MALE STERILITY IN TRANSGENIC MICE CAUSED BY EXPRESSION OF HERPES SIMPLEX TYPE 1 VIRUS THYMIDINE KINASE.

JOANNE BURKE

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1993
To my husband Andrew and my son Michael.
I declare that this work is my own, except where otherwise stated,

Joanne Burke.
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The mouse major urinary proteins (MUPs) are encoded of by a family of about 35 genes. The expression of the genes is under complex tissue-specific, developmental and hormonal control. Four groups of Mup genes have been identified, the largest of which are the group 1 genes and the inert group 2 pseudogenes. Most group 1 genes are expressed exclusively in the liver, while a small subset are also expressed in the mammary glands of females. In an attempt to identify the regions in Mup genes which lead to their tissue specificity and hormone responses, transgenic mice were generated containing the 2.2 kb 5' promoter region of a mouse Mup group 1 gene (BS6) linked to the coding region of the herpes simplex virus type 1 thymidine kinase gene (HSV1-tk). HSV1 thymidine kinase (HSV1-TK) assays revealed that the hybrid gene was expressed in the liver of 4 of the 5 transgenic lines examined, with expression first being detected at just over 2 weeks of age. In 3 of the 4 expressing lines examined expression was lower in female than male livers and when females of one of the sexually dimorphic lines were treated with testosterone, levels of HSV1-TK activity were elevated, although not to male levels. In these aspects of liver expression, the transgene exhibited many of the characteristics of the endogenous group 1 Mup genes. However, the transgene was
also consistently expressed at high levels in the preputial gland and testis. This is unexpected since Mup genes are not expressed in either tissue. Evidence is presented here which suggests that the causes of misexpression in the preputial gland and testis are different.

Testicular misexpression is independent of the attached tissue-specific promoter, and is observed in transgenic lines containing a promoterless HSV1-tk gene. In all cases the testis transcripts originated at positions in the HSV1-tk reporter gene downstream of the normal translation initiation codon. In agreement with this, the TK protein synthesized in the testis was shorter than the full-length protein, of a size consistent with initiation at Met46 and Met60, the second and third internal ATG codons. Thus the transcription of HSV1-tk in the testis is directed by sequences present in the HSV1-tk reporter gene itself, acting as a testis tissue-specific promoter. In most cases the TK activity in the testis rendered the transgenic male mice sterile. In lines with high levels of expression, sperm counts were low, malformed sperm were present and males were sterile. In lines with lower expression levels sperm counts were normal but sperm motility was reduced and males were again sterile. Low levels of HSV1-TK were detected before the first meioses occurred. However expression increased
greatly thereafter and most HSV1-TK is found in spermatids. In an attempt to restore fertility in transgenic male mice, synthesis of 5'-ethynyl-thymidine, a non-toxic competitive inhibitor of HSV1-TK, was undertaken. Preliminary results indicated that 5'-ethynyl-thymidine causes a decrease in HSV1-TK activity.
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Chapter 1: GENE EXPRESSION IN TRANSGENIC ANIMALS.

"Transgenic animals", a term introduced by Gordon and Ruddle in 1981, may be defined as those that have foreign DNA integrated into the chromosomes present in their somatic cells, and usually also their germ line, as a consequence of the experimental introduction of DNA (Palmiter and Brinster, 1985). Many cloned genes that have been introduced into the mouse germ line have shown appropriate tissue-specific and stage specific patterns of expression, despite their integration into apparently random sites in the host genome. Therefore, gene transfer into mouse embryos, combined with in vitro mutagenesis, can serve as an experimental assay for the cis-acting DNA sequences that dictate specific patterns of transcription in the developing animal. Furthermore, the ability to introduce and express cloned genes allows one to investigate the phenotypic effects of altered gene expression. One can cause the over-production of a normal gene product or direct its synthesis to an unusual cell type, or inhibit the expression of an endogenous gene in a developing mouse by introducing a gene encoding antisense RNA (Munir et al., 1990; Han et al., 1991; Pepin et al., 1992). These approaches can be used either with a gene whose normal function is already known, or with newly identified genes whose functions are being investigated. In addition, novel genes not normally expressed in the
mouse, or mutant forms of normal mouse genes, can be introduced. These approaches provide new experimental strategies for answering basic questions in many areas of mammalian biology, and in some cases make possible the production of animal models of human diseases.

Although most publications on transgenic animals have dealt with mice, DNA integration has also been achieved in pigs, sheep and rabbits (Hammer et al., 1985c; Hammer et al., 1986b). In addition, transgenic techniques have proved useful in plant breeding and one application is to confer plants with resistance to certain diseases (Loesch-Fries et al., 1987).

Transgenic mice may be derived in three different ways (1) direct injection of DNA into one of the pronuclei of the fertilized eggs; (2) infection of embryos with a retrovirus; (3) the use of embryonic stem cells previously transfected with the DNA of interest to produce chimaeras either via aggregation or blastocyst injection. To date method 1 is the most widely used and all of the results presented in this thesis were obtained from transgenic mice derived by this method. However, methods 2 and 3 have some special and interesting features that I will discuss.

Methods Used To Generate Transgenic Animals.

Gordon et al (1980) were the first to report the successful integration of foreign DNA into mice by microinjection. Since then many investigators have had
success with this method (Brinster et al., 1981; Costantini and Lacey, 1981; Wagner E.F. et al., 1981; Wagner T.E. et al., 1981). The method of microinjection is described in detail elsewhere (Brinster et al., 1985; Gordon and Ruddle, 1983; Hogan et al., 1986). Briefly, superovulated females are mated and single-cell embryos are harvested from the oviducts. After the removal of the cells that surround the embryo, 1 to 2 picolitres of the appropriate foreign DNA (containing about 1 ug/ml of linear DNA) is injected into one of the pronuclei. This is followed by implanting the microinjected eggs into the oviduct of a pseudopregnant mother and allowing gestation to proceed normally.

