophoresed in 1.5% agarose under denaturing conditions. Transferred RNA was probed with (a) a HRAS1 probe or (b) an actin probe as a control for loading.

EJ-18-8D and EJ-18-3E are human bladder carcinoma cell line subclones, EJNAC is a HRAS1-selected fusion hybrid (David Porteous and Veronica van Heyningen, pers comm), C127 is a mouse cell line and all the other cell lines are independent HRAS1-selected transfectants as described in Porteous et al, 1986a).

* There appears to be an association of the HRAS1 message with 28s RNA as judged by the presence of hybridising RNA in (a) above. It is noticeably absent from all the transfectant cell lines and may reflect some human-specific control mechanism.

* The actin control hybridisation in (b) above reveals that there are differences in the level of HRAS1 message between certain transfectants but by no more than a few-fold. The use of an actin control is perhaps a bad choice as it has been implicated in tumorigenicity.
3.2 HRAS1 P21 expression.

Figure 3.2 shows the levels of P21\textsuperscript{HRAS1} precipitated from rapidly growing cell cultures by the monoclonal antibody YA6-192 (Furth et al, 1983)(a kind gift from Dr A Balmain). The transfectant cell lines E65.5, E65.6, E65.7, E65.9 and E67.1 all express similar levels of protein. E67.4 contains a notably lower level of P21, the significance of which is not clear. Both EJ-18-8D and EJ-18-3E show the same low level of P21 expression.

It is clear that there is no correlation between the level of \textit{in vitro} P21 and \textit{in vivo} tumorigenicity. For example, tumorigenic variation in the transfectants is not reflected in a variation in P21 levels, nor is this the case for the two subclones of the bladder carcinoma cell line. Both EJ-18-8D and EJ-18-3E have identical level of P21 expression.

3.3 Levels of HRAS1 expression and tumorigenic variation.

Several observations can be made from the study of HRAS1 RNA and protein levels. Firstly, there is no direct relationship between either HRAS1 RNA or protein levels and \textit{in vivo} tumorigenic potential. Secondly, the levels of P21 are not directly related to the mRNA levels. For example, EJ-18-8D and -3E have as high a level of RNA expression as most of the transfectants yet they clearly express less than half the amount of protein. Clearly there are other cellular restraints placed upon the levels of translation in these cells. As this difference is seen with all the transfectants, it is unlikely to be a mutation which affects expression and is more likely to be the segregation of a controlling element upon transfection.

The precise role of HRAS1 expression in relation to tumorigenesis is unclear. As activated \textit{ras} genes are found frequently in tumours and tumour cell lines it would appear to play a contributory role towards stable transformation. Indeed, the overexpression of the normal HRAS1 (Chang et al, 1986b) and the correlation of HRAS1 P21 expression with malignancy in prostate carcinoma (Viola et al, 1986), bladder carcinoma (Viola et al, 1985) and colonic carcinoma (Hand et al, 1984) would appear to substantiate this. However, evidence that P21 levels in tumours reflect levels of expression in the normal tissue appears to contradict this view (Chesa et al, 1987).
Figure 3.2 HRAS1 protein expression in HRAS1-selected chromosome transfection.

[35S]-methionine labelled cell extracts were immunoprecipitated with antibody YA6-192 and anti-rat coated protein-A-sepharose and the complexes separated on 12% SDS-polyacrylamide gels. After enhancement, precipitated proteins were visualised by autoradiography. Shown below are two representative gels of independent precipitations.
More recently, Gilbert and Harris (1988) have shown that cells passaged from tumours derived from NIH3T3 cells carrying a transfected HRAS1 gene can remain tumorigenic in the absence of HRAS1 p21 expression. They suggest that tumorigenic variants arise by secondary changes from the pool of HRAS1 expressing cells. Once these secondary changes have occurred, expression of HRAS1 is no longer required for tumorigenicity.

Our observations are in agreement with those of Chesa et al (1987) and Gilbert and Harris (1988). The lack of correlation of HRAS1 expression with the tumorigenic potential of the transfectants and of EJ cell line subclones, suggests that it plays no direct role in this potential. The caveat to this is that only in vitro levels of RNA and P21 have been assayed. A histochemical analysis or direct protein measurement in fresh tumor material might show a positive correlation.

As the tumorigenic ability appears not to lie in the expression of HRAS1 message or P21 levels, and the elevated tumorigenicity of E65.5 is associated with a molecular rearrangement immediately 3' to the HRAS1 locus, a detailed analysis of the mechanism and potential consequences of the rearrangement was undertaken.
CHAPTER 4
CHAPTER 4 SECONDARY TRANSFECTION FROM E65.5.

4.1 Aims of secondary gene transfer.

We can use secondary gene transfer from E65.5 to provide valuable insights into the causes of its elevated tumorigenicity. DNA mediated gene transfer, with its inherent DNA fragmentation, will lead to the random segregation of sequences syntenic with the HRAS1 locus in E65.5. In contrast, chromosome mediated gene transfer will preserve the local DNA structure. Thus secondary gene transfer can be used to investigate the relationship of the Ha-ras locus and its surrounding DNA milieu to the E65.5 phenotype.

Both morphologically and in its ability to grow in soft agar, E65.5 is distinct from the other CMGT transfectants (see 1.5). The assessment of relative tumorigenic potentials required the use of a large number of mice in experimental mouse tumour assays. With this in mind, and with a view to the size of in vivo experimentation required to study a large panel of secondary transfectants for their tumorigenicity, the secondary lines were assayed first for their morphology and anchorage independent growth ability to see if any clear delineation existed at the molecular level between E65.5-alike and non-alike transfectants.

To determine if there have been any additional alterations at that locus which might affect its transforming abilities, I have compared the effect of the reintroduction of the cloned H-ras gene from E65.5 with the original cloned gene from EJ-18-81, pEJ. In a similar fashion, any direct role played by sequences translocated immediately adjacent to the Ha-ras locus in E65.5 has been tested by the reintroduction of a molecular clone for the region, lambda 10.21 (for cloning details see chapter 5).
4.2 Secondary chromosome transfectant 178C1.

Mitotic chromosomes from E65.5 were transferred into mouse C127 cells as described by Porteous et al (1986a). Transfection, primary focus isolation and primary transfectant culturing were performed by John Morten. Subsequent soft agar cloning and the generation of a clonal monolayer culture was performed by David Porteous and myself.

178C1 is a secondary chromosome transfectant derived from E65.5 mitotic chromosomes. As can be seen from figure 6.1, 178C1 is morphologically similar to E65.5. The cell line grows loosely attached to tissue culture plastic and produces large colonies in soft agar although never as large as those formed by E65.5 (see fig 4.1). Restriction analysis of 30Kb around the HRAS1 locus reveals no detectable site changes additional to those in E65.5 (see figure 4.3).

4.3 Secondary DNA transfection from E65.5.

High molecular weight E65.5 DNA was transfected into mouse C127 recipient cells and foci were isolated by ring cloning after four weeks. Ten clonal monolayer cultures were generated and the morphologies and abilities to grow in soft agar analysed. Two transfectants, D2B and D2C were generated by David Porteous in a similar fashion.

The secondary transfectants exhibit a wide variation in morphology. They range from flat, pavement-like cells to highly dendritic cells. Figure 4.2 illustrates the variation found in both morphology and ability to grow in soft agar.

4.4 Molecular analysis of the transfected HRAS1 loci.

Restriction and Southern blot analysis of the secondary DNA transfectants reveals that all contain the human Ha-ras locus. Only six transfectants retain the 18Kb E65.5 BamHI HRAS1 fragment, but further restriction mapping reveals that these six are in fact rearranged at more distant sites. Figure 4.3 shows the restriction maps of the twelve secondary DNA transfectants and of 178C1.
4.5 Reintroduction of the cloned E65.5 HRAS1 constructs.

Plasmid pTZ-RTB-27 and lambda 10.21 DNA was reintroduced into mouse C127 cells in a direct comparative focus forming assay with the activated HRAS1 oncogene, plasmid pEJ. Amounts of pEJ, RTB-27 and lambda 10.21 were adjusted to give equal amounts of the HRAS1 locus in each transfection.

RTB-27 generated 83 foci, lambda 10.21 89 and pEJ 68 foci per $10^6$ cells per $\mu$g equivalent of DNA. Transfections were carried out with C127 DNA as carrier. C127 DNA alone generated no foci. These results show no significant difference between the focus forming abilities of the E65.5 and EJ-18-8D HRAS1 locus and no apparent effect of immediately adjacent sequences.

4.6 Secondary transfection and the E65.5 phenotype.

Secondary gene transfer from E65.5 highlights severable notable points. Firstly, all twelve independant secondary DNA transfectants were isolated and all contain the human HRAS1 locus derived from E65.5. We can conclude from this that as we do not isolate any other oncogenic fragments eg activated mouse ras oncogenes, this is the major, if not only oncogenic sequence present in E65.5. This observation must be qualified by the fact that we cannot rule out the presence of other oncogenes which are incapable of transforming C127 cells. Additionally, this does not rule out the presence of sequences which act synergistically with HRAS1 and which are segregated upon DNA transfer.

Secondly, transfection has generated a series of cell lines which vary widely in morphology and in ability to grow in soft agar. As can be seen from figure 4.2, cell morphology ranges from very flat, C127-like (3B4 and 1B13) to cells which are highly dendritic in appearance (D2C and 2A6).

In relation to the retention of DNA immediately ajacent to the E65.5 HRAS1 locus, there is no correlation with either morphology or growth potential in soft agar. For example, 178C1 which is similar to E65.5 in morphology and retains an identical restriction map close to the HRAS1 locus does not grow in soft agar as well as E65.5. In contrast, D2C has lost most of the surrounding DNA but is highly dendritic and grows rapidly in soft agar. 3B4 has also lost surrounding DNA and is a flat pavement-like cell line and grows only poorly in soft agar. Only one secondary transfectant, 7B, grows to the same
extent in soft agar as does E65.5. A further analysis of this cell line in terms of its tumorigenicity might be fruitful.

The lack of correlation with DNA structure and morphology or soft agar growth potential did not justify an extensive analysis of relative tumorigenicity. However, it was established that all secondary transfectants, regardless of \textit{in vitro} phenotype, were tumorigenic in immunosuppressed mice.

The variation in morphology cannot be explained by variation in HRAS1 copy number. All the secondary transfectant cell lines have amplified copy number equivalent to that in E65.5.

My results for secondary gene transfer highlight the difficulties in the interpretation of such experiments. There is no strong evidence for a direct role of the sequences immediately 3' to the E65.5 HRAS1 locus contributing directly to the tumorigenicity. However, firm conclusions could only be drawn from a more extensive set of secondary DNA and chromosome transformants.

Nevertheless, the clear and extreme tumorigenic phenotype of E65.5 justifies a molecular investigation of the mechanism and possible consequences of the rearrangement.
Figure 4.1

Morphology in monolayer culture (a, c and e) and soft agar growth after 12 days (b and d) of E65.5 (a and b), secondary chromosome transfectant 178C1 (c and d) and mouse C127 (e) cells. All are viewed under phase contrast.
Figure 4.2

Morphologies in monolayer culture (a, c, and f) and growth in soft agar (b, d, and h) of secondary DNA transfectants 3B4 (a and b), 2A6 (c and d) and 7B (f and h). Morphologies are taken under phase contrast. Soft agar growth is seen under light field microscopy after 12 days growth. Scales as fig4-1.
Genomic restriction mapping around the HRAS1 locus in secondary transfection from E65.5.

Genomic restriction maps at the HRAS1 locus of twelve secondary DNA transfectants and one chromosome transfectant derived from E65.5. The HRAS1 coding exons are marked as solid boxes and the solid line represents DNA which is non-contiguous with HRAS1 in E65.5. Not all restriction sites are shown at the HRAS1 locus. Where the precise localisation of new sites is unknown, the restriction site differing from E65.5 is circled. B=BamHI. G=BglII, T=TaqI, S=Sacl and H=HindIII.
CHAPTER 5
5 MOLECULAR CLONING AT THE E65.5 HA-RAS LOCUS.

5.1 Strategy for cloning.

A rearrangement at the c-Ha-ras-1 locus in transfectant E65.5 was first observed as an altered ras-hybridising BamHI restriction fragment in a screen of the independently arising chromosome mediated gene transfectants (Porteous et al, 1986a, 1986b). Further genomic restriction mapping revealed that the rearrangement had occurred 3' to a SacI restriction site which lies immediately 3' to the last coding sequences (Morten et al, 1987). Absence of hybridisation to a VTR specific probe further localised the breakpoint region to the 2Kb of DNA lying between the VTR and the SacI site (Morten et al, 1987).

Figure 1.5 shows that the rearranged Ha-ras locus is contained within an 18Kb BamHI restriction fragment, which is inside the cloning capacity of the phage vector EMBL3 (Frischauf et al, 1983). Several libraries had been made previously from the transfectant E65.5 but had yielded no Ha-ras hybridising clones. Screening of a total BamHI insert library of 400,000 recombinants (Bob Hill, Christine Sime, David Porteous, pers comm), a 70,000 recombinant BamI size-selected library (David Porteous, pers comm) and a 80,000 recombinant Sau3A partial library (see section and Chapter 6) had all been unsuccessful. Several reports (Wyman et al, 1985; Nader et al, 1986) had suggested that certain sequences appear to be unstable in some strains of bacteria when cloned into a phage vector and that these sequences are subsequently underrepresented in the final amplified library. With these observations in mind and in order to maximise the chance of cloning the fragment, my approach was to make a size-selected library and screen it without the amplification step. To further aid my screen, after initial selection for recombinants on strain Q359, the positively hybridising clones were re-screened and plaque purified on the recombination deficient E.coli strain 1255.
5.2 Construction of an E65.5 Genomic Library.

5.2.1 Vector EMBL3.

The vector EMBL3 is a high capacity bacteriophage replacement vector (Frischauf et al, 1983). It carries a 13Kb 'stuffer' fragment which is removed prior to cloning and is replaced with a suitably sized genomic fragment. The stuffer is removed by digestion with an enzyme that cuts in two flanking polylinker sequences. It has the advantage over most other phage lambda vectors in that recombinant phage can be selected for both genetically and physically. Genetic selection uses the properties of the *spi* system, a system which is intimately involved with recombination pathways in the bacterium.

Phage that carry a functional *gam* gene cannot grow on bacteria carrying a P2 prophage. This is called the *spi* (sensitivity to P2 interference) phenotype. Although not fully understood, it depends upon the interaction of the lambda *gam* gene product with the host recBC protein. The bacterial host function recBC is responsible for inhibiting the transition to rolling circle replication of the lambda genome. The lambda function of the *gam* gene inhibits recBC, making the cell appear to have a recBC phenotype and enabling the mass production of phage DNA. The interference arises from the P2 *old* function. Hosts which are recBC are killed by P2 infection.

The central stuffer fragment of EMBL3 contains the *red* and *gam* genes, so in order to select against non-recombinant phage, the library is plated on a P2 lysogen. Wild-type, non-recombinant phage make the host recBC by the action of gam and are killed by the action of P2. To allow the production of recombinant phage DNA, as the rolling circle mechanism is non-functioning due to recBC inhibition, the host bacterium must be recA*. *E.coli* Q359 is such a host.

5.2.2 E65.5 Genomic library.

BamHI cut E65.5 DNA was size-separated on a sucrose gradient and the collected fractions screened for Ha-ras-hybridising DNA (see Fig 5.1) Positive fractions (41-47) were pooled and used for library construction in the BamHI site of the vector EMBL3.
Figure 5.1 Size selection of Ha-ras hybridising E65.5 DNA.

[A] 200μg of E65.5 genomic DNA was digested to completion with restriction enzyme BamHI and separated on a sucrose gradient. Fractions were collected (1-40, 50μl; 41-59, 250μl), aliquots run on a 0.6% agarose gel and the DNA visualised with ethidium bromide. Fractions 1-40 were pooled as 1-5, 6-9 etc.

[B] DNA was transferred to a nylon membrane and hybridised overnight with a nick translated Ha-ras probe. The filter was exposed to film at -70°C for 3 days. Positively hybridising fractions were pooled and used for library construction as described in materials and methods.
Figure 5.2 Screening of size selected E65.5 library.

Triplicate lifts were taken onto nitrocellulose filters from plates containing approx 80,000 recombinant EMBL3 clones. These were probed with nick translated total human, total mouse and Ha-ras DNA probes at 68°C. Filters were exposed at -70°C for overnight (human and mouse) and 5 days (Ha-ras).

Here a representative single plate is shown.
Ligation, packaging and selection on Q359 resulted in library of approx 400,000 recombinant clones. These were screened by plaque hybridisation (see fig 5.2) with a Ha-ras probe and 50 positives were identified. These were isolated and 5 of the strongest hybridising clones were rescreened by plating 50,000 pfu on strain 1255.