When foreign DNA molecules are injected into pronuclei, about 25% of the mice that are born carry one or more copies of the injected gene (Brinster et al., 1985). Because integration usually occurs prior to DNA replication, about 70% of the transgenic mice carry the transgenes in all of their cells, including the germ line cells (Costantini et al., 1985; Gordon and Ruddle, 1981; Palmiter et al., 1982; Stewart et al., 1982). In the remaining 30% of the transgenic mice, integration apparently occurs after one or more rounds of replication; hence, the transgenes are present in only a fraction of the cells (Costantini and Lacey, 1981). These mice usually show mosaicism in both somatic and germ cells, but in some
mice the germ line is deficient. The original transgenic mice are designated "founder" mice and, when it is present in germ line cells, the foreign gene is usually inherited as a Mendelian trait.

Integration within the host genome appears to be at random (Lacey et al., 1983). The number of copies of the foreign DNA sequence that are integrated ranges from one to several hundred. When multiple copies are present, they are usually found at a single chromosomal locus (Lacey et al., 1983). However, insertion at more than one site sometimes occurs and is detected by the segregation of sites in the G1 and subsequent generations. The copies of the foreign DNA at each integration site are arranged primarily in a head to tail tandem array (Brinster et al., 1981; Costantini and Lacey, 1981; Palmiter et al., 1982), although other arrangements have been documented. The efficiency of introducing foreign DNA into the eggs of mice has been studied by Brinster et al., (1985) who found that the optimal conditions for integration involve the injection of several hundred molecules into the male pronucleus of fertilized one-cell embryos. They also noted that linear DNA is integrated more efficiently than circular DNA. Furthermore, the efficiency of transgenic mouse production is influenced by the strain of mouse used.

The mechanism of integration of microinjected DNA is
unknown, although it is thought that the injected DNA molecules associated by homologous recombination before integration and in most cases insert subsequently at a single chromosome site (Brinster et al., 1985). Frequently, rearrangements, deletions, duplications (Mark et al., 1985; Covarrubias et al., 1987), or translocations (Mahon et al., 1988) of the host sequences occur at the insertion sites.

An alternative method for introducing genes into the mouse germ line is the infection of mouse embryos with retroviral vectors. Pre- or postimplantation embryos can be infected by either wild-type or genetically engineered retroviruses, leading to the stable integration of viral genomes into the host chromosomes (Jaenisch, 1976; Jaenisch et al., 1981; Stuhlmann et al., 1984; Jahner et al., 1985; van der Putten et al., 1985). In contrast to microinjected DNA, retroviruses integrate by a precisely defined mechanism into the genome of the infected cell. Only one proviral copy is inserted at a given chromosomal site and no rearrangements of the host genome are induced apart from a short duplication of host sequence at the site of integration (Varmus, 1982). Preimplantation mouse embryos can be exposed to concentrated virus stocks (Jaenisch, 1977) or co-cultivated on monolayers of virus-producing cells (Jahner and Jaenisch, 1980). Methods have also been devised to introduce virus into postimplantation
embryos between days 8 and 12 of gestation (Jaenisch, 1980). While this allows infection of somatic cells, germ cells are infected with a low frequency (Jaenisch et al., 1981).

The use of retroviral vectors as a means of obtaining transgenic animals that correctly express a gene that has been introduced into the vector, has several disadvantages. Firstly, there is a size limit of the foreign DNA inserted into the vector, although to a certain extent the use of cDNA-based genes circumvent this problem. Secondly, there is interference with expression of the inserted DNA due to methylation, which occurs when retroviral vector is passed through the germline. However, retroviral vectors have proven useful as an approach for expressing genes in a subset of cells in the adult animal. This work is motivated by an interest in delivering genes to specific cells in humans for gene therapy. For example gene therapy is being considered as a treatment for several diseases of the hematopoietic system, including sickle cell disease and beta-thalassaemia.

The integration of retroviruses into the genome may disrupt an endogenous gene and therefore serve as an insertional mutagen. This offers a way for obtaining mutants with the altered developmental processes. The virtue of such a strategy is that it not only produces mutations, but at the same time, tags a gene and therefore
offers a way of cloning the mutated gene. Several types of insertional mutations, after retrovirus insertion, have been obtained in transgenic mice (Schnieke et al., 1983; Soriano et al., 1987; Weiher et al., 1990).

A more recently developed technique involves the use of embryonic stem cell lines (ES cells), which are derived in vitro from explanted blastocysts and retain their normal karyotype in culture (Doetschman et al., 1985). When injected into host blastocysts, ES cells can colonise the embryo and contribute to the germ line of the resulting chimeric animal (Bradley et al., 1984; Gossler et al., 1986). Genes can be efficiently introduced into ES cells by DNA transfection or by retrovirus-mediated transduction, and cell clones selected for the presence of foreign DNA often retain their pluripotent character. By means of this approach, mice have been generated from clones that were selected in vitro for a specific phenotype. For example, hypoxanthine-guanine phosphoribosyl transferase (HPRT-) deficient ES cells were introduced into the developing mouse and subsequently contributed to the germ line to produce animals totally deficient in HPRT (Kuehn et al., 1987).