Only a small number (average 3) of these plaques were Ha-ras positive. Two clones, 10.21 and 7.5, were further purified and their DNA's isolated. These were identical clones as judged by initial restriction mapping (data not shown), and all further analysis was carried out on clone 10.21.

To detect an average of only 3 positive plaques from 50,000 screened indicates that the clone is inefficient at producing viable virions on the host Q359. Subsequent plaque purification steps on host 1255 appeared to alleviate this problem, although even on this strain DNA preparations gave consistently poor yields. This apparent instability on Q359 was probably responsible for the failure to isolate it on previous library screening, all of which used amplified phage stock.

Figure 5.2 also shows the library was screened with radiolabelled total human DNA as probe to identify clones of human origin. Positive regions were identified and an analysis of several of these is in chapter 8.

5.3 Lambda clone 10.21.

Lambda 10.21 detects a single 18Kb BamHI band in Southern blots of E65.5 DNA and a bands of approximately 20Kb (progenitor fragment of the translocated DNA) and 6.6Kb (Ha-ras) in total human DNA (see section 5.3.3) indicating that no major rearrangements have occurred on molecular cloning.

5.3.1 Restriction analysis.

Figures 5.3a, b and c show a hybridisation and multi-enzyme restriction analysis of lambda clone 10.21. Hybridisation to radiolabelled Ha-ras and lambda DNAs identifies restriction fragments and aids the construction of a full restriction map.
Figure 5.3a Restriction mapping of lambda clone 10.21.

1μg of lambda 10.21 DNA was digested with the restriction enzymes as shown for each panel and separated on a 0.8% gel. After visualisation with ethidium bromide (i) the fragments were transferred onto nylon filters and probed with radiolabelled Ha-ras (ii), lambda (iii) and total human (iv) DNA's respectively.
Figure 5.3b Restriction mapping of lambda clone 10.21 (cont).

1μg of lambda 10.21 DNA was digested with the restriction enzymes as shown for each panel and separated on a 0.8% gel. After visualisation with ethidium bromide (i) the fragments were transferred onto nylon filters and probed with radiolabelled Ha-ras (ii), lambda (iii) and total human (iv) DNA's respectively.
Figure 5.3c Restriction mapping of lambda clone 10.21 (cont).

1 μg of lambda 10.21 DNA was digested with the restriction enzymes as shown for each panel and separated on a 0.8% gel. After visualisation with ethidium bromide (i) the fragments were transferred onto nylon filters and probed with radiolabelled Ha-ras (ii), lambda (iii) and total human (iv) DNA's respectively.
Hybridisation to total human DNA reveals the presence of highly repeated DNA and serves as a guide for the cloning of repeat free fragments. The complete restriction map of the lambda clone (see fig 5.4) confirms and refines the genomic restriction map.

5.3.2 Subcloning clone 10.21.

The vector pTZ (Vierra and Messing, 1982) is a pUC derived cloning vector with several useful features: high copy number, small size (2.9Kb), polylinker cloning sites and an F1 origin of replication. This enables the plasmid to replicate in the single-stranded form when helper functions are provided by a helper phage. Inserts carried in this vector are therefore amenable to sequencing without the need to subclone into the single-stranded phage vectors derived from M13.

In an attempt to subclone the entire genomic insert of clone 10.21 BamHI/BglIII cut DNA was shot-gun cloned into the BamHI site of pTZ. Recombinant plasmids carrying lambda inserts were identified by colony hybridisation and discarded. Three clones carrying the Ha-ras locus and four carrying the 4Kb central BglIII fragment were identified by specific hybridisation to lambda 10.21 restriction fragments. No clones were obtained carrying the 3' 6Kb BglIII or 3' BamHI/BglIII fragments. Both orientations of the Ha-ras fragment were isolated for sequencing of the breakpoint (see section 5.5 and chapter 6).

EcoRI subcloning produced a 3' subclone, an insert of which was used to identify plasmids carrying the 3' 6KB BglIII fragment.

Therefore the complete insert was isolated within 4 genomic subclones. These are listed below and are represented graphically in figure 5.5a.

RTB-27 6.1Kb BamHI/BglIII fragment carrying the Ha-ras locus.
RTB-23 As for 27 above but in opposite orientation.
RTB-15 3' 2.8Kb EcoRI fragment.
RTB-16 3' 6Kb BglIII fragment.
RTB-5 Central 4Kb BglIII fragment.
Figure 5.4

Complete restriction map of the lambda clone 10.21, shown here as an 18Kb insert into the vector EMBL3, based upon the restriction fragment hybridisations in figures 5.3a-c.
Figure 5.5 Plasmid subcloning and fragment isolation from lambda 10.21.

[a] Complete isolation of the genomic insert of lambda clone 10.21 in 4 subclones in the vector pTZ. See section 5.3.2.

[b] Fragments isolated and used as hybridisation probes in the search for repeat-free regions of clone 10.21.
5.3.3 Analysis of repeat-free subclones.

Two approaches were used to isolate repeat-free subclones of lambda 10.21. The first was to identify and clone restriction fragments which failed to hybridise to radiolabelled total human DNA and use these as hybridisation probes back onto human DNA. The second approach was to isolate restriction sub-fragments of the primary subclones by preparative gel electrophoresis. These were then used as hybridisation probes directly by random priming. The latter method proved the most efficient method of studying fragments of interest rapidly.

By restriction analysis and hybridisation with total human DNA an EcoRI/HindIII fragment of RTB-5 was identified which was repeat-free. This fragment was force-cloned into pTZ and is called RF-5. Despite the fact that total human DNA fails to hybridise to it, use of RF-5 as a hybridisation probe against a digest of human genomic DNA gives a smear characteristic of highly repetitive DNA.

To further analyse RTB-5 it was divided into 4 restriction fragments as seen in Fig 5.5b. Both 5′ HindIII (550bp) and 3′ EcoRI (1.8Kb) fragments produced strong repetitive smears on human DNA (data not shown). Fragment HindIII/SacI (900bp), a subfragment of RF-5, gives a ladder of hybridising fragments when used as a probe against both E67-1 and E65.5 DNA. This fragment was used to map repeat repeat bands in E65.5 and a secondary chromosome transfectant 178C1 (see chapter 8). The pattern of hybridisation observed is indistinguishable from that achieved with a human alu repeat probe, except for the presence of a strong band coinciding with the fragment it was cloned from.

The central 750bp SacI/EcoRI fragment of RTB-5 is essentially single copy. It was used to screen both a chromosome panel and several total human gene libraries (see chapter 7).

The EcoRI subclone RTB-15 produces two fragments containing insert DNA upon digestion with HindIII. The 2Kb insert, which is the most 3′ fragment of 10.21, detects a subset of repeat bands identified by the HindII/SacI fragment. This insert also cross-hybridises to a single 20Kb mouse band in C127.
Figure 5.6 Analysis of genomic fragments.

Fragments pictured in figure 5.5 were used as hybridisation probes against a panel of BamHI cut DNA's (EJ-18-8D-total human DNA, 1W1/1B816-an equal mix of two chr11 only somatic cell hybrids, E67.1 and E65.5-two CMGT transfectants, and C127-mouse DNA).

i) RTB-15 500bp HindIII insert which is single copy. It detects two bands in human DNA (marked with triangles) at 20Kb and 5Kb.

ii) RTB-15 2Kb HindIII insert detects a series of 4 bands in E65.5 and multiple bands in human DNA.

iii) RTB-5 900bp ScaI/HindIII fragment detects a series of fragments in E65.5 and total human DNA.
The 400bp HindIII fragment of RTB-15 is essentially single copy and detects several bands in BamHI restricted human DNA.

5.4 Localisation of the breakpoint region by fine-point restriction analysis.

Lack of hybridisation to a VTR specific probe (J1-the gift of J. Heighway) (Morten et al, 1987) indicated that the breakpoint in E65.5 lay immediately within or 5' this region. Making use of the known sequence of the genomic Ha-ras gene (Capon et al, 1982), I was able to identify restriction fragments which would allow the breakpoint to be localised to a small enough region for sequencing. Four diagnostic restriction enzymes (SphI, AccI, XhoII and PvuII), all having sites within the 2Kb of DNA between the retained Sacl site and the VTR, were identified. By direct comparison of the refined restriction map of RTB-27 with the cloned Ha-ras gene (pEJ) the breakpoint could be localised to a region of 125bp.

As can be seen from figure 5.7a, diagnostic fragments resulting from both BamHI/SphI (4719bp) and BamHI/AccI (4468bp) are both absent from RTB-27 indicating that they have been lost in the rearrangement. Fragments resulting from PvuII (2538) and XhoII (776bp) digests are both present (figure 5.7b). Assuming that the loss of the AccI site is not merely a polymorphism between the Ha-ras alleles or is due to base-pair alterations near to the breakpoint, the breakpoint in E65.5 is thus localised to the 125bp of DNA lying between the PvuII and AccI sites of the normal Ha-ras locus. This is shown diagramatically in figure 5.8.

5.5 Subcloning of breakpoint region.

The use of pTZ to produce single-stranded DNA of high enough quantity and quality for direct sequencing is critical to its use as a genetic engineering vector. My own experience is that the production of ssDNA from the 6.1Kb inserts of RTB-27 and RTB-23 is poor (data not shown). Therefore, with the localisation of the breakpoint to a very specific region of the RTB-27 clone, subcloning a smaller fragment was found to give much better single stranded template.
Figure 5.7a Breakpoint analysis with diagnostic restriction fragments.

Plasmids pTZ, RTB-27 and pEJ were digested with SphI and BamHI/AccI, the fragments separated on a 1% agarose gel and stained with ethidium bromide. Position of the diagnostic fragments based upon the sequence of Capon et al (1982) are marked. See section 5.4 for details.
Figure 5.7b Breakpoint analysis with diagnostic restriction fragments.

Plasmids pTZ, RTB27 and pEJ were digested with XhoII and PvuII, the fragments separated on a 1% agarose gel and visualised with ethidium bromide (left hand panels). DNA was transferred to nylon membranes and was probed with the radiolabelled BamHI genomic insert from pEJ. Filters were exposed to film at -70° for 4 hours (right hand panels). Diagnostic fragments based upon the sequence of Capon et al (1982) are marked. See section 5.4 for details.
The strategy for subcloning the breakpoint region was one of cutting and religation. Cutting at a polylinker restriction site which leaves the breakpoint region still attached to the vector, followed by religation generates a subclone in one rapid step. Subclone RTB-23 was used as the substrate for this strategy as this was in the correct orientation to produce ssDNA of the correct strand for sequencing using an oligonucleotide primer complementary to a region immediately 5' to the retained PvuII site.

As is shown in figure 5.9, RTB-23 was subcloned with two cutting and self-ligation steps. These two steps reduced the size of DNA insert from 6.1Kb to 1.3Kb in two rapid steps. Plasmid RTB-23.31 was used for sequencing (see chapter 6).
Figure 5.8 Breakpoint localisation in RTB-27 by fine point restriction mapping. Diagramatic representation of the data presented in figures 5.8a and 5.8b. The figure shows the diagnostic restriction fragments of pEJ and their sizes in base-pairs used to analyse the breakpoint. See section 5.4 for details.
Figure 5.9 Subcloning of the breakpoint region of RTB-23 for sequencing.

The figure shows the strategy by which the breakpoint region of RTB-23 was isolated as a 1.4Kb insert by two consecutive cutting and religation steps. See section 5.5 for details.

Introduction into bacterial host NM522. Superinfection with helper phage M13K07. ISOLATION of single-stranded DNA
CHAPTER 6
CHAPTER 6 ANALYSIS OF THE BREAKPOINT REGION.

6.1 Sequencing of the breakpoint.

As discussed in section 3.4, the breakpoint region was localised to a region of 125bp between the PvuII and AccI sites of the normal Ha-ras locus. This region was subcloned into pTZ to give RTB-23.15 (see 5.5) from which template ssDNA for sequencing was produced.

The strategy for sequencing was to use a 17mer oligonucleotide primer complementary to a region 10bp 5' to the retained PvuII site. The oligo 5'-TGCCTCCTGGAGTGGAC-3' was made and the region sequenced using the dideoxy chain termination method of Sanger (Sanger et al, 1977). Sequencing was performed simultaneously with dITP to alleviate potential problems of sequencing through runs of guanine residues. A region of 156bp was sequenced and the consensus sequence obtained from 5 runs can be seen in figure 6.1a. Figure 6.1b shows that the region sequenced encodes an open reading frame.

By comparison with the Ha-ras sequence of Capon et al (1982) it can be seen that a point of divergence is reached 30bp 5' to the lost AccI site (see figure 6.2). Past this point of divergence only 12 out of 75bp are the same. This confirms the localisation by restriction mapping of the breakpoint to this region. The breakpoint lies 310bp 5' to the first repeat of the VTR and 700bp 3' to the poly-A site of the Ha-ras transcription unit.

The position of the breakpoint relative to the Ha-ras coding regions is shown diagramatically in figure 6.2. The sequence of the translocation breakpoint (TBR) is aligned with the sequence of the normal Ha-ras gene.

The localisation of the breakpoint to a region outwith the transcription unit of the Ha-ras gene confirms that the rearrangement does not appear to alter the gene structurally.
Figure 6.1 Sequence of 23.15.

a) Sequence of clone 23.15 was obtained by chain extension from an oligonucleotide primer and is the consensus of five sequencing runs.

b) The sequence of 23.15 was analysed for open reading frames (ORF's). Only one frame contains an ORF. Amino acids are abbreviated as single letters and stop or non-sense codons are marked by !.

a) GCTCTGCCC CACTCTCCCC CGCCCCCTGCC CTCACCCCTAC CCTTGCCCAC GCCTGCTCTCA
TGGCTGGTTG CTCTTGGAGC ACTAAGACAT TTAAAGTGTA TCCCTGACGT TCTGACTTGG
CAACTACGGC TTTATAGCAG GTTGAAGAGA ACGTC

b) GCTCTGCCCACCTCTCCCGCCCGCCCTGCCCTACCCCTTGGCCCACGCCCTGCTCTCA
APAPLSAPAPALTLPPLPTPA
LLPHSPPPTPLPSYPCCPRLPHPH
SCPTLPRPCPHPTHPLAHACLM

TGGCTGGTTGCTCTTGGAGCACTAAGACATTTAAAGTGATCCCTGACGTCTGACTTGG
WLVALGALRHLKCIIPDVLTW
GWLLEH!DI!SVSTFLG
AGCSWSSTKTFKVFYP!RSDLA

CAACTACCGCTTTATAGCAGGGTTGAAAAGAAGTC
QLRLYSRLKRT
NYGFIAKG!KER
TTAL!QVEKNNV
Figure 6.2 Alignment of 23.15 with HRAS1.

The figure illustrates the position (relative to the coding exons of HRAS1 (black boxes) and the VTR region) and the sequence of the breakpoint region of E65.5.

The sequence of 23.15 is shown (hatched shading) aligned with the HRAS1 sequence of Capon et al (1982) between the PvuII and AccI restriction sites (boxed). The oligonucleotide primer used for sequencing is underlined. Divergence of the two sequences is shown by a broken line under the sequence of 23.15.
6.2 Analysis of the breakpoint sequence.

The sequences of the Ha-ras gene surrounding the breakpoint and the newly sequenced TBR were analysed for several features.


[2] Similarity to cloned genes and known repetitive DNA elements.


6.2.1 Base composition surrounding the breakpoint.

Figure 6.3 shows a base-pair composition analysis of the Ha-ras coding strand surrounding the breakpoint region. The region of 200bp around and between the PvuII and AccI sites of HRAS1 is 64% GC rich in base composition. It is clear from this analysis that immediately 5' to the breakpoint lies a region that is highly rich in cytosine residues. This region contains a series of runs of C residues which resemble the core element of the minisatellite repeat (Jeffreys et al, 1985) (see figure 6.4 and section 6.2.3).

Figure 6.3 also shows that as you cross the point of diversion between the 23.15 and HRAS1 sequences, there is a switch from high GC content to a higher AT content. 5' to the breakpoint, the region of common sequence between HRAŚ1 and 23.15 is 70% (57/81bp) G+C, whilst 3' to the breakpoint 23.15 is 57% A+T (compared to 40% A+T for HRAS1).
Figure 6.3 Base-pair composition around the breakpoint.

The sequences of 23.15 and HRAS1 between the PvuII and AccI sites (Capon et al, 1982) were analysed for base-pair composition using the Amersham Staden DNA analysis package. The heights of the graphs for each individual base-type represent the % of their presence within a 10bp window.

i) A and T base pair composition in HRAS1 at the breakpoint.

![A and T base pair composition graph]

ii) G and C base pair composition in HRAS1 at the breakpoint.

![G and C base pair composition graph]

iii) (A+T) and (G+C) base pair composition of 23.15.

![A+T and G+C base pair composition graph]
6.2.2 Similarities to other known sequences.

The sequence of TBR was used to screen the consensus sequence of the *alu* repetitive element and to cloned human L1 repeats. No homologies were found with these two repeated DNA's. We can conclude from this that, unlike other DNA translocation events (de Klein et al, 1986), the breakage event was not mediated by a repetitive DNA element.

The sequence of TBR encodes a short open reading frame (see figure 6.1b) and was therefore used to screen for previously cloned genes and other DNA elements. The Genbank and EMBL sequence data-bases were searched with the sequence of TBR using the wordsearch program of Devereuax et al (1984). No sequences of significant homology were detected above the level of background random sequence similarity.

6.2.3 Presence of potentially recombinogenic sequences.

Figure 6.4 shows the sequences of both Ha-ras and TBR around the breakpoint. As can be seen, there are no regions of similarity between the two divergent sequences after the breakpoint.

No clusters of the element CAGG, which is found repeated at a recombination hotspot in the mouse major histocompatibility complex (Steinmetz et al, 1986) were found. Neither the motif GAGG, which has been found near to translocations at the c-myc locus in mouse plasmacytomas (Picolli et al, 1984), nor the motif CGGC, which is found near the breakpoint of the X:21 constitutional translocation *dilck* in a female muscular dystrophy patient (Bodrug et al, 1987), are present in the vicinity of the breakpoint.

Using the criteria of Boehm et al (1987), the region was searched for sequences bearing a loose homology to the immunoglobulin recombination heptamer signal (5'-CACAGTG). Sequences matching GTG (where * = any base) were searched for in the sequence of 23.15. Two such putative heptamers were found, both of which are close to the breakpoint, as shown in figure 6.4.

One is present 2bp from the breakpoint in the Ha-ras sequence and is conserved at 4/7 base pairs compared to the normal heptamer (CACAGTG vs GGTAGTG). The other is found 11bp 3' to the breakpoint in the 23.15 sequence and is conserved at 5/7 positions (CACAGTG vs TAAAGTG). No
elements resembling nonamer sequences (AGTTTTTGT) were detected.

6.2.4 Presence of Topoisomerase I sites.

An analysis of DNA rearrangements has shown that around 90% of illegitimate recombination events contain a site for topo-isomerase I (CAT, CTY, GTY, RAT where Y=pyrimidine and R=purine) within the 10bp surrounding the site of breakage (Picolli, 1988) suggesting a role for Topo I in non-homologous recombination events. As can be seen from figure 6.2 the sequence of 10bp surrounding the point of divergence in 23.15 contains no such sites.

6.2.5 Presence of potential hairpin loops at the breakpoint.

A structural analysis of the 23.15 sequence with the Staden DNA package revealed the presence of several potential hairpin loops around the breakpoint in E65.5.

Figure 6.5a shows that a potential 8-pair loop is present only 16bp 3' to the breakpoint in the Ha-ras sequence. The only other hairpin of this magnitude in the 200bp of sequence surrounding the breakpoint lies immediately 5' to the first minisatellite-like repeat but this relies on 4 G-T mispairings.

Figure 4.5b shows that at the point of divergence of the HRAS1 and 23.15 sequences, a potential double hairpin exists. This consists of a hairpin in TBR and a chimaeric hairpin formed from the sequences of both 23.15 and HRAS1.
Figure 6.4 Presence of recombinogenic elements at the breakpoint.

a) The HRAS1 sequences of Capon et al. (1982) and 23.15 are shown aligned as in figure 6.2. Putative heptamer-like motifs are indicated in both the HRAS1 and 23.15 sequence by stars above to elements corresponding to ****GTG (see 6.2.3).

Underlined sequences are regions of similarity to the minisatellite core of Jeffreys et al (1985) and are shown in (b).

<table>
<thead>
<tr>
<th>HRAS1</th>
<th>GCCATCCTGCTG</th>
<th>CTCCTGGGAG</th>
<th>TGAGACAGGT</th>
<th>TGCCAGCTGG</th>
<th>TCCGTCTGCT</th>
</tr>
</thead>
<tbody>
<tr>
<td>RTB-215</td>
<td>GCT</td>
<td>GCCATCCTGCTG</td>
<td>CTCCTGGGAG</td>
<td>TGAGACAGGT</td>
<td>TGCCAGCTGG</td>
</tr>
<tr>
<td>PvuII</td>
<td>GCCATCCTGCTG</td>
<td>CTCCTGGGAG</td>
<td>TGAGACAGGT</td>
<td>TGCCAGCTGG</td>
<td>TCCGTCTGCT</td>
</tr>
</tbody>
</table>
| Underlined sequences are regions of similarity to the minisatellite core of Jeffreys et al (1985). The number of base pair matches is indicated for each.

b) Four putative minisatellites are shown aligned with the core sequence of Jeffreys et al (1985). The number of base pair matches is indicated for each.

<table>
<thead>
<tr>
<th>Core 5'-CXTCCCTGCCCACCTCC</th>
<th>Match</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCTCCCTGCCCACCTCC</td>
<td>12/16</td>
</tr>
<tr>
<td>TCCCCCCTGGCGGCCCTCT</td>
<td>9/16</td>
</tr>
<tr>
<td>GCCCTGCCCACCTCGCC</td>
<td>11/16</td>
</tr>
<tr>
<td>ACCCTTGGCCCACGCC</td>
<td>10/16</td>
</tr>
</tbody>
</table>
Figure 6.5 Presence of potential hairpin loops at the breakpoint.

a) Sequence of the Ha-ras coding strand encoding a hairpin loop near to the breakpoint (marked by a vertical line). The element with homology to the heptamer recombination signal is underlined. See 6.2.5.

\begin{verbatim}
T
C.T
C-G
G-C
A-T
C-G
T.G
C-G
G.T
\end{verbatim}

\begin{verbatim}
CCTGCTAGTGTCACTG ATACACA
\end{verbatim}

b) Sequence of 23.15 showing the formation of two hairpin loops across the breakpoint. Non-Ha-ras sequence is boxed with hatched lines. See 6.2.5.

\begin{verbatim}
T·:·A·
T.G·:T-A:
C-G·:T-A:
T-A·:T.G:
C-G·A-T:
G-C·:C-G:
T·A·:A·T·
GCTGTT·CTAAG·ATCCCTG
\end{verbatim}
6.3 Do sequences at the breakpoint identify possible mechanisms of rearrangement?

The rearrangement at the HRAS1 locus of E65.5 could be the product of either a random breakage and reunion event or the product of an enzyme based recombination reaction. In the latter case we would expect to find some evidence for elements that are known to be recombinogenic in other molecular systems such as the immunoglobulin system, although as we have seen in section 1.6 the products of recombination events seem to vary widely. However, a systematic search of the sequence around the breakpoint, presented in section 6.3, has identified several elements that could have played a role in the breakage event.

The analysis has shown that elements known to be recombinogenic such as repetitive DNA elements eg the L1 and alu repeats, and recombinogenic sequences such as CAGG (Steinmetz et al, 1985) are absent from the breakpoint region. However, the appearance of three other structural motifs may be significant: i) the region of high GC content 5' to the breakpoint, ii) the secondary hair-pin structures at the breakpoint and iii) the presence of heptamer-like elements immediately 3' to the breakpoint.

As the activity of a V-D-J recombinase has been suggested to play a role in many chromosomal translocations (Boehm et al, 1988a and 1988b and refs therein), their involvement in the rearrangement of the E65.5 HRAS1 locus merits careful consideration. The presence of two heptamer-like elements very close to the breakpoint is provocative. There are several noticeable points about the two elements. Firstly, the two heptamers are only 4/7 and 5/7bp conserved with consensus heptamer. Whether they could function as active heptamers is not known, although this degree of variation in functional heptamer sequences has been observed. Secondly, normal V-D-J rearrangement usually involves both a heptamer and a nonamer element, but no nonamer-like elements are present around the breakpoint. Boehm et al (1988a) have identified a translocation event that appears to be mediated on one half of the translocation by a single heptamer element, so the absence of a nonamer on one half of the translocation does not rule-out its possible role. There would of course be a prerequisite for a full complement of signals on the reciprocal fragment.

Thirdly, the position of the two heptamers relative to the breakpoint is incompatible with them both playing a role in the rearrangement if it was mediated by such a recombinase reaction. The
heptamer on the HRAS1 sequence is not present in E65.5 and therefore a rearrangement directed by this element would involve interaction with heptamer and nonamer sequences which lie 5' to the breakpoint in the progenitor TBR DNA. Therefore the rearrangement in E65.5 would be the reciprocal half of a translocation event. The presence of such signals in the progenitor of TBR would be strong evidence for such a mechanism.

We would expect the putative heptamer that is encoded in 23.15 would interact with sequences that lie 5' to the breakpoint in the HRAS1 sequence. Since no such sequences are present, this possibility seems most unlikely, as it would mean the event had been driven only by a single heptamer. No such event has been reported previously.

The second structural motif present near to the breakpoint is the highly GC-rich region lying approximately 30bp 5' to the breakpoint. This region contains repeated elements which have homology to the core element of the minisatellite repeat (see figure 6.4). Evidence that such elements play a role in the initiation of recombination comes from several sources.

In bacteriophage lambda, chi sites are regions of DNA which enhance the frequency of recBC promoted general recombination (reviewed by Stahl, 1979). Recombination is maximal close to these sites but has been found up to 10Kb away. The base pair composition of these sites (GCTGGTGT, Smith et al, 1981) has homology (5/8 base pairs) to the core of the minisatellite suggesting a role for these elements in eukaryotic recombination (Jeffreys et al, 1985). The second source of evidence comes from a study of a recombination hot spot in the murine major histocompatibility complex. The recombinogenic region has a high degree of homology to the the minisatellite core (Steinmetz et al, 1986). It is interesting to note that chi, the CAGG repeat (Setinmetz et al, 1986), the GAGG motif (Picolli et al, 1984), the heptamer element \*\*\*GTG and the GGC found near the muscular dystrophy breakpoint (Bodrug et al, 1987) are all sub-elements of the core sequence of the Jeffreys minisatellite GGAGTGGGCAGGAXC (Jeffreys et al, 1985). This may reflect a common element in recombination pathways such as a motif specific endonuclease, as has been identified for the heptamer recombination element (Hope et al, 1986).
More recently San and Gilbert (1988) provided evidence that guanine rich motifs are capable of forming four-strand DNA complexes \textit{in vitro}, an event which, if it occurred \textit{in vivo}, might play a role in the initiation of a recombination event.

Whether these GC rich motifs played a role in the rearrangement in E65.5 can only be confirmed with the isolation and sequencing of the progenitor region of TBR, as a requirement for these types of translocation mechanism is the presence of homologous sequences on the other strand to be exchanged.

The presence of hairpin loops at the breakpoint is provocative. Any mechanism of general recombination requires the formation of single-stranded DNA at some stage eg at the centre of a Holliday cruciform. One would envisage that the presence of secondary DNA structure in the DNA might influence the termination of a recombination event.

The switch in base-pair composition upon crossing the breakpoint (70% GC to 57% AT) is similar to the case of the X:21 translocation in muscular dystrophy ( >90% GC vs 66% AT, Bodrug et al, 1987), and may reflect the influence of overall DNA structure upon recombination breakpoints.

In summary, the sequence analysis of the breakpoint region of E65.5 reveals interesting features, but one can only summarise as to their relevance and potential contributions. This is particularly so in the light of the observations that translocation events are often not conservative in nature, but involve the random addition and/or the deletion of DNA at the breakpoint. Firm conclusions as to the precise mechanism of rearrangement depends upon further evidence of the origin of the translocated DNA and the cloning and sequencing of the progenitor fragment.
CHAPTER 7
CHAPTER 7 CHARACTERISATION OF THE PROGENITOR

BREAKPOINT REGION.

The presence of a translocation at the E65.5 HRAS1 locus and its association with elevated tumorigenicity makes the isolation of the original translocated DNA important. Such a rearrangement might identify an existing or new oncogenic or co-oncogenic element.

7.1 Chromosomal origin of TBR.

To initially determine the origin of the translocation breakpoint region (TBR), a chromosome 11 mapping panel was used. This was because every random lambda clone to date that had been isolated from both E65.5 and the other chromosome transfectants had mapped back to chromosome 11 (see chapter 8 and David Porteous, pers comm.). With lambda 10.21 as a probe, a band of hybridisation is seen in human DNA, but no repeatable hybridisation could be detected to two chromosome 11 only hybrids (see figure 7.1). This result suggested that the rearrangement involved neither chromosome 11 or mouse material. A full panel of somatic cell hybrids carrying various human chromosomes (see figure 7.2) was therefore used to localise the TBR sequence to a specific chromosome.

Using the complete lambda 10.21 as a hybridisation probe, non-HRAS1 human specific bands could be detected in hybrids 2, 3, 7, 8 and 22 (see figure 7.3). The human HindIII HRAS1 band is 20Kb in size and is unresolved on most gels. This pattern of hybridisation for lambda 10.21 confirms that the rearrangement is with human DNA and is most compatible with a localisation to human chromosome 6. If hybrid 3 is giving a false positive result then the localisation could also be to chromosome 8. The lack of hybridisation to single and low copy chromosome hybrids positively excludes chromosomes 1, 7, 9, 10, 11, 13, 14, 21, 22, X and Y.
To confirm that the translocation involves human chromosome 6, hybridisation of a non-HRAS1 single copy lambda 10.21 probe to hybrids MCP6, EDAG3R and EDAG2.4 (all kindly donated by Dr Nigel Spurr) was tested (see figure 7.4). Hybrid MCP6 contains chromosome 6 DNA cytogenetically determined to be from bands 6p21-qter, EDAG3R from 6pter-6q21 and EDAG2.4 from 6q21-qter. The hybridisation seen in figure 7.4 confirms the presence of TBR DNA in hybrids 3, 7, 8 and 22 but unexplainably, I detect hybridisation to both EDAG hybrids (which is contradictory to a unique chromosome 6 position). This suggests that TBR either maps to more than one site on chromosome 6 or, more likely that the cytogenetic assessment of the hybrids used is incomplete. Assuming that the chromosome 6 localisation is correct, then the lack of hybridisation to MCP6 suggests that TBR lies telomeric to 6p21. These results need to be substantiated by an analysis of further hybrid cell lines.

7.2 Cloning of the progenitor sequence to TBR.

7.2.1 Screening of total human genomic libraries.

Single copy markers isolated from plasmids RTB-5 and RTB-15 and the complete lambda clone 10.21 hybridise to a single 20Kb non-HRAS1 fragment in BamHI digested human DNA. These single copy probes were used to screen genomic libraries for this progenitor TBR fragment. Screening of both 10^6 pfu of a total human EMBL3 library (kindly donated by Dr Roland Wolf) and 10^5 clones from a total human cosmid library (kindly donated by Dr Hans Lehrach) failed to yield positive clones.

However, screening of 10^6 pfu of a Sau3A partial library of total human DNA (kindly donated by Dr Wendy Bickmore) with a single copy probe from RTB-15 identified two positive clones, 4.11 and 4.21. Upon secondary screening with a single copy probe from RTB-5, only clone 4.11 was positive. This clone is likely therefore to contain the progenitor regions of DNA around the breakpoint. Clone 4.21 is presumed therefore to be a Sau3A partial fragment with an overlap in the region of RTB-15 which does not extend as far 5' as RTB-5. Further restriction analysis of 4.21 suggests that it identifies at least 3Kb of DNA not present in 4.11 (data not shown). Lambda clone 4.11 was plaque purified and DNA isolated for further characterisation.
7.2.2 Characterisation of lambda clone 4.11

Lambda clone 4.11 DNA was digested with various restriction enzymes and, in combination with hybridisation with cloned fragments of lambda 10.21 (data not shown), a restriction map was built up (see figure 7.5).

As can be seen from figure 7.5, clone 4.11 and clone 10.21 have several restriction fragments in common including the 2.8Kb EcoRI and 6.8Kb BglII fragments. Most noticeably, fragments up to 500bp from the breakpoint region (1.9Kb HindIII fragment) appear intact. This is evidence that no gross changes have occurred to the DNA in the region upon rearrangement in E65.5, and it also confirms that all the non-HRAS1 DNA in clone 10.21 is indeed from one contiguous DNA fragment.

7.2.3 Subcloning of the breakpoint region.

Restriction digests of lambda 4.11 were probed with an insert from plasmid RTB-27 containing the breakpoint and a fragment was identified (data not shown) which was isolated by shot-gun cloning BglIII fragments into BamHI cut vector pTZ. Clone pTZ-TBR-1, carrying this progenitor breakpoint region was identified by colony hybridisation. pTZ-TBR-1 DNA was isolated, purified and the breakpoint region sequenced using oligonucleotide-primed double-stranded DNA sequencing.