A very promising use of ES lines is made possible by recombination between exogenous DNA sequences transferred into these cells and homologous endogenous sequences (Doetschman et al., 1987; Thomas and Capecchi, 1987).
Several reports have shown that the selectable Hprt locus could be targeted by homologous recombination in ES cells at frequencies around 1 in 1000 cells that integrated DNA (Doetschman et al., 1987; Thomas and Capecchi, 1987). More recently, clever selection schemes for the selection or detection of these rare homologous events has been developed. These include the use of the polymerase chain reaction to identify target clones (Joyner et al., 1989; Zimmer and Gruss, 1989; Koller and Smithies, 1989), use of promoterless or enhancerless neo constructs to enrich for gene targeted events in genes expressed in ES cells (Jasin and Berg, 1988; Doetschman et al., 1988; Schwartzberg et al., 1989; Sedivy and Sharp, 1989), and application of a positive-negative selection system to enrich for targeted events in expressed or nonexpressed genes (Mansour et al., 1988). Successful transmission into the mouse germ line has now been published for HPRT+ targeted cell lines (Thompson et al., 1989; Koller et al., 1989). Other targeted genes have now been transmitted through the mouse germline, these include lines carrying targeted alterations in c-abl (Schwartzberg et al., 1990, 1991), Hox-2,6 homeobox (Hasty et al., 1991), N-myc (Charron et al., 1990), c-src (Soriano et al., 1991) and c-engrailed-2 homeobox genes (Joyner et al., 1991). A way is now open to site-directed mutagenesis of any gene and subsequent transfer of the genetic modification in the mouse germ
line, thus allowing the investigation of the functional role of developmentally important genes, for example.

**Gene Expression In Transgenic Animals.**

The expression of genes that are transcribed in a tissue-specific manner is controlled by *cis*-acting regulatory sequences. Such sequences can be identified by introducing suitably manipulated genes into the mouse genome.

The usual strategy is to harvest various tissues from the transgenic mice and assay for specific mRNA or protein. In order to distinguish the products of the injected gene from the endogenous counterpart, it must be marked in some way. For example, it may be from a different species, it may be a minigene with some exons deleted, or it may be modified by altering, inserting or deleting a few nucleotides. Alternatively, hybrid genes can be constructed in which the regulatory region (promoter) of the gene of interest is used to direct the expression of the protein coding region (reporter gene) of another gene. Reporter genes might code for an easily assayable enzyme that is not normally found in the mouse, a polypeptide that is easily identified immunologically, or an oncogene that could lead to unrestrained growth of the cell type in which it is expressed (Brinster and Palmiter, 1985; Overbeek *et al.*, 1985; Hammer *et al.*, 1985a,b; Readhead *et al.*, 1987).

Specific transgene expression, which is also stably
transmitted, has been found for many genes either active in only one cell type, in a few cell types, or in many cell types. Among the great number of studies utilizing transgenic mice, I have chosen to discuss the analysis of two different genes for which the results obtained are the most complete: the elastase I and alpha-fetoprotein genes.

The elastase I gene. Elastase I is a digestive enzyme synthesized and secreted by the acinar cells of the exocrine pancreas. Initially, a 27 kb genomic fragment containing 7 kb of upstream flanking sequence, 11 kb of exon and intron sequences, and 5 kb of downstream flanking sequence was used to generate transgenic mice (Swift et al., 1984). The integrated gene exhibited the physiological pattern of tissue-specific expression. Analysis of a series of deletions showed that 205 bp of 5' flanking sequence were sufficient to selectively direct the expression of an heterologous structural gene (the human growth hormone gene) to the pancreas of transgenic mice (Ornitz et al., 1985a). More recently, Hammer et al (1987a) showed that an even shorter sequence of 134 bp, 5' of the gene, containing an enhancer like sequence, is both necessary and sufficient for the specific expression of the reporter gene. Furthermore, this sequence was able to confer the correct timing in developmental expression on the fusion gene. Thus in the case of the elastase I gene, a small genomic region contains the information required
for tissue-specific regulation of gene expression. Similar results have been obtained in the case of other genes, including the gamma-crystallin (Goring et al., 1987; Kondohn et al., 1987), and the protamine genes (Peschon et al., 1987).

The alpha-fetoprotein gene (AFP). Although many genes analysed in transgenic mice thus far have contained only one defined tissue-specific enhancer, some genes expressed in several tissues may contain multiple enhancers. Expression of the alpha-fetoprotein gene is directed by no less than three enhancers with over-lapping tissue specificities. AFP is expressed during development in three different tissues, namely the visceral endoderm of the embryonic yolk sac, and the foetal liver and intestine. Its expression is very different from one organ to another. To discriminate both at the mRNA and DNA levels between transgene and endogenous gene expression, an AFP minigene was constructed. This consisted of 7 kb of 5'flanking sequence, together with the first 3 and last 2 exons (out of the 15 normal exons) and 0.4 kb of 3'flanking sequences (Krumlauf et al., 1985). This AFP minigene directed expression to all three tissues that normally express the endogenous AFP gene, but the liver overexpressed the transgene several-fold over endogenous expression, while in the intestinal tract expression was often greater than 5000-fold higher than normal. This
expression was seen even when more 5' flanking sequence sequence was introduced, suggesting that any repressing elements are not within the 14 kb upstream region of the AFP gene. In a subsequent analysis of the 5' flanking sequences, three enhancer domains spanning several kilobases could be defined. At least one of these was necessary for the transcription of AFP gene in one of the three embryonic tissues. Moreover, the analysis of the mice transgenic for constructs that included the different enhancers suggest that these enhancers act in combination to determine the level of expression in the different organs (Hammer et al., 1987b).