7.3 Sequencing of the progenitor breakpoint region.

Following denaturation, double stranded plasmid DNA can be successfully sequenced alleviating the necessity to produce high quality single-stranded template DNA (Chen and Seeburg, 1985). Using an oligonucleotide complementary to a region 32bp 3' to the breakpoint in the sequence of 23.15 (for an exact location see figure 7.6b), a region of 90bp of pTZ-TBR-1 was sequenced. The consensus of two sequencing runs is shown in figure 7.6a and is shown combined with the 23.15 TBR sequence aligned with that of HRAS1 in figure 7.6b.

7.3.1 The TBR sequence.

Previously, in the sequence analysis of 23.15 (chapter 6), the point of divergence, and hence the assumed breakpoint, had been an A residue neighbouring the C residue marked by a single star in figure
7.6b. However, the presence of a C residue in both HRAS1 and TBR at that point means the exact breakpoint is uncertain. The overall base composition of TBR is 48% G+C and 52% A+T.

As can be seen from figure 7.6b, in the region of TBR DNA 5' to the breakpoint, there is little homology with HRAS1 except for one short region which lying 22bp upstream. This is marked by shaded circles in the figure. Within this short 10bp region, HRAS1 and TBR have seven base pairs in common. This region of HRAS1 is one previously identified as having homology to the minisatellite core region (see chapter 6).

7.3.2 Sequence motifs around the breakpoint.

Within the region of TBR having homology to HRAS1 are the sequences CCCT and CCT (both of which are underlined in figure 7.6b). These appear frequently in the surrounding regions of DNA. CCCT appears four times within the minisatellite regions of HRAS1 and CCT several times. It is interesting to note that immediately adjacent to the breakpoint are 4 C residues, which bear an obvious homology to the C-rich regions of HRAS1. The immediate region of the breakpoint contains no CAGG or GAGG motifs or any potential topoisomerase I sites as were discussed in chapter 6.

7.3.3 Recombination signals in TBR.

As detailed in chapter 6, the presence of a heptamer like signal element in HRAS1 immediately 3' to the breakpoint suggested that a possible rearrangement mechanism might utilise such recombination signals. The progenitor sequence of TBR does contain a heptamer-like element, TCATGTG which is four base pairs from the breakpoint, and also a T-rich nonamer-like element 40bp 5' to the heptamer.

7.4 Possible mechanisms of rearrangement at the E65.5 HRAS1 locus.

Does the sequence analysis of the progenitor TBR provide any insights into the mechanism of the rearrangement which occurred at the HRAS1 locus of E65.5? Two possible mechanisms appear possible; i) the involvement of a V-D-J recombinase and ii) the involvement of recombinogenic sequence motifs and a short stretch of homology between HRAS1 and TBR.
7.4.1 Immunoglobulin-like rearrangement signals.

As was discussed in section 7.3.3, TBR does contain both heptamer and nonamer-like elements, however several points must be made to qualify these observations. Firstly, both signals are divergent from the consensus signals; TCATGTG versus CACAGTG for the putative heptamer, and TGTTTTTGG versus GGTTTTTGT for the nonamer. However, as previously discussed, a degree of divergence is often found in in vivo T- and B-cell neoplasias (Haluska et al, 1986; Finger et al, 1986; Boehm et al, 1988a). Secondly, for the heptamer and nonamer signals to function as recombination promoters in B- and T-cell neoplasias, there is a stringent spacer prerequisite placed upon them. This states that the signals should be 23 or 12 bp apart. In the case of the putative signals in TBR they are separated by 40bp. To date adherent V-D-J recombinase activity has only been associated with rearrangements which occur when one half of the translocated DNA's is of immunoglobulin origin. No evidence exists to suggest that TBR encodes an immunoglobulin-like locus and to date none have been identified on the suggested chromosomal origin of TBR. This and the atypical spacing of the putative signal motifs argues against V-D-J recombinase activity being the mechanism of rearrangement. It will nevertheless be of interest to determine how frequent and with what spacing such degenerate heptamer and nonamer signals are found as more legitimate and illegitimate recombination events are cloned and sequenced.

7.4.2 Small regions of homology and their putative role in recombination.

As described in section 7.3, the sequence motifs CCCT and CCT are found both within the small region of homology to HRAS1 and distributed within the region of HRAS1 near to the breakpoint. The fact that both a short region of homology exists and that the motif CCCT is present argues for a direct role in the mechanism of rearrangement.

Firstly, short regions of homology have been suggested to play a role in the X:21 constitutional translocation in a female with muscular dystrophy (Bodrug et al, 1987). In that case, the 4 base pair motif CGGC was found near to the breakpoint and was repeated six times in the translocation partner DNA.
The motif CCCT has been reported elsewhere to be present near to DNA rearrangements. Nalbantoglu et al (1986 and 1988) reported the presence of runs of the motif GAGG which is the homologue of CCCT, near to deletions and insertions in the hamster APRT locus.

It seems likely therefore that the presence of this motif and its homology to a minisatellite-like element have mediated or promoted the recombination event.

7.5 Lack of expression of TBR-encoded sequences.

Lambda clones 4.11 and 4.21 were used as hybridisation probes to screen for transcripts in total and polyA RNA panels but none were detected (data not shown).

7.6 The E65.5 translocation.

The analysis of TBR and HRAS1 sequences around the translocation in E65.5 has revealed possible mechanisms of rearrangement. We do not wish to exclude the possibility of a contributary effect of the heptamer sequence in the recombination event, although the short regions of minisatellite-related homology would appear the most likely explanation for promoting or initiating the event.

To continue the analysis of E65.5 at a more global level, a molecular fingerprint of the DNA has been built-up by the use of L1 repeated DNA's (see chapter 8).
Figure 7.1.

Southern blot hybridisation of radiolabelled lambda 10.21 DNA to a panel of somatic cell hybrid and transfectant DNA's digested with restriction enzyme BamHI. 1W1 LA4.9 and 1B816 are two chromosome 11 only hybrids and EJNBC is a transformation-selected fusion cell line containing 11p13-11pter (David Porteous, pers comm). E67.1, E65.5 and E65.7 are HRAS1-selected chromosome transfectant cell lines.
Table of somatic cell hybrid DNA's used to determine the chromosomal origin of TBR sequences. For each hybrid (numbered 1-24), '+' indicates the presence of that particular chromosome. The hybrids used were: 1= SK82/B82/E4/5, 2= CTP 41/3, 3= ADP 3.10.7, 4= ADP 3.10.9, 5= HROP 25/14, 6= H22.6/13, 7= SK81/WG3H, 8= CTP 41.7, 9= SK81/RAG65, 10= H22.6/9, 11= WEHT7, 12= WEHT8, 13= CL21, 14= IW1 LA4.9, 15= PGME9, 16= PGME8, 17= THYB1-6, 18= THYB1-12, 19= 3E7, 20= DF37, 21= IB816 and 22= POR4.

| Hybrid | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  | 15  | 16  | 17  | 18  | 19  | 20  | 21  | 22  | X   | Y   |
|--------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1      | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 2      |     | +   | +   | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 3      | +   | +   | +   |     | +   | +   |     | +   | +   |     | +   | +   |     | +   |     |     |     |     |     |     |     |     | X   | Y   |
| 4      | +   | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 5      |     | +   | +   |     | +   |     | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 6      |     |     |     | +   |     | +   |     | +   |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 7      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 8      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 9      |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 10     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 11     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 12     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 13     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 14     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 15     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 16     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 17     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 18     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 19     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 20     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | X   | Y   |
| 21     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | Y   |
| 22     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     | +   |

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Figure 7.3.

Southern blot localisation of radiolabelled lambda 10.21 DNA to HindIII digested DNA from the hybrids listed in figure 7.2. Bands detected are labelled as 'M'=mouse and 'H'=human.
Figure 7.4

Southern blot localisation of the single copy marker RF-5 to a panel of somatic cell hybrid DNA's digested with restriction enzyme HindIII. For hybrid names and their respective chromosomal content see figure 7.2 and section 7.1.

(\text{NB}) RF5 hybridising DNA is not visible in this figure due to poor photographic reproduction.
Figure 7.5

The restriction map of progenitor TBR clone 4.11 is shown aligned with that of lambda 10.21 to show the common restriction fragments. Probes 1 and 2 are the single copy probes derived from lambda 10.21 used to isolate 4.11 from a genomic library (see section 7.2). Plasmid subclone pTZ-TBR-1 is the subcloned BgIII fragment of lambda 4.11 containing the progenitor breakpoint region.

Figure 7.6a

Sequence of the progenitor breakpoint region obtained from double-stranded dideoxy sequencing of plasmid subclone TBR-1.

5' - TTTGAATGTC TTAGAGGGGA CACATGATT CTAGATTGCA GCTGGCTGTA AGGAAGGGAG GCAATTAACC AAAAACAGGA XCCATTTTTG CAGC
Figure 7.6b.

The sequence of TBR across the breakpoint made up of the combined sequences of 23.15 and TBR-1 which overlap by 15bp, is shown (hatched) aligned with the sequence of HRAS1. The region complementary to the oligonucleotide primer 5' AGCCGTAGTTGCCAAGTCA used for sequencing TBR-1 is underlined (see section 7.3). Sequence data derived from 23.15 is underlined by a broken line. A region of homology between HRAS1 and TBR is indicated by a closed circle between the conserved residues. The presence of sequence motifs CCT and CCCT is indicated in both sequences (see section 7.3.2).
CHAPTER 8
8.1 A repeat analysis of E65.5.

The human genome contains two major families of repeated DNA sequences; the short interspersed nuclear elements (SINES) and the long interspersed nuclear elements (LINES). SINES, or as they are more commonly referred to in human DNA, *alu*, repeats are very abundant in the human genome, occurring on average once every 5-10Kb. The mouse genome contains a smaller number (10-20,000) of *alu* equivalents which make the analysis of amplified subchromosomal fragments of human DNA in mouse cells difficult. LINES, or *Li* repeats are less abundant in the human genome, occurring on average every 150Kb of DNA but probes which show no cross hybridisation to mouse DNA are available for studying sub-chromosomal lengths of human DNA in a background of mouse DNA. The *Li* repeated sequences have been particularly useful in this respect.

A detailed *Li* repeat analysis of E65.5 was undertaken to measure accurately its human DNA content, and to serve as a reference against which to compare subsequent secondary transfectants. E65.5 DNA was digested with various enzymes and the DNA probed with the *Li* 1.2 and 1.8 human repeated DNA elements. Figures 8.1 and 8.2 show that E65.5 contains up to 22 fragments (BglII, 1.2 probe). Assuming that an *Li* repeat occurs approximately every 150Kb (Shafit-Zagardo, 1982) and that each band represents essentially a single-copy hybridising element (see also 8.2), then we can estimate the human DNA content of E65.5 to be between 3 and 4Mbp.

8.2 Human DNA content of 178C1.

Figure 8.3a shows a comparison of E65.5 and 178C1 when probed with the human *Li* 1.8 probe. It is noticeable that every band that is present in 178C1 has a homologue in E65.5. There appears to be no amplification or rearrangement of bands.
Figure 8.1 L1 repeat mapping of E65.5.

10μg of E65.5 genomic DNA was digested with various enzymes and Southern blotted onto a nylon membrane. L1 hybridising fragments were visualised by autoradiography after hybridisation with a nick translated L1 1.2 probe. Size markers are fragments of lambda DNA cut with HindIII.
Figure 8.2 L1 repeat mapping of E65.5.

10μg of E65.5 genomic DNA was digested as described in figure 8.1 and probed with an L1 1.8 probe.
That all other bands are of either equal intensity or are absent, indicates that these are likely to be single copy units. The variation in the degree of hybridisation intensity is therefore most likely to arise from varying degrees of homology to the L1 probe DNA. This is true also of the 1.2 probe (see figure 8.3b). This is in contrast with several of the primary chromosome transfectants, including E65.5, several of which showed banding alterations compared to the normal chromosome 11 consistent with major rearrangements and amplifications (Porteous et al, 1986a).

Based upon a comparison of L1 hybridising fragments, 178C1 appears to contain one third to one half of the human DNA present in E65.5. This is estimated by comparing numbers of hybridising fragments. For example, in BglIII digests, the 1.2 probe detects 10 bands in 178C1 compared to 22 bands in E65.5 giving us an estimate of 2Mbp for the size of the 178C1 transgenome. The absence of rearranged bands in 178C1 is striking and contrasts with degree of rearrangement accompanying this primary chromosome transfer.

Homologous bands in 178C1 and E65.5 hybridise with equal intensity. This must mean that the copy number of each band in each transgenome is identical. This confirms the earlier observation that 178C1 has an equal copy number of HRAS1 genes to E65.5. Taken together with the L1 data, this indicates that the 178C1 transgenome is a subfragment of the E65.5 transgenome which has been amplified to give an equal copy number of HRAS1 elements.

Figure 8.4 shows a comparison of E65.5 and 178C1 DNAs probed with a subfragment of RTB-5 which corresponds to an alu repeat fragment. This substantiates what we observe with the L1 repeat probe. All the bands present in 178C1 are present in E65.5 and appear to be unrearranged and unamplified. It is noteworthy that the number of bands which are present in 178C1 compared to E65.5 indicates that the transgenome is much larger than was estimated from L1 repeat analysis. The alu hybridisation suggests that 178C1 contains much closer to 3/4 of the human DNA content of E65.5. As previously discussed, the presence of background hybridisation and the merging of multiple bands into a smear means that accurate estimates of the transgenomes based upon alu repeat bands is impossible.
Figure 8.3 L1 repeat mapping in secondary chromosome transfer.

10μg of E65.5 and 178C1 DNA's were digested with BamHI and BgIII and probed with L1 1.2(a) and 1.8(b) probes.
Figure 8.4 Alu repeat mapping in secondary chromosome transfer.

10μg of genomic 178C1 and E65, DNA's were digested with BamHI and the fragments probed with a radiolabelled human alu repeat probe.
It is clear that secondary chromosome transfer has reduced the complexity of the E65.5 transgenome. This reduction does not appear, on the basis of repeat mapping, to be accompanied by the gross rearrangements seen on primary transfer.

8.3 An analysis of random lambda clones from E65.5

Random human lambda clones were isolated from two recombinant libraries made from E65.5 DNA. Human clones from a Sau3A partial library, which was unsuccessfully screened for the rearranged HRAS1 locus (for construction details see figure 8.5), and clones from the size-selected BamHI library described in chapter 5 were plaque purified using hybridisation to human and mouse DNA's (see fig 8.6). After pre-annealing with total human and mouse DNA's to exclude repeated DNA from the hybridisation reaction (see 2.9.3), each was used to probe a panel of HRAS1 selected chromosome transfectants. Figure 8.7 and 8.8 show three such random lambda clones and the hybridisation localisation data for others is summarised in table 8.1.

8.3.1 Specific lambda clones.

Lambda M12 (fig 8.8a) detects multiple human bands, all of which are present in a chromosome 11 only hybrid (1W1-LA4.9). The cotransfer of bands in the other transfectants is not uniform, with only E65.5 and E67.1 containing all four bands. E65.9 and E65.6 each contain different subsets of the bands. This may indicate that M12 detects some type of dispersed element on chromosome 11.

Lambda M3 shows cross-hybridisation to mouse DNA and therefore could represent a coding sequence. This is also the case with three other random clones (see table 8.1).

Screening with radiolabelled total human DNA, and counter-screening with mouse DNA does not remove all the mouse clones reaching a tertiary screening stage. This is due to the low level of mouse homologues of the alu repeat. This was the case with six clones isolated by positive hybridisation to total human DNA. One such clone, B1, which was isolated as a clone carrying human DNA, is specifically rearranged in E65.5 (see figure 8.8b). None of the other five mouse clone isolated show any rearrangement.
Figure 8.5 Construction of a Sau3A partial library of E65.5.

E65.5 DNA was digested with Sau3A under conditions predetermined to give the largest number of fragments in the size range 23-18Kb. These were size-selected on a sucrose gradient (see photo) and the library constructed as described in materials and methods.
Figure 8.6 Random lambda clone isolation by hybridisation.

Random human lambda clones were isolated and plaque purified by repeated hybridisation screening with radiolabelled total human and mouse DNA's.
Figure 8.7 Random lambda clone cotransfer in CMGT.

Random human lambda clones 3(a) and 12(b) were used as hybridisation probes against a panel of DNA's. EJ-18-8D=total human, 1W1-LA4.9=chr11 only somatic cell hybrid, EJNAC and EJNBC=HRAS1 selected fusion hybrids containing 11p13-11pter (D Porteous and V van Heyningen, pers comm), C127=mouse DNA and E65.5, E65.6, E65.7, E65.9, E67.1 and E67.4 are independent HRAS1 chromosome transfectants. All digests are BamHI except H=HindIII, which was used to discriminate between clones of similar sized BamHI fragments.
Figure 8.8 Random lambda clone cotransfer in CMGT.