Modulation of transgene expression by environmental stimuli. Promoters of genes subject to modulation by hormonal or other environmental stimuli have also been shown in several instances to properly control expression of transgenes, as long as the cis-acting elements are included in the construct. The metallothionein promoter, for example, has been used to direct expression of many different reporter genes, and in some cases expression could be stimulated by feeding the animals with heavy metals (Palmiter, 1987). Hormone-inducible promoters that function in transgenic mice include the mouse mammary tumour virus (MMTV) long terminal repeat (LTR) (Stewart et al., 1988; Leder et al., 1986), the transferrin gene (Hammer et al., 1986a), and the H-2 Ealpha gene (Pinkert
et al., 1985). These results indicate that transgene expression can be modulated in vivo by external signals. Variable gene expression in transgenic animals. As a rule, tissue-specific genes are expressed appropriately, despite being integrated at an abnormal chromosomal location (Palmiter and Brinster, 1985; Shani, 1986; Sifers et al., 1987). This phenomenon is particularly common with unaltered genes and genes that have been only slightly modified. A difference in gene expression between a founder animal and its offspring is frequently due to mosaicism of the founder animal (McKnight et al., 1983). Mosaicism occurs when integration of the foreign DNA follows one or more cycles of DNA replication in the embryo. On average about 30% of the transgenic mice generated exhibit mosaicism. One expects to observe higher levels of expression in the offspring relative to the founder, and this increase usually corresponds to that expected based upon the germline transmission frequency (Ornitz et al., 1985a). In subsequent generations gene expression becomes stable.

Different lines of transgenic mice, generated by microinjecting the same DNA construct, exhibit varying levels of transgene expression (Chada et al., 1985; Ornitz et al., 1985a,b; Palmiter et al., 1982; Townes et al., 1985) and correlation between copy number and expression is generally poor. This effect is assumed to be due to the
exact position of the transgene in the chromosome. Transgenes are thought to integrate relatively randomly in the genome, placing the injected gene in different regions of the chromosome in different founder mice. The site of integration affects the level of transcription of a transgene in different mice. In some cases a mouse will have the transgene integrated but not expressed; the same transgene in a different founder may express to very high levels. Therefore, it is possible to study the effects of varying level of transgene expression by studying different lines of transgenic mice.

Variability in gene expression between individuals within a given transgenic line may be due to variable DNA methylation (Palmiter et al., 1982) and/or may include effects of modifier genes that could be segregating as a result of using a cross of two inbred strains of mice (e.g. C57 X CBA) to establish and propagate the transgenic lines. Alternatively, some genes may be very sensitive to nucleosome phasing and during development of the somatic cells the chromatin configuration might be "reset".

Developmental Specificity.

Fusion genes transferred into the germ line by pronuclear injection may also be used to study specific developmental lineages. For example Alpert et al., 1988, created transgenic mice carrying a fusion gene comprising the regulatory sequences of the insulin promoter linked to the
coding sequences of the SV40 T antigen (Tag). This can be used as a cell marker because it is a nonsecreted nuclear antigen. They examined the appearance of Tag immunoreactivity at different times of embryonic development and could deduce the existence of a common precursor for the various endocrine cells of the pancreas. Furthermore, the results support the idea of a common precursor for neuronal and pancreatic tissue, an idea that has long been debated (Le Douarin, 1988).

It has been shown that subpopulations of cells can be ablated by targeting to them the expression of a cell toxin (e.g. diphtheria toxin A chain) (Palmiter et al., 1987; Breitman et al., 1987; Behringer et al., 1988). Mice carrying an elastase promoter/toxin construct lacked a normal pancreas as a result of expression in pancreatic acinar cells, whereas expression of the diphtheria toxin gene under the control of the gamma-2 crystallin promoter resulted in mice with lens defects (Palmiter et al., 1987). This strategy should allow the elimination of any cell type to which expression of the toxin can be directed by a specific promoter. Live animals can be obtained in which targeted lineages, such as lens or pancreatic exocrine cells, are ablated. More recently, drug-inducible ablation of specific lymphoid cells was reported in mice expressing the herpes thymidine kinase gene under Ig gene promoter control (Heyman et al., 1989). Because dose and
time of drug delivery can be experimentally controlled, this approach can permit the ablation of any lineage, including those that are essential for survival of the animal.

Unusual Aspects Of Gene Expression In Transgenic Animals.

While many genes can be expressed in transgenic mice in an appropriate manner, there are some examples of genes that have not been expressed at all or with very low frequency. Prokaryotic vector sequences prevent the appropriate expression of some genes. The presence of plasmid or lambda phage vector sequences severely inhibited both the level and frequency of expression of beta-globin genes (Townes et al., 1985). Plasmid sequences also inhibited MT-hGH, alpha-actin and alpha-fetoprotein gene expression (Hammer et al., 1985a; Krumlauf et al., 1985; Shani, 1986). Furthermore, intronless cDNA genes are less efficiently expressed than complete genes (Brinster et al., 1988). The lack of expression of other constructs may be due to the exclusion of certain sequences that are required for expression. Alternatively, the hybrid gene could contain a new combination of cis-acting sequences which has an inhibitory influence on expression.

Occasionally, hybrid gene expression may occur consistently in inappropriate tissues, in addition to or instead of normal expression. Possibly the native gene is transcribed but the mRNA is not stable, whereas the hybrid
gene transcript may be sufficiently stable to allow detection. Alternatively, the hybrid gene could contain new regulatory specificities which activate its transcription in novel tissues (Swanson et al., 1985), or lacks cis-acting "silencer" sequences which are required for repression of the endogenous gene and which are located outside the hybrid construct. Silencers might be sites for covalent modifications (e.g. methylation), they might initiate condensation into an inactive chromatin conformation or they might phase nucleosomes in an inappropriate manner.