Random lambda clone B1 localisation to a panel of DNA's described in Figure 8.7 (see 8.3.1).
This is consistent with clone B1 being indicate it to be a junction fragment. It is noticeable that it is also rearranged in 178C1, which if it is indeed a junction fragment would indicate that the secondary transfer may have transferred intact a complete block of human DNA in the surrounding mouse chromatin.

8.3.2 Cotransfer and rearrangement of fragments in CMGT.

As can be seen from table 8.1, 5 out of 9 random human clones from E65.5 are rearranged with respect to EJ-18-8D. This indicates that a degree of rearrangement has occurred upon primary transfection, something was predicted from adherent L1 fingerprinting (Porteous et al, 1986a) and which has been observed with several other transfectants (David Porteous, pers comm).

It is apparent from the transfer of known chromosome 11 markers that the chromosome 11 content of the transfectants is varied (Porteous et al, 1986a). From the analysis of random lambda clones it is clear that several transfectants have blocks of chromatin in common (Porteous et al, 1987). For example, on the basis of such random clone isolation, E65.5 shares extensive chromosome 11 sequences with E65.6 and E67.1 (7/9 random clones in each cell line). In contrast, E65.7, to which no E65.5 random lambda clone cross hybridise, and E65.9 and E67.4 which contain only 1/9 clones each have few sequences in common. Thus the isolation of random clones can be used to assay the degree of cotransfer and to illustrate the degree to which CMGT can be used as a method for producing enriched cloning resources.

All nine random human lambda clones map to human chromosome 11 as judged by either hybridisation to the hybrid 1W1-LA4.9 and/or their presence in EJNAC. If the clone isolation is indeed random then the proportion of chromosome 6 DNA in the transfectant would appear to be <10%. Six of the nine lambda clones (B25, M15, B7, M6, B20 and M14) are absent from the hybrid 1B816. As this hybrid contains a single translocated chromosome 11 which lacks the terminal band 11p15 (including the HRAS1 locus) but is apparently otherwise complete (David Porteous, pers comm) this would indicate that these clones map in the region of 11p15.
### Table 8.1 Random lambda clone co-transfer in HRAS1-selected CMGT.

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**Notes**

All hybridisations were carried out at 68°C in the presence of 10% dextran sulphate. Filters were washed to a stringency of 0.5xSSC.

The presence of a BamHI hybridising fragment in each cell line is indicated by ' + ', absence by ' - '. When a band has an altered mobility this is indicated by ' R ' and when multiple bands are present the symbol EJ-18-8D, P= partial presence of fragments when multiple fragments are present, XH= cross hybridisation with mouse DNA and NT= not tested.

B1 is a mouse clone which is rearranged in E65.5 (see figure 8.8).

M12 detects 3 bands (figure 8.7) and M3 detects 4 bands (figure 8.8a).

The rearrangements detected by M14 in E67.4 and E65.6 are different.
CHAPTER 9 DISCUSSION

The analysis of the transfectant E65.5 has revealed much about the possible mechanisms underlying the rearrangement at the HRAS1 locus. However, what, if any, evidence exists to demonstrate a causal role for the rearranged HRAS1 oncogene in the elevated tumorigenicity?

9.1 HRAS1 expression and tumorigenicity.

As the HRAS1 oncogene is known to play a role in the transformation of certain cell lines in vitro, we should ask whether variations in the level of expression is responsible for the quantitative variation in in vivo tumorigenic potentials seen in the transfectant cell lines. With such a large range in tumorigenic potential, for example over a 100 fold between E65.5 and E65.7 (see figure 1.4), we might expect to see a large difference in the levels of HRAS1 encoded messenger RNA or protein. However, as we have seen in chapter 3, no such variation in expression is found in either messenger RNA or HRAS1-encoded P21. The disparity seen between levels of P21 expression and tumorigenic potential is further substantiated by the comparison of the of P21 expression in the two bladder carcinoma cell line subclones, EJ-18-8D (tumorigenic) and EJ-18-3E (non-tumorigenic). It seems, therefore, that P21 expression is not the underlying cause of the observed variations in tumorigenicity in E65.5 and the other transfectants.

While the results of some studies have been interpreted to suggest a direct link between P21 levels and tumorigenicity (Chang et al, 1986b; Viola et al, 1985; Viola et al, 1986 and Hand et al, 1984) my own observations are in agreement with those of Chesa et al (1987) who have shown that tumour P21 levels, rather than reflecting varying degrees of tumorigenicity, reflect the levels found the corresponding normal tissue. Similarly, Gilbert and Harris (1988) have also shown that in NIH3T3 cells transformed by HRAS1, P21 expression is not a necessity for tumorigenicity.
As the elevated tumorigencity of E65.5 is not due to a simple increase in the level of P21 expression, is the rearrangement affecting oncogenicity in some other dose-independent way? A molecular analysis of the cloned E65.5 HRAS1 gene showed that no structural interruption has occurred to the transcription unit, an observation that is substantiated by the lack of detection of any transcript or protein of altered mobility. Without sequencing the entire gene we cannot be certain that no secondary changes, such as those observed by Cohen and Levinson (1988), have occurred which may affect the RNA stability or the biochemical activities of the encoded protein in such a way as to potentiate its effects. Studies to investigate these properties and the ras involved second messenger pathways such as the inositol-phosphate intermediates (Wakelam et al, 1986) may reveal such functional alterations.

9.2 The presence of HRAS1 modulating sequences in E65.5.

It is now well established that oncogenes can cooperate to fully transform normal cells into tumorigenic cells (see section 1.3.2). Less is known, however, about other elements which, once this primary transforming event is established, contribute to the transformed phenotype and in doing so modulate the degree of tumorigenicity. An example would be the factors responsible for the acquisition of the ability to invade surrounding normal tissues. In the case of the transfectant E65.5, it is defined as being highly tumorigenic because of its ability to form tumors at low cell inocula in experimental mouse tumour systems. As we have seen, HRAS1 expression appears not to distinguish it from the other transfectants (all of which are also tumorigenic), so one might predict that the underlying cause of the elevated tumorigenicity is a co-oncogenic or modulating sequence or sequences which bestow upon E65.5 the ability to grow more successfully even at such low cell numbers, in a manner independent of cell cycle time. It should be emphasised that E65.5 was selected first by focus formation, not by tumour selection, and that consistent variations in morphology and transformed phenotypes were only distinguishable after clonal isolation and propagation in monolayer culture.

For E65.5, the presence of a rearrangement at the HRAS1 locus suggests that some modulating element may have been introduced. Direct DNA cloning, DNA reintroduction and genomic DNA transfection have been used as tools to investigate this region.
Secondary genomic DNA transfection from E65.5 has established two main points. Firstly, that no correlation exists between the DNA locally contiguous with the HRAS1 locus and the characteristic extreme \textit{in vitro} growth properties of the E65.5 cell line, by which E65.5 was first observed as being different to the other transfectants. This indicates that if modulating sequences are present in E65.5, they are not immediate to the rearranged region. This is supported by the observation that neither lambda 4.11 and lambda 4.21 detect transcripts. Secondly, transfection of genomic DNA has only ever led to the transfer of the human HRAS1 oncogene as the focus-forming unit. This implies that no other oncogenes, such as activated mouse oncogenes or others capable of inducing focus formation on their own (see table 1.3), are present. The use of other recipient cell types such as NIH3T3 would be a useful extension of this analytical tool and may detect a sub-set of oncogenes not detectable by transfection into C127 cells. Additionally, it would be interesting to study the cotransfer of groups of oncogenes with E65.5 DNA into primary cells as an attempt to detect co-operating or modulating sequences.

The observation that reintroduction of the clone E65.5 HRAS1 gene in both plasmid and lambda clone forms shows a non-elevated focus forming ability over the "normal" activated allele, provides further evidence that it is not the direct focus forming activity of the HRAS1 oncogene which has been altered, but rather it is a phenotype of the transformed cell. We would not expect an element which modulated the cellular influences HRAS1 to increase its intrinsic focus-forming ability."

Secondary chromosome transfer was used to generate the transfectant 178C1, which by L1 analysis contains a third to one half of the human DNA content of E65.5 and contains no detectable site alterations at the HRAS1 locus. Little rearrangement appears to have occurred upon transfection but on the basis of reduced anchorage independant growth it appears to have segregated the modulating element. This may mean that such an element lies some distance from the HRAS1 locus. The study of a more extensive range of secondary chromosome transfectants with long-range restriction analysis using pulsed-field electrophoresis could be used to look at more distant loci. Analogous to the study of contiguous DNA cotransfer by secondary DMGT, this could be used to assay for the presence of contiguous modulating elements.
9.3 The transfection process.

If modulating elements are responsible for the elevated tumorigenicity of E65.5, and having failed to noticeably detect any such elements in transfection assays, we need to address the question as to how they may have arisen. There are four main areas from which such elements could have arisen, all of which are products of the transfection process itself: i) the cotransfer of a human modulatory sequence or sequences, ii) the activation of co-transferred human material by transfection associated rearrangements, iii) the alteration of an inherent mouse DNA element or an alteration in the expression of a mouse gene, iv) the coincidental transformation of a "super-responsive" host C127 cell, and v) the deletion of a repressor.

Post-transfectional stabilisation of the transgenome must involve an interaction with host DNA sequences. An indirect "hit and run" mutational action by the stabilising transgenome or the necessary insertion event needed for its maintenance as a viable transgenome are both possible causes of damage to the host cell DNA. Similar observations have been found with SV40 transformed rat 3T3 cells (Seif et al, 1983) and have been suggested for transformation by the Herpes Simplex virus (Galloway and McDougall, 1983).

The transfection process itself is known to be capable of inducing permanent cellular alterations. Kerbel et al (1987) showed that treatment of a poorly metastatic mouse adrenocarcinoma cell line with calcium phosphate precipitate containing no DNA was associated with a high frequency emergence of both more and less aggressive tumorigenic variants. These changes were heritable and so must have involved permanent alterations at the genetic level. Others (Lau et al, 1985 and Gilbert and Harris, 1988) have reported the induction of genetic instability and permanent genetic changes during transfection of plasmids into Chinese hamster embryo fibroblasts and NIH3T3 cells. Gilbert and Harris (1988) also suggest that such instability and its resultant genetic consequences are the direct result of transfection with the HRAS1 oncogene. On the basis of these observations it is clear that secondary cellular changes are frequently associated with transfections. Thus the rearrangement of HRAS1 in E65.5 may be coincidental to the indirect effect upon tumorigenesis of the transfection process itself. With this in mind, a more detailed cytogenetic investigation of the mouse chromosomes in the transfectant cell lines and an analysis of such things as growth factor and general oncogene expression may reveal any secon-
9.4 The E65.5 HRAS1 rearrangement.

The isolation of both the rearranged HRAS1 and the progenitor TBR region as genomic clones has allowed me to investigate the mechanism of chromosomal rearrangement. This analysis has showed that no gross structural alterations have occurred to alter the HRAS1 transcription unit. However, it does suggest a role for short stretches of DNA homology between the two translocated partners in the mechanism of rearrangement.

9.4.1 The breakpoint.

Sequence analysis has localised the breakpoint in E65.5 to a position lying between the 3' SacI site and the variable tandem repeat region (VTR) of the HRAS1 gene. The VTR, which was suggested to play a role in the transforming ability of HRAS1 (Seeburg et al, 1984) is completely absent and thus non-HRAS1 locus DNA is introduced in close proximity to the gene. A report studying the promoter control of HRAS1 showed that the complete removal of the VTR region from the cloned activated HRAS1 allele in approximately the same position as it is removed in E65.5, had no effect on its focus forming ability (Honkawa et al, 1987). As we have already discussed in section 9.2, this is also the case for the HRAS1 locus of E65.5.

The sequence of TBR obtained from lambda clone 4.11 shows the presence of several short motifs of homology (see figure 7.6b) with HRAS1. Although such short regions of homology might appear to be non-specific, a precedent for an important role for tetranucleotide repeats in recombination events has already been set. Bodrug et al (1987) have examined both reciprocal products and parental sequences of an X:21 translocation in a female muscular dystrophy patient. The only DNA homologies around the breakpoint were short runs of CGGC. It was found near the breakpoint on the X chromosomal half and was repeated several times on the chromosome 21 half. They suggest that this limited homology led to the reciprocal tranlocation. Unfortunately, in the case of E65.5, we do not have the reciprocal half of the rearrangement (if indeed one ever existed) which means we infer the mechanism from an analysis of only the parent and one product strand. We cannot tell whether deletions have
occurred concurrently with the rearrangement event.

As was pointed out in chapter 6, there is a striking degree of similarity between elements and sequence motifs involved with or implicated in recombination events. The elements GAGG (Picolli et al, 1984), CAGG (Steinmetz et al, 1986) and Chi all show a degree of homology to internal motifs within the core minisatellite element described by Jeffreys et al (1985). The motifs CCCT and CCT which are found near to the breakpoint in E65.5 also have a similarity to the minisatellite-like elements found in HRAS1, which suggests they played a role in the recombination event. The fact that all these elements appear near to regions involved in DNA rearrangements, would suggest that they act as recognition motifs for DNA cleavage or strand nicking enzymes. The apparent clustering of such recognition motifs in minisatellite-like regions may be the cause of their high recombinational activity and may act to generate new core regions. The rearrangement of DNA fragments via recombination at these sites would lead to expanding and contracting clusters of such motifs, as was suggested by Jeffreys et al (1985).

More evidence for the role of such short simple motifs comes from positions of insertions and deletions within the hamster APRT locus (Nalbantaglu et al, 1986 and 1988). Both these type of events appear to be frequently associated with runs of the tetranucleotide GAGG. It is interesting to note that runs of GAGG are equivalent to runs of CCCT on the complementary strand.

Recently, San and Gilbert (1988) suggested that regions of the immunoglobulin switch region could interact by the formation of four-stranded parallel DNA complexes. Such an interaction would bring the paired regions into close proximity and may promote their recombination. Other elements which have suggested recombinogenic activity such as the minisatellites (Jeffreys et al, 1985), the telomeres of lower organisms such as Tetrahymena (see Henderson et al, 1987) and the closely related telomere repeats in human and mouse (Allshire et al, 1988) and the tetrameric motifs GAGG, CAGG and CCCT are also GC rich. Interactions between G-rich elements in a manner similar to that suggested by San and Gilbert may may contribute to the natural recombination events which appear at such loci.
9.4.3 Rearrangement and transfection.

Chromosome transfection is frequently associated with molecular rearrangement of the transferred DNA. The study of both L1 repeat fingerprints and random lambda clone isolation has revealed that such rearrangement extends beyond the HRAS1 locus to affect much of the transgenome of E65.5. By what mechanisms do these occur?

The creation of somatic cell hybrids by cell fusion results in intact chromosomal fragments being retained. This indicates that there is nothing intrinsically unstable or mutable about the structure of human chromatin in a mouse background. Chromatin instability or packing problems are therefore probably not the cause of the transfection associated rearrangements. The fact that L1 rearrangements are also found in chromosome transfer using electroporation (Dr Elizabeth Weir-Thompson, pers comm.) implies that it is not the co-precipitation with calcium phosphate which is responsible, but suggests rather, that the method of chromosome isolation or the presence of naked chromatin in a mouse cell is the source of rearrangement. As host cell encoded nucleases and DNA repair enzymes are most likely to be involved at some stage in transgenome degeneration and stabilisation, the role in the generation of the rearrangements observed appears the most likely explanation.

9.5 Does E65.5 HRAS1 rearrangement pre-exist in EJ-18-8D?

I have demonstrated that the rearrangement at the HRAS1 locus in E65.5 is with human DNA of non-chromosome I1 origin. This is the first and only example of such an inter-chromosomal rearrangement. Over 200 random clones isolated from three other transfectant cell lines all map onto chromosome 11 (David Porteous and Wendy Bickmore, pers comm.).

Conventional Southern blot analysis of a series of EJ-18-8D tumours has failed to identify a pEJ hybridising band of the size seen in E65.5 down to a level of detection of approximately 1/1000 cells (Morten et al, 1987-see bound copy). If the rearrangement pre-existed in EJ-18-8D it must be present at a very low level. Furthermore, if pre-existing, it cannot impose a significant tumorigenic advantage on EJ-18-8D as we failed to detect the rearrangement in any EJ-18-8D tumours (Morten et al, 1987).

A degree of chromosomal instability is clearly present in the bladder carcinoma cell lines, as
judged by the presence of several marker chromosomes (Hastings and Franks, 1983; Judy Fletcher, pers comm.), but a rearrangement at 11p15 occurring in less than 1/1000 cells would be undetectable in cytogenetic screening. The investigation of the breakpoint using oligonucleotide probes to the sequences of 23.15 and TBR-1 and the polymerase chain reaction may be the only way of determining absolutely whether it pre-existed or was a product of the transfection process.

Summary and concluding comments.