Synthesis Of Important Proteins.

Genes have also been microinjected into rabbit, sheep and pig embryos (Hammer et al., 1985c, 1986b). The success rate of generating transgenic domestic farm animals is lower than that obtained with mice. Hammer et al (1985a) demonstrated the technical feasibility of gene insertion into rabbits, sheep, and pigs by transferring growth hormone (GH) genes. While it is not clear that such experiments will lead to more rapid growth of economically important livestock, it is likely that transgenic farm animals may become a source of economically valuable proteins. Efforts have been made to direct the synthesis of human secretory proteins to the milk of mice (Gordon et al., 1987) and sheep (Simons et al., 1988). To this effect, the promoter of a milk protein gene is coupled to
the coding region of a gene which specifies a plasma protein (tissue plasminogen activator, blood clotting Factor IX) and the hybrid gene is introduced into the germ-line. Expression has been correctly directed to the lactating mammary gland. In the first instance protein concentrations were low, but recent observations, consisting of a fusion between the 5' sequence of the beta-lactoglobulin gene and a genomic fragment of alpah-antitrypsin, revealed the production of concentrations of protein that are suitable for commercial exploitation (Archibald et al., 1990). These and other experiments indicate that large animals can be used as "factories" for the production of complex proteins that, because of extensive post-translational modifications, are difficult to synthesis in bacteria. These proteins would be free of a variety of human diseases that could be acquired by harvesting such compounds from human serum.

Future Prospects.
The transgenic animals analysed thus far represent first steps in working out the technical and practical approaches to addressing questions in such fields as developmental biology, endocrinology, oncogenesis, and neurobiology, and it seems inevitable that there will be a tremendous increase in the generation of transgenic animals to study these questions further.
Chapter 2: CONTROL OF EUKARYOTE GENE EXPRESSION.

A central problem in molecular biology is to understand the mechanisms by which specific genes are expressed in a temporal tissue-specific manner or are activated in response to extracellular inducers. The regulation of gene expression can occur at various stages from transcription initiation to post-translational modification and protein transfer. It appears, however, that transcriptional control is the most common (reviewed by Darnell, 1982). In contrast to prokaryotes where one type of RNA polymerase catalyses the synthesis of all three types of RNA (messenger RNA, mRNA; transfer RNA, tRNA; and ribosomal RNA, rRNA), in eukaryotes a separate polymerase species is responsible for the synthesis of each type. That catalysing the synthesis of mRNA is called RNA polymerase II (polII; Sentenac and Hall, 1982).

DNA Sequences And Protein Factors.

Two different classes of promoter structure and function have been described for RNA polII transcription in eukaryotes. The first class includes the majority of genes and is characterized by the presence of a TATA element which specifies the site of transcription initiation, and is located about 30 bp upstream of it. Extensive genetic and biochemical evidence has shown that the TATA element plays a significant role in start site selection and in specifying the frequency of transcriptional initiation (Benoist and Chambon, 1981; Breathnach and Chambon, 1981; Dierks et al., 1983; Eisenmann et al., 1989; Ghosh et
al., 1981; Hahn et al., 1989; Horikoshni et al., 1989; Nakajima et al., 1988). By itself, a TATA element is sufficient to stimulate specific transcription in vitro, though it does so with low efficiency in vivo in the absence of auxiliary elements (McKnight et al., 1981; Sassone-Corsi et al., 1981).

Based on these properties, the TATA element can reasonably be considered to constitute a type of core promoter. In vivo reconstituted basal level transcription from polII promoters requires at least five distinct general factor activities. TFIIA, TFIIB, TFIID, TFIIE, and TFIIF, in addition to polII, all of which have been partially purified (Buratowski et al., 1989; Reinberg et al., 1987; Reinberg and Roeder, 1989). The TFIID fraction contains an activity that binds specifically to the TATA box (Buratowski et al., 1989; Van Dyke et al., 1988). This TATA binding factor is thought to play a pivotal role in orchestrating transcription initiation from polII promoters. In vitro, the binding of TFIID to the TATA box is a prerequisite for the subsequent binding of other factors and polII to form an active transcription complex (3,6). In addition to factors needed for basal levels of transcription initiation, some factors stimulate and/or repress transcription initiation. Such factors may bind to transcriptional enhancers, as well as more proximal upstream promoter elements (UPEs). In the current view, enhancers and UPEs mediate environmental and developmental regulation of transcription initiation by positively or negatively modulating the activity of the core
promoter complex (Horikoshni et al., 1989; Mitchell and Tjian, 1989).

A second class of core promoters lacks a detectable TATA element but in some cases these promoters have a discrete element overlying the start site itself which helps to fix the place of initiation (Smale and Baltimore, 1989; Ayer and Dynan, 1988). The promoters for these genes are found primarily in housekeeping genes (for review see Sehgal et al., 1988). They are usually GC-rich, contain several transcription initiation sites spread over a fairly large region and possess several potential binding sites for the transcription factor SP1 (Dynan and Tjian, 1983). The nature and extent of mechanistic differences between TATA-dependent and TATA-independent modes of transcription initiation are not presently known.

UPEs are a group of transcriptional control elements that are clustered in the region 30-110 bp upstream of the start site, although a number of promoters have recently been shown to contain functional elements downstream of the start site as well (Stenlund et al., 1987; Ayer and Dynan, 1988). These elements consist of short consensus sequences that bind to regulatory proteins. Many viral promoters contain multiple copies of a particular upstream element, the 'GC box'. Examples are the viral promoters of the SV40 early (Benoist and Chambon, 1981), and the HSV1-tk promoters (McKnight and Kingsbury, 1982). GC boxes are also found upstream of a number of cellular genes, especially in many housekeeping genes. These include the
mouse hypoxanthine phosphoribosyl transferase gene (Melton et al., 1984), the mouse adenine phosphoribosyl transferase gene (Dush et al., 1985), the human adenosine deaminase gene (Valerio et al., 1985), and the rat type II procollagen gene (Kohno et al., 1985).

DNase I footprinting and protection studies on mature and mutant versions of the SV40 early promoter have shown that the transcription factor Sp1 binds in a sequence-specific manner to the GC rich hexanucleotides that occur twice in each of the three 21 bp repeats of the SV40 early promoter (Dynan and Tjian, 1983; Gidon et al., 1985). Steric constraints and differences in binding affinities at each site limit Sp1-DNA interactions to five out of six sites at any one time. Not all GC boxes bind Sp1 equally well and sequences outside the core hexanucleotide seem to modulate the efficiency of binding (Kadonaga et al., 1986; Jones et al., 1986). Sp1 appears to act in conjunction with cellular factors such as CTF/NF-1 (Jones et al., 1985), which suggests that these factors may act in conjunction with each other to modulate the level of transcription.

A region of about 24 bp containing a binding site for a protein factor distinct from Sp1 is positioned between the two Sp1 binding sites of the HSV1-tk promoter. The pentanucleotide sequence CCAAT is found within this binding domain and is known to be a crucial component of several eukaryotic promoters (McKnight and Tjian, 1986). Two cellular factors that interact
with CCAAT sequence have been described. These include C/EBP, isolated from rat liver cells (Graves et al., 1986) and the CTF/NF-1, isolated from HeLa cells (Jones et al., 1987). The first interacts not only with several CCAAT sequences but also with the core enhancer of SV40 and polyomavirus (Johnson et al., 1987). The second is identical to NF-1, a protein required for adenovirus DNA replication (Nagata et al., 1983) and interacts with the promoters of alpha and beta-globin genes (Efstradiadis et al., 1980) and HSV and chicken tk genes (McKnight and Kingsbury, 1982; Merrill et al., 1984). Although these various CCAAT-containing elements appear grossly similar in sequence, evidence suggests that different DNA binding factors are capable of distinguishing between them (Dorn et al., 1987; Oikarinen et al., 1987). Other UPEs such as the metal regulatory element (Karin et al., 1984; Stuart et al., 1984; Carter et al., 1984) or the heat shock regulatory element (Pelham, 1982) have a more specialized role.

The activity of many viral and cellular promoters is also modulated by an enhancer. Like an UPE, an enhancer is composed of a group of transcriptional control elements. An enhancer must be on the same molecule of DNA, but can be 1,000 bp or more from the promoter. The most extensively studied of these elements are the two 72 bp repeats present between the early and late transcription units of the SV40 virus. Although most enhancers are found between 100-500 bp upstream from the TATA box, some are located within the transcription unit itself. For
example, the mouse immunoglobulin heavy chain enhancer is situated in the intron between the J and C segments of the gene (Gillies et al., 1983; Banerji et al., 1983). Other enhancers may be located great distances from the TATA box, e.g. that of the mouse albumin gene is 10 kb upstream of the TATA box (Pinkert et al., 1987).

As more enhancers and UPEs were analysed it became clear that they resemble each other. Certain short consensus sequences typical of enhancers are also found as UPEs in certain other genes. An example is the octamer element which is found within immunoglobulin upstream promoter and enhancer regions (Falkner and Zachau, 1984). The operational distinction between enhancers and UPEs is action at a distance from the TATA box. For example, the SV40 enhancer can functionally replace the beta-globin UPEs when placed in a position normally occupied by the UPEs (Treisman and Maniatis, 1985). Similarly particular sequence motifs originally classified as UPEs, for example the hsp70 gene heat shock regulatory elements of Drosophila melanogaster, are able to act as inducible enhancers when present in more than one copy (Bienz and Pelham, 1986).

Elements within UPEs and enhancers can be classified as basically two types: constitutive elements and regulatory elements. Constitutive elements are often well conserved between different genes which are functionally unrelated, and are found in many diverse organisms. Examples include the GC box, the CAAT box, the core enhancer element, the octamer and
the AP1 binding site (Lee et al., 1987). These elements are involved in controlling the basal rate of transcription.

Regulatory elements are capable of activating or repressing the level of transcription. Examples of regulatory elements include those present within enhancers responsible for induction of certain genes by the action of specific agents including heat shock, viral infection, exposure to heavy metals and response to steroid hormones. Inducible enhancers are found in the heat shock inducible genes originally studied in detail with the hsp70 gene of Drosophila melanogaster (Pelham, 1982) and since analysed in the hsp70 genes of other organisms including Xenopus laevis (Bienz and Pelham, 1986), the heavy metal inducible metallothionein genes of mice and man (Serfling et al., 1985; Carter et al., 1984), the steroid hormone responsive LTRs of MMTV (Chandler et al., 1983) and a number of other cellular genes including the chicken lysozyme gene (Renkawitz et al., 1984). The cis-acting elements within enhancers and UPEs are generally short. They are well conserved between different genes responding to the same stimuli, and are often present in multiple copies. The number of copies often dictates the strength of response (Topol et al., 1985; Searle et al., 1985). Examples include the metal response element (Stuart et al., 1984) of the heavy metal inducible genes, the heat shock response element (Pelham and Bienz, 1982) of heat shock inducible genes, and the glucocorticoid response element of glucocorticoid inducible genes (Jantzen et al., 1987).
Other regulatory elements may be conditional in that they are only active in particular cell lines or at specific stages of development. These are present in tissue- and developmental stage-specific enhancers, and bring about specific expression. Two of the best characterised examples are the immunoglobulin light and heavy chain enhancers which are active in B mature cells (Gillies et al., 1983; Banerji et al., 1983).