I have investigated the role of the HRAS1 oncogene in the highly tumorigenic transfectant cell line E65.5. I have demonstrated that the molecular rearrangement does not interfere with HRAS1 expression and have shown that the tumorigenicity of E65.5 cannot be explained by a simple P21 dose-dependant mechanism. Indeed, from an analysis of other cell lines it appears that HRAS1 expression itself is a poor indicator of tumorigenicity. A molecular analysis of the breakpoint has suggested that the rearrangement was a result of a recombination system which operates via specific common recognition motifs and which has been implicated in DNA rearrangements analysed in several other experimental systems.

In conclusion, I feel that this study has highlighted the difficulties in drawing firm conclusions about the causes of the complex in vivo and in vitro phenotypes of cellular transformation. However, if the rearrangements found in CMGT are a true reflection of the illegitimate recombinatory pathways associated with spontaneous translocations and deletions in vivo, then along side its use as an enrichment resource for human gene cloning, CMGT could hold a very prosperous future in dissecting the human genome and unravelling the recombinogenic mechanisms directly relevant to cancer and disease aetiology.
REFERENCES
REFERENCES


Lane DP and Crawford LV.(1979) T antigen is bound to a host protein in SV40-transformed cells. Nature 278, 261-263.


Inst 77, 697-701.


Yunis JJ, Soreng AL and Bowe AE. (1987) Fragile sites are targets of diverse mutagens and carcinogens. Oncogene 1, 59-69.


During the duration of this thesis, the following paper and abstracts were presented:
Abstract. Transfection of DNA derived from a variety of tumours can induce morphological transformation of certain immortalised but normally contact-inhibited cell lines. This important technique has been instrumental in the identification and subsequent molecular cloning of a number of oncogenes, including Harvey-ras. We can extend this approach for studying neoplastic potential by performing chromosome-mediated, as distinct from DNA-mediated, gene transfer. This modification offers two potentially important advantages, both of which stem from the fact that sub-chromosomal lengths of DNA are transferred. Firstly, the expression of the oncogene can be studied in its normal chromosomal milieu; potential modifying effects of linked and unlinked sequences can be evaluated. Secondly, new chromatin segments with transforming potential but too large to be transferred as naked DNA may be revealed. Our experiments illustrate some of the new insights into the molecular basis of neoplastic change which can be gained by this technique. They also demonstrate the power of the technique as a genetic tool for the isolation and detailed molecular analysis of oncogene-associated, sub-chromosomal regions of the human genome.

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1. The c-Harvey-ras-1 oncogene and the focus-forming assay

The impact of molecular biology upon our understanding of neoplastic change has been as quick as it has been dramatic; the promise for the future is no less exciting. The identification, isolation and characterisation of a set of mitigating genetic factors, the oncogenes, has been of singular significance. Various criteria have been used to assign oncogenic roles to specific gene sequences (reviewed by Varmus (1) and, most recently, by Bishop (2,3)). These include, a) transduction of cellular sequences by the oncogenic retroviruses, b) insertional activation of cellular proto-oncogenes by retroviruses, c) demonstration of sequence homology with known oncogenes or genes involved in the normal transduction and regulation of growth stimulatory signals, and d) correlation with tumour specific translocations and fragile sites. In some instances, the cumulative evidence for the implicated gene sequence playing a direct oncogenic role is overwhelming, but in others the evidence can best be described as circumstantial. In all cases, what we most want, but most often lack is a functional assay for neoplastic potential.

The mouse NIH3T3 (or C127) focus-forming assay is a functional test which has played a central role in the identification of oncogenes (4-6). In this assay, DNA extracted from tumours or tumour cell lines is coprecipitated with CaPO₄ and applied to monolayers of NIH3T3 cells. Transfection with the oncogenic component is scored as focus-formation, or the ability to induce morphological transformation of...
the recipient cells. Unfortunately, the assay is both exciting and subjective, capable of detecting only a sub-set of oncogenic activities (2,3). This spectrum can be broadened by complementation studies utilising primary fibroblast cultures as indicators of transforming activity (7,8). Nevertheless, the majority of tumour DNA's remain refractive in focus forming assays. The purpose of the present article is to discuss modifications to the assay, aimed at widening the scope still further. Principal amongst these is the use of isolated mitotic chromosomes rather than naked DNA as the donor genetic material. Before detailing the procedure, it is worth considering some of the limitations and interpretative difficulties associated with the DNA transfection procedure and the manner in which these shortcomings might be overcome by the alternative technique of chromosome mediated gene transfer.

The DNA sequences shown to have transforming activity in focus-forming assays are commonly referred to as «dominantly-acting». The term may be mis-leading as it implies that, under otherwise normal physiological conditions, activation of a single allele is in itself sufficient for cellular transformation. Several lines of evidence argue for factors beyond the mutational activation of the oncogene being important in malignant transformation. For example, tumorigenicity is typically suppressed following fusion between transformed and untransformed cells (9), even when the fusion partners are the same cells used successfully as oncogene donor and indicator cell in DNA transfection focus-forming assays (10,11). Re-expression of tumorigenicity is only seen following specific chromosome loss or rearrangement (12,13). Tumorigenic conversion may depend critically upon the effective level of oncogene expression, as determined by the action of putative «anti-oncogenes» or simply the balance between chromosome homologues carrying either the activated oncogene or the normal proto-oncogene (11). There is good experimental evidence for trans-acting factors suppressing the expression of oncogenic retroviruses by modifying the chromatin structure surrounding the site of integration (14). Whatever the final mechanistic explanation for tumorigenic suppression, observations such as these highlight the importance of the cellular environment and re-emphasise the multi-step nature of carcinogenesis.

The same, and more, is true for the «dominantly-acting» oncogenes identified by DNA transfection; the transforming activity of primary DNA-mediated transformants is often significantly higher than that of the primary tumour DNA, a consequence of either (a) amplification of the transforming DNA sequence following transfection, (b) integration of the transforming DNA at a high expression site in the recipient chromatin or (c) segregation from negatively controlling sequences flanking the oncogene in the donor DNA.

How then does the technique of chromosome mediated gene transfer (CMGT) differ from that of DNA mediated transfection and how do we envisage these differences contributing to a fuller understanding of the neoplastic process? In the DNA transfection technique, the donor material comprises naked DNA with an average length of ≈200Kbp or less. Random concatamerisation and rearrangement of the transfected DNA means that sequences longer than 50 Kbp are rarely transferred intact, although each cell can incorporate as much as 2,000 Kbp of DNA (15,16). This homogenisation process has the effect not only that flanking controlling sequences will be segregated, but also that large genes will be transferred inefficiently, if at all. There is one advantageous consequence in that independent transformants will have very few DNA sequences in common. This greatly aids the identification (17) and subsequent isolation (18, 19) of the shared transforming principle.

In contrast, the donor material in CMGT comprises whole chromosomes isolated from mitotic cells. The amount of chromatin which is transferred and stably maintained in any one transformant ranges from gene sized to sub-chromosomal (20-23). Markers known to be syntenic with the selected gene are frequently co-transferred (21, 24). This feature offers the opportunity to study the expression of an oncogene in its normal chromosomal milieu, uninfluenced by the site of integration within the chromosomal apparatus of the recipient cell. We might also expect the chromosome transfer technique to select for specific chromosomal rearrangements which modify the neoplastic potential of the primary transforming oncogene. Furthermore, we hope to establish, through careful assessment of the technique with known oncogenes, the extent to which CMGT might offer a novel approach to the identification and isolation of a new and extensive set of oncogenes, ones which are too large to be transferred intact and functional by DNA transfection. CMGT would then comprise a powerful genetic tool for the detailed analysis of many oncogene-associated, sub-chromosomal regions of the genome.

Given the various unknowns and uncertainties concerning the CMGT process, it was essential to test the chromosome transfer strategy with a well characterised oncogene which located to an equally well mapped region of the human genome. The obvious first choice was the oncogene activated in human bladder carcinoma, c-Harvey-raz-1, which maps to band 11 p15 on the short arm of chromosome 11 (25). We hope that by reviewing and expanding upon our previous work (21, 22, 26-29), in the form of a detailed practical guide to the use of oncogenes in chromosome transfer, that both the potentials and the limitations of this approach will become apparent.

2. The chromosome mediated gene transfer protocol

Our transfection protocol, summarised in Table I, is a simple adaptation and combination of chromosome isolation (30, 31) and calcium phosphate co-precipitation (24, 32) techniques. Several factors determine the overall efficiency of the procedure. The following are amongst the most important:
Table 1. A Protocol for chromosome mediated gene transfer.

(i) Arrest donor cells in mitosis by the addition of Colcemid (0.1 µg/ml for 18 hr) to the culture medium.

(ii) Shake off mitotic cells, chill on ice and pellet by centrifugation (=200g for 7 min).

(iii) Resuspend to =10⁶ cells per ml in fresh 0.075M KCl (in deionised water) and leave to swell (10-20min at 37°C or 30-40 min at room temp).

(iv) Chill on ice and spin down gently (=150g for 7 min) at 4°C. Aspirate the supernatant and resuspend to =5×10⁶ cells per ml in 0.1% digitonin (Fluka AG), 15mM Tris. HCl, 3mM CaCl₂, pH7.0. Keep at 4°C until step vii).

(v) Gently draw the cell suspension 3 or 4 times through a 21 g needle. Check for chromosome release and integrity by light microscopy or, preferably, by fluorescence microscopy of a distamycin DAPI or ethidium bromide stained sample.

(vi) Remove intact nuclei, whole cells and debris by centrifugation (7 min at 100g). Dilute out the digitonin from the chromosome suspension with 15mM Tris. HCl, 3mM CaCl₂, pH7.0. Add sucrose to 5% and pellet chromosomes by centrifugation at 1,300g for 20 min.

(vii) Aspirate off all the supernatant and gently resuspend the chromosomes in 5% sucrose, 15mM Tris. HCl, 3mM CaCl₂, pH7.0. Repellet the chromosomes by centrifugation at 1,300g for 20 min.

(viii) Aspirate off all the supernatant and resuspend the chromosomes gently and evenly in 2 × HeBS (1 × HeBS is 25mM HEPES, 140mM NaCl, 0.75mM Na₂HPO₄, pH 7.5) at room temp in a clear plastic centrifuge tube. Add an equal volume, dropwise, of 2×CaCl₂ (1×CaCl₂ is 0.125mM) while gently bubbling air through to mix. A bluish and lightly floculent co-precipitate of chromosomes and CaPO₄ will form.

(ix) Aspirate the medium from the recipient cells (5 × 10⁶ cells per 100 mm dish, seeded 18hr previously) and add the freshly formed co-precipitate (0.8ml per plate).

(x) Leave for 20min at room temperature, agitating occasionally.

(xi) Add ten volumes of Dulbecco's Minimal Essential Medium (DMEM) supplemented with 5% fetal calf serum (FCS) and incubate at 37°C for 6-8hr.

(xii) Aspirate the medium and co-precipitate and shock the cells with gycerol (15% in HeBS, 2-3min at 37°C). Aspirate off the glycerol, wash one with 5% FCS, DMEM and refeed with same.

(xiii) Reseed cells at 24 hr post-transfection in low gelling temperature agarose for anchorage independent growth or in tissue culture flasks for focus formation or prior to direct tumour selection. Medium change monolayer cultures twice weekly. Supplement agarose plates with fresh FCS weekly.

(i) Donor chromosomes must be prepared from actively growing, mycoplasma-free cultures and handled gently. Optimum conditions for colcemid arrest, KCl swelling and detergent lysis must be determined for each donor line. Some cell lines, for example the C127 cell line, require brief pretreatment with trypsin before swelling in KCl. The addition of sucrose (or fetal calf serum) to 5% avoids compaction and clumping of chromosomes on pelleting and allows for even resuspension. When carefully prepared, the chromosomes are quite stable if held on water-ice in 0.1% digitonin, 15mM Tris.HCl, 3mM CaCl₂, pH7.0. Biological activity is retained for at least 7 days. Chromosomes isolated in 15mM Tris. HCl, 2mM EDTA, 0.5mM EGTA, 80mM KCl, 20 mM NaCl, 14mM β-mercaptoethanol, 0.5mM spermidine, 0.2mM spermine, pH7.2, the isolation buffer preferred for chromosome sorting on the flourescence activated cell sorter (33), are biologically inactive in transfection, at least for chromatally integrated pSV2neo and for Harvey-ras. However, full activity can be restored by dialysis against the 15mM Tris.HCl, 3mM CaCl₂, pH 7.0.

(ii) The chromosome technique is more sensitive than the DNA technique with respect to the quality of the transfection material and the calcium phosphate co-precipitate. Chromosomal integrity can be judged by flourescence microscopic examination of an ethidium bromide or distamycin DAPI stained sample. Preparations with poor morphology, «tailings» of DNA or excess co-purified non-chromatin will give poor results. The form of the calcium phosphate co-precipitate is also critical. This tends to be slightly «heavier» than for naked DNA but should still be lightly flocculent with a bluish tinge. Excessive clumping, a consequence of poor resuspension or too concentrated a preparation of chromosomes, reduces the transformation frequency by reducing the effective dose of co-precipitate per cell. We aim for a final concentration of between 5 × 10⁷ and 2 × 10⁸ chromosomes in 0.8 ml co-precipitate per plate. Clumping tends to increase with standing so co-precipitates are applied to aspirated monolayers as soon as they are formed. The co-precipitate is diluted ten-fold with medium after 20 min and incubated at 37°C. We have noted a marked cytotoxic effect if, as in some DNA transfection protocols, the calcium phosphate co-precipitates are left on overnight; 4 to 8 hours incubation gives.
Figure 1. Oncogene selected chromosome mediated gene transfer. The figure outlines the chromosome transfer protocol and our selection strategies for malignant transformation. See Table 1 and Section 3 for details.

near optimum results. DMSO can substitute for glycerol to «shock» the cells, but the conditions for obtaining a significant increase in transfection frequency, without overall loss of cell viability, are more stringent.

(iii) It is equally important that the recipient cells are completely mycoplasma-free and grow rapidly and exponentially towards confluence, thereafter maintaining a flat monolayer for at least 4 weeks. The best efforts can still be thwarted by batch variability in fetal calf serum and tissue culture plastic. However, it is our general experience that mouse C127 cells are more robust than NIH3T3 cells. Monolayers can be maintained for longer, with a lower background level of spontaneous focus formation (26).

3. Selection for cellular transformation

The ultimate success of the CMGT technique depends on the effectiveness of the selection for the minority of transfection events which result in cellular transformation. The selections we employ are illustrated in Figure 1. They serve both to identify and to classify each independent transformation event. The salient features are described below.

3.1. Focus formation. When cloned oncogenes, for example c-Harvey-ras-1, are transfected into mouse C127 or NIH3T3 cells, foci of transformed cells can be detected against the monolayer background at 3-10 days post-transfection. With naked tumour DNA, for example from the EJ bladder carcinoma cell sub-line, EJ-18-8D (34), foci appear a little later, at about 7-14 days post-transfection. However, with chromosomes from EJ bladder bladder carcinoma, foci are rarely seen before 21 days post-transfection and may take as long as 42 days to appear (26). This extended lag is almost certainly due to the much longer time taken to establish stable incorporation and expression of chromatin compared
Figure 2. Phenotypic variation between HRAS1-selected CMGT’s. The figure is adapted from Figures 1 and 4 in ref. 26 and shows the characteristic differences between three independent transformants, E65-5 (Fig. 2 a, b and c), E65-6 (Fig. 2 d, e and f) and E65-7 (Fig. 2 g, h and i), with respect to their morphology of monolayer culture (Fig. 2 a, d and g), potential for anchorage independent growth (Fig. 2 b, e and h) and tumour histology (Fig. 2 c, f and i). See Sections 3 and 4.1 for details.
Table II. Tumorigenic potential of HRAS1-selected CMGT's. The data is abstracted from Tables I and II in ref. 26 and shows the effect both of absolute cell inoculum and of co-inoculation with excess untransformed mouse C127 cells upon the tumorigenic potential of independent HRAS1-selected CMGT's.

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with cloned or naked DNA. We will return to this point again in Sections 3.3 and 4.3. Because of the initial instability of the chromosome mediated transformants, it is essential to clone and sub-clone foci as soon as is practical. Individual colonies are usually isolated and reisolated with cloning rings. Advantage can also be taken of the relative reduction in cell adhesion of the transformed cells compared with untransformed C127 cells to the tissue plastic. The transformed cells can be «washed off» with a pastette and used to reseed a fresh flask, leaving the co-isolated, but more firmly attached, untransformed cells behind.

Although the C127 cells are contact inhibited, their exponential growth rate is similar to that of most transformed derivatives. Primary foci picked with cloning rings can still be lost if dilution leads to outgrowth by co-isolated untransformed cells or revertants. An effective way to minimise this risk is to apply an independent and positively selectable criterion for cellular transformation, as discussed below.

3.2. Anchorage independent growth. Anchorage independent growth, or the ability to grow in semi-solid medium (methocell or low gelling temperature agarose) is one independent criterion by which to judge neoplastic change. We routinely select for growth in 0.25% low gelling temperature agarose set on a 0.5% base (Seaplaque, FMC Colloids Inc.). Although it is clear that not all morphologically transformed cells have the capacity for anchorage independent growth (34) (and vice-versa (35)), we have yet to observe an EJ DNA- or chromosome-mediated transformant which can not. Indeed, if the products of primary transfection dishes are plated either in monolayer culture or directly in agarose, foci and anchorage independent colonies arise in equal numbers (26). Agarose colonies picked with fine-tip plastic pastettes and propagated in monolayer culture is a convenient way to clone primary foci and obtain cultures devoid of untransformed C127 cells.

The form and extent of anchorage independent growth, however, is highly variable between independent clones (see Section 4.1 and Figure 2). The characteristic behaviour of each transformant in agarose is, in itself, a useful index of neoplastic change (26). We do not exclude the possibility that a small proportion of transfection events will give rise to cells which, although capable of anchorage independent growth, remain overtly untransformed in monolayer culture. The opposite may also be true. Finally, it is of interest that, whenever we have been able to test it, we have not detected any phenotypic or genotypic changes, before and after selection for anchorage independent growth.

3.3 Tumour selection. A third and, perhaps, the most relevant
survivors is sufficiently high for them to be directly selectable, proportionately increased.

ing cells (now as individual clones rather than as a confluent monolayer) will contain the transforming gene of interest is shown recently that the same sub-population of cells compe-

tent to incorporate transfected chromatin are also susceptible to transfection with plasmid DNA (26, 41). Secondary chromosome mediated gene transfer shows that the plasmid sequences become physically linked with the donor chromosome fragments (41). The advantage of this co-selection strategy is that we can use resistance to G418 as an initial selection for the minority of cells which have stably incorporated transfected material. The frequency with which surviving cells (now as individual clones rather than as a confluent monolayer) will contain the transforming gene of interest is proportionately increased.

In fact, the proportion of transformed cells amongst the survivors is sufficiently high for them to be directly selectable, frequencies for Harvey-ras are typically of the order of 1 in 5 \times 10^5 C127 cells.

Any co-selection technique which reduces the background level of non-transformants is of particular value. The plasmid pSV2-neo encodes bacterial resistance to the antibiotics neomycin and kanamycin; when transfected into eukaryotic cells, the plasmid confers resistance to the neomycin anologue Geneticin G418 (GIBCO Biocult) (40). It has been shown recently that the same sub-population of cells competent to incorporate transfected chromatin are also susceptible to transfection with plasmid DNA (26, 41). Secondary chromosome mediated gene transfer shows that the plasmid sequences become physically linked with the donor chromosome fragments (41). The advantage of this co-selection strategy is that we can use resistance to G418 as an initial selection for the minority of cells which have stably incorporated transfected material. The frequency with which surviving cells (now as individual clones rather than as a confluent monolayer) will contain the transforming gene of interest is proportionately increased.

by mass inoculation of G418 resistant cells into immunosuppressed mice, with tumour formation as the end point, but it is important to inoculate the products of transfection dishes singly, so that not only strong, but also any weaker transforming activities and events can be revealed (26). This approach removes the difficult and subjective criterion of morphological transformation in the focus forming assay (42,43). The co-selection strategy has revealed transforming activity in tumour DNA which was previously refractive in standard focus-forming assays (43).

Alternatively, the individual clones can be screened visually, with the possibility that subtle morphological differences, not detectable in the standard focus-forming assay, are detected. Exocrine factors produced by untransformed cells can have a suppressive effect upon morphological transformation. These effects are most pronounced in focus forming assays in confluent monolayer culture, but will be reduced, if not completely removed, in co-selection assays for clonal growth (44).

4. Characterisation of transfectants and tumours

The phenotypic criteria which we have used to select trans-
Figure 4. Translocational and transpositional rearrangement of the c-Harvey-ras 1 locus. Genomic restriction maps of the c-Harvey-ras-1 locus in EJ-18-8D (indicating the four coding exons and the VTR region) and the HRAS1-selected CMGT’s, E65-6 (no restriction site changes), E65-5 (all sites 3’ to exon 4 changed) and E65-7 (all sites 3’ and 5’ to the coding region altered). E, EcoRI; B, BamHI; S, SacI; K, KpnI; T, TaqI; Bg, BglII; X, XhoI; H, HindIII.

formed cells also distinguish independent transformants and serve as a basis for comparing the neoplastic potential of each transformant. Certain criteria turn out to be particularly useful predictors for assessing new transformants. Molecular genetic analysis augments the phenotypic characterisation to provide a comprehensive and unique description of each transformant.

4.1 Phenotypes. Beyond the longer latent period between transfection and focus-formation in chromosome as opposed to DNA transfection, the first noticeable difference between the techniques is in the morphology of independent transformants. Whereas those of pEJ- or EJ DNA-mediated transformants are rather uniform, those of EJ chromosome-mediated transformants are characteristically diverse (ref. 26 and Fig. 2). There is a corresponding variation in cell adhesion to tissue culture plastic and in cell density at confluence. E65-5 grows to a higher cell density and E65-7 to a lower cell density than any of the others. Again, it is E65-5 which shows the highest and E65-7 the lowest growth potential in semi-solid medium (Fig. 2). The colonies formed by E65-6 in low-gelling temperature agarose are often as large but never as dense as those formed by E65-5 (Fig. 2).

It was of particular interest to establish whether these, at times quite subtle, differences in cellular transformation were reflected in tumorigenic differences. We have tested this in our experimental mouse tumour assay (Section 3.3). We have quantitated the tumorigenic potential of each HRAS1-CMGT by both stepwise reduction in the number of transformed cells inoculated per site and by co-inoculation of reducing numbers of transformed cells in the presence of a constant excess of normal C127 cells. The salient observations from a detailed analysis (26) are summarised in Table II. While the tumorigenic potential of the HRAS1-selected CMGT’s is universally high (essentially 100% at inoculi of 10^6 or more cells), the level of spontaneous tumour formation following inoculation with untransformed C127 cells is exceedingly low (less than 1 in 28 × 10^7 cells). However, we do see pronounced differences in tumorigenic potential between independent HRAS1-selected CMGT’s at reduced inoculi. E65-7 fails to form tumours at inoculi of 5 × 10^4 or less, whereas E65-5 forms tumours consistently when as few as 10^3 cells are inoculated. E65-5 derived tumours also develop more rapidly than from E65-7. E67-1 and E65-6 display an intermediate potential.

The same trend is observed when decreasing numbers of transformed cells are co-inoculated each time with 10^7 C127 cells. Interestingly, the number of transformed cells necessary to give rise to tumours is reduced by about 100-fold under the co-inoculation condition. This protective effect probably arises from overloading residual immune responsiveness in the experimental mice and contrasts with the suppressive effect of untransformed cells in monolayer focus forming assays (Section 3.4). Our results also show that if even a weakly transformed cell is present within a population of untransformed cells at a frequency of 1 in 10,000 or greater, then direct selection by tumour formation should be possible (Section 3.4). However, if independent transformants are present within the same population at equal frequencies, then tumour formation will inevitably select for
Table III. Co-transfer of syntenic gene markers in HRAS1-selected CMGT. The markers are arranged, left to right, according to their consensus order on chromosome 11 (21, 25, 29, 54, 70, 71), with c-Harvey-ras-1 (HRAS1) most telomeric on 11p and PGA (plasminogen A), MIC8 (cell surface antigen recognised by monoclonal antibody TRA1.10) and APOA1 (apolipoprotein A1) mapping at or below 11q13. Other abbreviations are INS, insulin; HBB, β-globin; PTH, parathyroid hormone; CALC1, calcitonin; LDHA, lactate dehydrogenase A; FSHB, β-subunit of follicle stimulating hormone; MIC11 & MIC 4, cell surface markers recognised by monoclonal antibodies 165.A5 and F10.44.2 respectively; CAT, catalase.

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the most aggressive event.

The histopathology of the tumours formed by each transformant is also illustrated in Fig. 2. Again, this phenotypic criterion is strikingly consistent within and variable between independent HRAS1-CMGT's. E65-5 always produces clearly demarcated tumours with a very dense inner cell mass, which shows no signs of host tissue infiltration. At the other end of the spectrum, E65-7 forms a much less dense inner cell mass, with characteristic <<sworling>> and extensive infiltration of the host connective tissue, fat and voluntary muscle. E67-1 and E65-6 display an intermediate phenotype. Whilst the tumorigenic potential per se of E65-7 is clearly reduced compared with E67-1 and E65-6 and dramatically less than E65-5, it turns out to be the only HRAS1-CMGT to show invasiveness. Secondary tumours develop in the abdominal cavity in about 10% of cases. We saw no such invasive spread amongst 44 tumours from E65-5, 17 tumours from E67-1 or 27 tumours from E65-6. With the notable exception of E65-7, the absence of invasive potential amongst our HRAS1-CMGT's contrasts with the high levels of metastatic (45) and, more importantly, invasive (46, 47) potential reported for NIH3T3 cells transfected with a variety of ras constructs. This could reflect pre-selection (46) or selection in vivo (47) for highly malignant transformants, but a more interesting possibility is that we are seeing intrinsic differences in expression between transfected DNA and chromatin and between NIH3T3 and C127 cells.

4.2. Genotypes. The variations in phenotypic aspects of tumorigenicity are stable characteristics of each HRAS1-CMGT for which we now seek a genetic basis. The first clue came from a molecular analysis of the c-Harvey-ras-1 oncogene in each transformant (ref 21 and Fig. 3). The actively transforming c-Harvey-ras-1 oncogene in the human EJ bladder carcinoma cell line is contained within a 6.6 kbp BamHI restriction fragment and the same is true for all but two of the HRAS1-CMGT's. The exceptions are E65-5 and E65-7 where the pEJ hybridising fragments are =18kbp and =12kbp in size, respectively (Fig. 3a). We have tested every transformant for restriction site changes up to 20kbp 5' and 3' to c-Harvey-ras-1. In some cases, the restriction map is indistinguishable from EJ-18-8D. In others, there are individual restriction site changes, but only in E65-5 and E65-7 is the c-Harvey-ras-1 region directly involved. A genomic restriction map of these rearrangements is shown in Fig. 4. Although coding exons 1 to 4 appear to be intact in both cases, all sites 3' to the gene are altered in E65-5, whereas in E65-7 sites both 3' and 5' to the gene are changed. Thus, E65-5 has the appearance of a translocational rearrangement and E65-7, a transpositional rearrangement. It is tempting to speculate that the molecular rearrangements and neoplastic characteristics are causally related. Molecular cloning and reconstruction experiments are now being undertaken to test this.

The entire tandem repetition region immediately 3' to c-Harvey-ras-1 (variation in which generates the high frequency restriction fragment length polymorphism (RFLP) for this gene in the population (48)) is completely absent from E65-5 (Fig. 3b and c). An unexpectedly high frequency of longer VTR regions has been reported associated with a diverse range of tumours, leading to the speculation that c-Harvey-ras-1 might play a contributory role in the aetiology of these tumours, through VTR-enhanced expression of the proto-oncogene (49). Whilst a high frequency of rare c-Harvey-ras-1 alleles has also been reported associated with breast cancer (50), an extensive study from our own laboratory has failed to find any such correlation (J. Mackay et al, in
preparation). A similar lack of correlation between normally rare RFLP alleles and a variety of previously implicated tumour types has now been reported by a number of independent investigators (51-53). We have not detected any selective amplification of the VTR region in any of the HRAS1-selected CMGT's but our observations with E65-5 demonstrate that complete absence of the VTR is compatible with a highly tumorigenic phenotype, questioning further the role of the VTR region.

It is most likely that the rearrangements in E65-5 and E65-7 both arose as a consequence of the transfection process, rather than by selection for pre-existing, clonal rearrangements in the EJ-18-8D cell line. Mixing experiments show that we would have detected each rearrangement if present at the 1% level, but neither rearrangement has been detected in any sub-population of EJ-18-8D, nor in any derived, primary tumour material (Fig. 3).

We wished to establish the extent to which sub-chromosomal DNA fragments could be co-transferred intact with the c-Harvey-ras-1 locus. Table III summarises the co-transfer data for a number of defined gene markers on the short arm of chromosome 11 (21, 54). The high frequency cotransfer of markers known to map some distance from c-Harvey-ras-1 indicates that the CMGT process facilitates the uptake of extensive lengths of chromatin. However, interstitial deletion events also occur. For example, in no instance do we see either PTH or CALC1 co-transferred. Analysis of a more extensive set of primary transformants will be necessary to determine whether there is selective loss of particular syntenic loci.

Despite the short arm of chromosome 11 being one of the better mapped regions of the human genome, the distances separating adjacent genes is still immense in molecular genetic terms. We have augmented the single-copy gene co-transfer data with a general approach for assessing the amount and form of human DNA co-transferred (21). The technique, which we have termed L1 «fingerprinting», is illustrated in Fig 5. It utilises cloned members of the L1 (formerly KpnI) family of repeated DNA sequences, of which there are ≈20,000 copies dispersed throughout the human haploid genome, an average of one for every 150kbp (55). An internal consensus restriction site has been lost for many individual L1 repeats. This gives rise to restriction fragment length variants (RFLV's), creating a completely diagnostic hybridisation profile or «Fingerprint» for each transformant (21, 22, 26). The overall human DNA component can be accurately estimated from the intensity of hybridisation to the consensus sequences and the total number of RFLV's.

We can also estimate the extent of molecular rearrangement consequent upon chromosome transfection by comparing the L1 «fingerprints» of independent HRAS1-CMGT's with each other and with somatic cell hybrids which contain chromosome 11 as their sole human component. Thus, the L1 «fingerprints» for E67-1 and E67-4 are strikingly similar, both carrying simple subsets of the complete chromosome 11 pattern. There is no evidence for gross DNA sequence rearrangement. In contrast, the L1 «fingerprints» for E65-5 and E65-6 both suggest significant local amplification and rearrangement of DNA sequences. This is consistent with the translocational rearrangement of c-Harvey-ras-1 in E65-5. The aberrant co-transfer of single-copy genes in E65-6 indicated rearrangement, but not to the extent now suggested. The L1 «fingerprint» for each transformant is invariant with cell passage, following agar cloning and tumour formation and thus, once formed, the HRAS1-CMGT's are very stable (21).

These findings have been substantiated and extended through the isolation of an extensive set of lambda and cosmids human DNA recombinants from E65-6 and E67-1 (27, 29). The E67-1 clones derive exclusively from chromosome 11. We have found only one molecular rearrangement compared
with EJ-18-8D DNA amongst the forty four clones isolated from E67-1. In contrast, many of the clones isolated from E65-6 contain partial rearrangements or are amplified compared with the donor DNA.

4.3. Transgenome structure. The amount of human DNA co-transferred with HRAS1, although substantial, is rarely within the resolving power of conventional histocytochemical techniques. Human DNA can be covalently modified with N-acetoxy-2-acetylaminofluorene (AAF) and DNA hybridisation to mitotic chromosome spreads visualised with fluorescently labelled monoclonal antibodies directed against the AAF moiety (56). The resolving power of this technique is excellent, with a sensitivity limit of \( \approx 1,000 \text{kbp} \) of DNA (22). By this method, we have shown that E67-1 and E67-4 each contain a single hybridising fragment, but that E65-6 contains two discrete fragments inserted interstitially on one mouse chromosome, with a third, smaller fragment associated centromERICally on a second mouse chromosome (22, 28). This analysis provides a physical basis for the molecular rearrangements discussed above.

Most recently, we have developed a double-labelling technique, with biotinylated-human DNA to visualise the human transgenome and tritiated pEJ to localise the HRAS1 oncogene (28). We find discrete hybridisation of pEJ to all three blocks of human chromatin in E65-6. Significantly, we also see two sites of pEJ hybridisation in the E67-1 and E67-4 transgenomes, consistent with head-to-tail and head-to-head tandem transgenome duplication respectively. The transgenome duplications must be essentially perfect as we see no heterozygosity for any of the defined gene or CMGT derived DNA markers mapping to either E67-1 or E67-4. At present, we require gold-enhanced biotin labelling to visualise the transgenome in E65-5, which precludes double-labelling with tritiated pEJ. Nevertheless, it is again clear that the transgenome is duplicated, with usually two copies of the relevant mouse chromosome and, thus, four copies of the transgenome per cell (28).