Finally, certain promoter elements are able to mediate negative regulation. One such element was identified in the upstream region of the inducible beta-interferon gene on the basis that its deletion resulted in high levels of gene expression in the absence of the inducer (Goodbourn et al., 1986). Other such elements have been found in the retinol-binding protein, insulin, alpha-fetoprotein, c-myc, and immunoglobulin heavy chain genes among others (Camper and Tilghman, 1989; Hay et al., 1989; Imler et al., 1987; Nir et al., 1986).

Transcriptional control in hepatocytes. The liver plays many crucial roles in body function, balancing carbohydrate and lipid metabolism, providing the great majority of serum proteins, detoxifying exogenous chemicals and removing internally produced products that can be toxic in excess. Special proteins are required to carry out these functions and a large number that are made in hepatocytes are unique to this cell type.

Analysis of liver-specific gene regulation has led to the
identification of two classes of required cellular transcription factors, some that are widely distributed and others that display a more limited cellular distribution (Babiss et al., 1987; Cereghini et al., 1988; Costa et al., 1988; Costa et al., 1989; Courtois et al., 1988; Grayson et al., 1988a,b; Herbst et al., 1989; Lichsteiner et al., 1987; Liu et al., 1988). These factors interact with multiple DNA sequence-specific motifs present in the promoter and enhancer elements of hepatocyte-expressed genes so that a single gene (for example, transthyretin) can employ as many as five different proteins acting on as many as 10 independent sites (Costa et al., 1989). These factors may dictate liver specificity by either acting directly to positively stimulate transcription complex formation or, alternatively, by functioning to suppress the activity of negative-acting ubiquitous factors, thereby allowing expression in hepatocytes while preventing transcription in inappropriate cell types.

Albumin is a prototypical tissue-specific gene product whose gene contains a highly liver-specific promoter segment (from -175 to -30bp) regulated by the ubiquitous proteins NF1 and CAAT together with the liver enriched C/EBP and hepatocyte nuclear factor-1 (HNF-1) proteins (Babiss et al., 1987; Cereghini et al., 1988; Cereghini et al., 1987; Lichsteiner et al., 1987). The nuclear protein HNF-1 (also named LFB1) was detected as a protein that bound to sites required for cell-specific expression of fibrinogen and alphal-antitrypsin genes.
Its binding site is also present in the promoter and/or enhancer regions of several liver-specific genes (Courtois et al., 1988; Cereghini et al., 1988; Grayson et al., 1988b; Kugler et al., 1988; Hardon et al., 1988; Vaulont et al., 1989; Frain et al., 1989). The HNF-1 binding site has been shown to be essential for cell type-specific transcription in vitro (Monaci et al., 1988; Lichsteiner and Schibler, 1989), in transfection assays (De Simone et al., 1987; Heard et al., 1987; Schorpp et al., 1988; Feuerman et al., 1989), or in transgenic mice (Tripodi et al., 1991). In the case of the albumin promoter, the HNF-1 binding site is not only essential, but also sufficient to drive liver-specific transcription (Ryffel et al., 1989; Lichsteiner and Schibler, 1989; Tronche et al., 1989). In addition, the albumin gene contains an upstream enhancer element, consisting of both positive and negative regulatory domains, which contributes both to tissue specificity and to maintaining the high rate of albumin transcription in hepatocytes (Herbst et al., 1989; Liu et al., 1988; Pinkert et al., 1987).

Structure And Possible Action Of Trans-acting Proteins.

Many sequence-specific transcription factors are comprised of two critical regions, typically located on separable domains (reviewed by Ptashne, 1988), a DNA binding region responsible for affinity between the transcription factor and the DNA response element, and a transcriptional activation region. The transcription activation region is believed to function by
directly or indirectly interacting with one or more of the general transcription factors and either recruiting that factor into the transcription complex or altering its conformation to a form that is better able to participate in the initiation of transcription (reviewed by Lillie and Green, 1990; Lin et al., 1990). A number of different motifs have been identified among DNA-binding and transcription activation regions (reviewed by Ptashne, 1988). DNA-binding motifs include Zinc-fingers, leucine zippers, helix-loop-helix and helix-turn-helix. A major class of activation regions has a highly acidic region enriched in glutamate and aspartate (Hope and Struhl, 1986; Ma and Ptashne, 1987). Alteration of the sequence of acidic residues, without changing the negative charge, strongly affected activation potential (Giniger and Ptashne, 1987; Hope et al., 1988). This result indicated that a specific structure in the acidic domain is required for function. Other activators have nonacidic regions, including the glutamine-rich activation domain of Sp1 (Courey et al., 1989) and the proline-rich CTF/NF-1 (Mermod et al., 1989).