5. Other oncogenes in chromosome mediated gene transfer

The chromosomal transfer of the Harvey-ras oncogene is the first to be molecularly proven and analysed in detail, but the idea of using CMGT as a functional assay for cellular transformation is not new. Shani et al (57) obtained SV40-TAg positive cells, capable of producing infective virus, after transfer of chromosomal SV40 from transformed Chinese hamster cells to permissive monkey BSC1 cells. Genomic Chinese hamster DNA was not found to be effective, but the conditions used were sub-optimal for DNA transfection. Spandidos and Siminovitch (58,59) reported the chromosomal transfer of anchorage independent growth and tumorigenic potential from Chinese hamster ovary cells to primary Chinese hamster lung cells and to Syrian hamster fibroblasts, but because the nature of the genetic factor responsible was unknown and the transfer was between like or identical species, it was impossible to confirm successful transfer at the cytogenetic or molecular level. The studies of Cassingena et al (60) confirmed and amplified the qualitative findings of Shani et al (57) and of Spandidos and Siminovitch (58, 59).

Shih et al (61) described the transfer of focus-forming ability and anchorage independent growth from chemically transformed mouse cells to the mouse NIH3T3 cell line. In one instance, chromosome transfection, but not DNA transfection, produced foci at high frequency. Further molecular analysis was limited because the transfer was between mouse strains, but the characteristic pattern of endogeneous retrovirus confirmed that the transformants were of NIH3T3 and not donor origin.

More recently, Minden et al (62) showed that chromosomed isolated from human acute myelogenous leukemic cells could induce focus formation in mouse NIH3T3 cells. They were able to use a human specific repeated DNA probe to demonstrate the consistent transfer of specific human DNA sequences to the mouse cells. In this case, the transforming principle is almost certainly the N-ras oncogene, which transfers efficiently with naked DNA as the donor material (63).

Most recently, two chromosome transformation systems, one using the activated met oncogene (64, and our unpublished results), the other using chromosomal SV40 (our unpublished results), have been analysed at the molecular level, but both were developed primarily as genetic tools. These will be discussed in the next Section.

6. Implications for genetic analysis

An early hope for the CMGT technique was as a fine-structure mapping tool (65). This was based upon the observation that syntenic markers were frequently co-transferred with the selected locus (20,24). In testing the c-Harvey-ras-1 oncogene in CMGT, we were conscious of a growing list of human cell lines carrying cellular oncogenes or chromosomally integrated viruses (1-3). We considered that CMGT, with selection for malignant transformation, might provide an effective general mapping strategy, which would allow us to concentrate immediately upon regions of the genome directly implicated in neoplasia and inherited disease.

Our detailed analysis of the c-Harvey-ras-1 selection system has shown that the CMGT process is accompanied by a high level of intrachromosomal rearrangement. Thus, although tightly linked markers do tend to cosegregate, these are not necessarily the ones most closely linked to the selected locus on the donor chromosome (ref 21 and Table III). Our observations caution against the reliance on co-transfer data for constructing sub-chromosomal maps.

Intrachromosomal rearrangement is not a peculiarity of the oncogene selection system; we and others (66) find the same types of rearrangements accompanying biochemical and
immunological selections. Nevertheless, the CMGT approach still has an enormous potential in the context of human genome mapping, by providing enriched sources of molecular probes for predetermined, sub-chromosomal regions of the genome (27,29,67).

6.1 From c-Harvey-ras-1 to Wilms' tumour. The clinical association of Wilms' tumour with aniridia (irislessness) is well established (68). Cytogenetic analysis of these patients reveals variable length, constitutional deletion of the short arm of chromosome 11 and localises the relevant genetic region to band 11p13 (69). We (70) and others (71) have used defined gene markers which map to the short arm of chromosome 11 to start to define the precise location of the Wilms' tumour gene. Although certain of these markers map close, none are universally deleted (29). To get to the Wilms' gene, we need new markers.

The forty four human DNA recombinants isolated from the HRAS1-selected CMGT, E67-1 (Section 4.2) have been sub-localised on chromosome 11 by mapping to somatic cell hybrids carrying either deletions or translocations involving the 11p13 region of chromosome 11 and associated with Wilms' tumour or clinically related conditions (29). The CMGT derived markers provide a detailed fine-structure map of the whole of the short arm of chromosome 11. In particular, they define a smallest region of overlap for the Wilms' tumour gene locus, which is unlikely to exceed a megabase length of DNA (29).

These markers also serve to define the molecular content and extent of rearrangement in other HRAS1-CMGT's (Porteous et al., in prep.). In this way, transformants likely to provide sources of markers enriched for particular sub-chromosomal regions have been identified and successfully tested (D.J. Porteous, M.C. Hirst, W. Bickmore and P. Little, unpublished results). With the density of markers already achieved, we can start to construct a long-range restriction map for the region using <<pulsed-field>> gel electrophoresis (72), to determine accurately the length of the smallest region of overlap and to map precisely the Wilms' tumour associated deletion and translocation breakpoints (W. Bickmore, J. Maule, V. van Heyningen, N. Hastie and D.J. Porteous, unpublished results). Several of our CMGT-derived clones recognise high frequency DNA restriction fragment length polymorphisms (RFLP's), which will be useful for constructing a complementary recombinational map of the region and tracking disease gene segregations in affected families (P. Boyd and D.J. Porteous, in preparation).

As a method for enrichment cloning, the CMGT strategy certainly matches, if not exceeds, any previous approach, such as library construction from somatic cell hybrids (73), sorted chromosomes (74) or subtractive hybridisation cloning of DNA sequences complementary to X chromosome deletions (75).

6.2 From met and SV40 to cystic fibrosis. MNNG-HOS is the only cell line in which the met oncogene has been found activated (76). The mode of activation was chemical carcinogenesis with MNNG. The mechanism of activation appears to be a complex translocational rearrangement between the met proto-oncogene on the long arm of chromosome 7 and an as yet undefined locus on chromosome 1 (77). The met oncogene is of special interest to the human geneticist because it turns out to map very close to the primary genetic defect in cystic fibrosis (CF) (78). Scambler et al (64) have tested met-selected CMGT as a way of <<homing in>> on the CF gene (79). Extensive clone isolation from a transformant which retains about 5 megabases of human DNA and includes the pJ3.11 marker, which flanks immediately to the other side of the CF locus from met (80), has brought the team of St. Mary's team very rapidly to within sight of the CF gene (Scambler & Wainwright, personal communication).

There were good reasons to question met-selected CMGT as a reliable approach for cloning the CF gene (79). There was an equal chance that the CF locus would turn out to be on the <<wrong>> side of the met-activating translocation breakpoint. Furthermore, the rearrangement is complex, affecting independent, but closely linked loci. Until the CF gene is cloned, the worry will remain that the physical linkage between CF and met has been altered in the MNNG-HOS cell line. We have investigated an alternative approach which promises to complement the met-selected CMGT approach towards cloning the CF gene. The human chromosome 7-only somatic cell hybrid, CI21, is transformed by virtue of SV40 genomes integrated immediately below the CF locus (22). Transfection of CI27 cells with CI21 chromosomes gives rise to transformants which show anchorage independent growth and are weakly tumorigenic. They carry the CI21 configuration of SV40 genomes, together with variable amounts of human DNA of chromosome 7 origin, which can include the CF region. These experiments point to a general mapping strategy, exploiting SV40 or other transforming viruses integrated randomly throughout the human genome.

7. Implications for neoplasia

Many of the advantages of the CMGT technique stem from it's capacity to bridge the complexity gap between the genome and the gene. The function of chromosomal elements can be examined in a manner comparable with whole cell or cell fusion studies, whilst DNA sequences of particular relevance can be identified and analysed with the same precision possible in DNA transfection experiments.

Our observations with c-Harvey-ras-1 point to many areas in which CMGT can contribute to our understanding of neoplastic change. Our experiments also emphasis the value of an integrated analytical approach. For example, whereas the initial molecular and cytological analysis was entirely consistent with a single copy of chromosomal c-Harvey-ras-1 being sufficient for transformation (21,22), double-labelling
in situ hybridisation analysis now indicates that at least two copies are essential for stable transformation (28). It is of interest that the molecular evidence also points to a doubling, through mitotic recombination, of the dosage of activated c-Harvey-ras-1 in the EJ-18-8D cell line; several DNA markers, including c-Harvey-ras-1, which map near the tip of chromosome 11 and are highly polymorphic in the population, but are all homozygous in EJ-18-8D, whereas catalase and other more centromeric markers are heterozygous (J.E.N. Morten and D.J. Porteous, unpublished observations). We can use the heterozygous markers to show that both chromosomes 11 must carry a copy of activated c-Harvey-ras-1. Homozygosity for the activated c-Harvey-ras-1 oncogene may have been an essential part of the pathway to EJ bladder cancer. Again, experimental studies clearly show the importance of amplification of the activated c-Harvey-ras-1 oncogene in the pathway from establishment through tumour progression to metastasis (81-84). Each set of observations relates to the sense in which the term "dominantly-acting" oncogene is misleading (Section 1).

The elevated tumorigenic potential of E65-5, in association with a chromosomal translocation involving c-Harvey-ras-1, suggests a synergistic effect for the newly associated DNA sequences. The rearrangement of the c-Harvey-ras-1 locus selected in E65-7 and associated with invasive potential urges further analysis. In other CMGT-associated rearrangements, we have noted a repeated failure to co-transfer certain regions of the short arm of chromosome 11. Perhaps there are certain syntenic loci which modulate malignant transformation negatively.

In contrast with EJ-18-8D, on which our CMGT studies have so far concentrated, the tetraploid sub-line of EJ bladder carcinoma, EJ-18-3E, shows only limited capacity for anchorage independent growth and is not tumorigenic in nude mice (34). However, we find that naked DNA and chromosomes isolated from EJ-18-3E are equally effective as the diploid EJ-18-8D sub-line at transforming mouse C127 cells. Whereas the EJ-18-3E cell line is also non-tumorigenic in our experimental mouse tumour assay, the derived chromosome mediated transformants are strongly tumorigenic (unpublished data). Detailed comparison of the EJ-18-3E and EJ-18-8D derived transformants may illuminate the possible role of negative effectors upon malignant transformation.

Transpositional, translocational, deletional and duplicative rearrangements can each accompany the CMGT process. However, it is worth emphasising that, once established, the transfectants are remarkably stable upon cell passage, growth in semi-solid medium and in tumour formation (21, 26). The techniques which we have used to reveal rearrangements (L1 «fingerprinting», genomic restriction mapping, co-transfer analysis and clone isolation exercises) also show that, compared with DNA transfection, the level of molecular scrambling is remarkably low and that very extensive lengths of DNA are co-transferred intact. The prospects must be very good for CMGT to reveal new transforming elements, beyond the size which can be transferred intact by conventional DNA transfection. The total number of oncogenes would effectively double if as much as a quarter of the tumours which are refractive in DNA transfection were effective in CMGT. CMGT might also provide a useful functional assay for molecularly identified but complex loci, such as the BCR / c-abl rearrangement associated with the 9:22 Philadelphia translocation, characteristic of chronic myelogenous leukemia (85, 86).

Loss of heterozygosity for polymorphic markers on the short arm of chromosome 11 has been used to study the aetiology of a variety of tumour types, including Wilms' tumour (87-90), breast (91) and bladder (92) carcinoma, but only telomeric markers, which map at or close to the c-Harvey-ras-1 locus, have been used. The identification of DNA segments which recognise high frequency restriction fragments length polymorphisms (RFLP's) has been an important by-product to arise from the isolation of an extensive set of human recombinants from E67-1 (P. Boyd and D.J. Porteous, in preparation). These new markers are spaced along the short arm of chromosome 11 and will therefore be invaluable for determining the mechanism of allele loss not only in bladder carcinoma, but also in cancer of the breast and kidney. They can also be used for testing linkage and assessing risk in cases of familial cancer.

8. Conclusions

Our studies with the c-Harvey-ras-1 oncogene in chromosome mediated gene transfer have established the potentials and limitations of this new functional assay for neoplastic change and provide a reference point for experiments with other selectable markers and oncogenes. There are two major and distinct ways in which the CMGT approach can contribute towards an understanding of the molecular basis for neoplastic change. Firstly, CMGT can illuminate the functional role and constraints of previously defined or putative oncogenes. Secondly, and perhaps most excitingly, CMGT is ideally suited to the functional identification of new transforming genetic elements.

Our findings point to the importance of gene dosage (or chromosomal duplication), of the local chromosomal milieu and of independent, but syntenic gene loci in modulating the expression of the malignant phenotype. Primary and secondary chromosome transformants which segregate quantitative and qualitative aspects of the malignant phenotype will allow such contributory effects to be analysed in detail.

The CMGT technique may reveal new oncogenes by facilitating the intact transfer of extensive and contiguous DNA lengths. Even megabase sized transforming genetic elements could be defined quite precisely by molecular «fingerprinting» and cloning exercises on primary and secondary chromosome transformants.

Finally, the CMGT approach in general and with
oncogene selection in particular offers a powerful new approach for human genome mapping which enables the experimenter to concentrate immediately upon regions directly implicated in genetic disease and neoplastic change.

Acknowledgements

We thank Prof. H.J. Evans for his support and encouragement, our colleagues in the Molecular Genetics Section for useful discussions, Drs M.E. Foster and C.M. Steel and Mrs M. Wilkinson for help with the experimental mouse tumour assays, Miss G. Cranston and Mr. C. de Angelis for technical assistance and Mr N. Davidson and Mr A. Bruce for photographic preparation. This work was supported in part by a MRC Recombinant DNA Training Fellowship awarded to D.J.P. and a CRC Project Grant awarded to J.E.N.M. M.C.H. is supported by an M.R.C. Research Studentship.

References

28 Gosden JR and Porteous DJ: HRAS1-selected, chromosome mediated gene transfer; in situ hybridisation with combined biotin and tritium label localises the oncogene and reveals duplications of the human transgenome. Cytogenet Cell Genet (in press).
38 Loyer A, Scangos GA and Ruddle FH: Mechanisms of DNA uptake by mammalian cells: fate of exogenously added DNA monitored by

Received May 8, 1987
Accepted May 14, 1987
MOLECULAR CLONING OF THE CHROMOSOMAL BREAKPOINT ASSOCIATED WITH INCREASED TUMORIGENICITY IN A HRAS1-SELECTED CHROMOSOME-MEDIATED GENE TRANSFORMANT

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Introduction of mitotic chromosomes from the human EJ bladder carcinoma cell line into mouse C127 cells, with selection for cellular transformation, generated a series of transformants all of which carry and express the HRAS1 oncogene on varying lengths of human chromatin.

One transfectant, E65.5, shows a 100 fold increased level of tumorigenicity over the other transformants but with no detectable change in expression of the HRAS1 gene.

Genomic restriction analysis shows that E65.5 is exceptional in that although all restriction sites 5' to and within the coding regions are retained, all sites that lie just 3' to the HRAS1 locus have been lost. A variable repeat region (vtr) probe shows no detectable hybridisation.

As an initial step towards understanding the nature and possible mechanism of the chromosome translocation, particularly with a view to the raised tumorigenicity, the rearranged HRAS1-hybridising fragment has been isolated from an size-selected genomic library cloned into the phage vector EMBL3. Fine restriction mapping of a subcloned fragment establishes that the coding sequences are intact and that the complete vtr has indeed been lost, the breakpoint lying within 700bp from the HRAS1 polyA addition site.

Co-transfer of defined genes and cloning of anonymous DNA markers following HRAS1-selected chromosome mediated gene transfer.


We have tested chromosome mediated gene transfer (CMGT) as a fine-structure mapping tool and cloning resource, using the activated HRAS1 oncogene on chromosome 11 (band 11p15.4) as a dominant selectable marker (Porteous et al (1986) Mol.Cell.Biol.,6,2223-2232). Defined markers known to be syntenic with HRAS1 on the short arm of chromosome 11 are frequently co-transferred, but not always those shown by other physical mapping studies or linkage analysis to be the ones most closely linked to HRAS1. **In situ** hybridisation analysis and dispersed repeat DNA mapping ("finger-printing") provides corroborative evidence for chromosomal rearrangement accompanying the CMGT process. To investigate this further, we have constructed lambda and cosmid libraries from several independent HRAS1-selected CMGT's and isolated over 100 human DNA recombinants, the first 44 of which have been localised to sub-regions of chromosome 11 and provisionally assigned as D11S38 to D11S82 (Porteous et al (1987) Proc.Natl.Acad.Sci.USA, in press). The human DNA recombinants derived from certain transformants show significant sequence amplification and rearrangement while others show negligible molecular rearrangement compared with the original donor DNA. However, they all show long-range rearrangements, as detected by pulsed-field gel electrophoresis analysis (Bickmore, Maule, Porteous and Hastie, HGM9, submitted). The observation of singular importance is that the HRAS1-selected CMGT derived human DNA recombinants are highly enriched for sub-regions of chromosome 11, predicted on the basis of single copy co-transfer analysis and therefore provide a powerful cloning and fine-structure mapping resource. These new markers have been used in a directed approach towards fine-structure mapping of the short arm of chromosome 11 and, in particular, for "homing in" on the WAGR region (Porteous, Bickmore, Boyd, van Heyningen and Hastie, HGM9, submitted).