The mechanism by which a protein bound many base pairs from a gene activates transcription remains unclear. This stimulation is associated with an increased density of polII on a linked gene (Weber and Schaffner, 1985). Both in vivo and in vitro transcription factors bound to enhancers/UPEs are thought to interact with polII and/or other factors, in order to promote transcription (Hai et al., 1988; Horikoshni et al.,
The most plausible model which has been invoked to explain this interaction is the looping model. This proposes that initiation of transcription is facilitated by the interaction of enhancers/UPEs with proximal promoter elements via proteins bound to the DNA. The DNA between them is thereby looped out. DNA looping has been described in prokaryotes in several cases, including cooperative repressor binding over a distance, site-specific recombination and replication (Dunn et al., 1984; Hochschild and Ptashne, 1986; Kramer et al., 1987; Wang and Giaever, 1988; Gralla, 1989). There is also some evidence for DNA looping in eukaryotes, where cooperative action or binding of transcription factors over a distance has been observed (Theveny et al., 1987; Schule et al., 1988; Cohen and Meselson, 1988).

The 5' flanking region of transcriptionally active genes can often be structurally distinguished as nucleosome-free deoxyribonuclease-hypersensitive zones occupied by DNA binding proteins (Gross and Garrard, 1988). Experiments indicate that formation and stabilization of transcription initiation complexes with sequence-specific transcription factors can competitively exclude histones and prevent inhibitory effects of nucleosome assembly on transcription in vitro (Wolffe and Brown, 1988; Workman et al., 1988). It is likely that there are multiple mechanisms by which initiation complexes are stabilized in vivo, and that different activation domains of
DNA binding transcription factors underly some of these mechanisms. Specific association between regulatory regions of active genes and nuclear scaffold proteins have also been demonstrated (Gasser and Laemmli, 1986); perhaps some activation domains contact proteins of the nuclear matrix. These interactions could facilitate transcription if, for example, they serve to tether genes to nuclear regions with locally high concentrations of other essential transcription factors.

**Future Prospects.**

Studies of DNA binding transcription factors in animal cells have led to the discovery that they are a remarkably diverse group, constructed from a variety of combinatorially arranged functional domains. The mechanism by which sequence-specific DNA binding factors affect different rate-limiting steps in transcription initiation are largely unknown, and future experiments will be aimed at understanding, at the biochemical level, how different transcription factors activate initiation and which proteins they interact with in order to accomplish this.

It is sometimes possible to gain a greater understanding of the factors affecting gene expression by examining multigene families because subtle differences which lead to changes in regulation are not obscured. In certain cases the pattern of expression within a multigene family varies during development in different tissues and under the control of different
inducers. Examples include the tissue-specific expression of different mouse kallikrein genes (Mason et al., 1983; van Leeuwen et al., 1986), varying patterns of globin synthesis during development, and the induction of metallothionein genes by glucocorticoids and heavy metals (Karin et al., 1984). The gene families encoding the rodent urinary proteins appear to be regulated in all three ways and an investigation of gene expression within this family is presented within this thesis.
Chapter 3: THE MOUSE MAJOR URINARY PROTEIN GENE FAMILY.

The mouse major urinary proteins (MUPs) are encoded by a family of about 35 to 40 highly conserved genes (Hastie et al., 1979; Bishop et al., 1982). Most of these are members of two distinct groups, the group 1 genes and the inert group 2 pseudogenes (Bishop et al., 1982; Clark et al., 1982; Held et al., 1987; Shahan et al., 1987a; Al-Shawi et al., 1989). Most group 1 genes are expressed exclusively in the liver, while a small subset are also expressed in the mammary glands of females (Shahan et al., 1987b; Shaw et al., 1983). Outwith group 1 and 2, some have been placed in two further groups (3 and 4). The group 3 genes are also expressed in the liver, but under hormonal control different from the group 1 genes (Kuhn et al., 1984), and the group 4 genes are expressed in the lachrymal and salivary glands (Shahan et al., 1987a; 1987b).

Mup Gene Organisation And Structure.

The group 1 and group 2 genes are organised in head to head pairs about 15 kb apart. With the 3' flanking regions each pair constitutes a 45 kb imperfect palindrome (Clark et al., 1984b; Bishop et al., 1985; see Figure 3.1A). The 45 kb units are located on chromosome 4 (Clark et al., 1982), and it is estimated that there are thirteen to fifteen 45 kb units, many of which are adjacent to each other.
A) Organisation and structure of the Mup genes.

Diagrammatic representation of the 45 kb unit. Group 1 and group 2 genes are approximately 3.9 kb long (shaded). Open rectangles are regions of homology between the flanking regions of the two groups of genes. The homology is not continuous over the 11 kb 3' flanking region but is interrupted by regions of non-homology. Genes are in a head to head (divergent) orientation. The 45 kb gene pair structure constitutes the evolutionary unit of the Mup locus.

B) A diagram to show the two main forms of the MUP mRNA.

The two main MUP RNAs showing their structural relationship to the gene BS6 are shown. Short mRNA contains exons 1-6. Long mRNA contains exons 1-5, a short 5' region of exon 6 and the whole of exon 7. The greater part of liver MUP mRNA is in the long form and nearly all of this is transcribed from the group 1 genes. Exons are shown as boxes and the coding regions are shaded. Adopted from Clark et al., 1984a.
The upstream region of five group 1 and four group 2 genes have been sequenced. Both group 1 and group 2 genes possess a TATA consensus sequence. The cap site of a group 1 gene (Bs6) has been accurately mapped by primer extension and nuclease protection experiments, and is positioned at 31 bp downstream of the TATA box (Clark et al., 1985b). Approximately 25 bp upstream of the TATA box is a sequence CCATAC, which shows limited homology to the consensus for the CAAT box (Ghazal, 1986; Held et al., 1987). The major differences observed in these genes are in the length and composition of a hypervariable A-rich region, 15 bp upstream from the TATA box. The promoter region of Mup genes contain a number of sequences showing homology to sites known to bind trans-acting regulatory factors. These include the glucocorticoid regulatory element (GRE), metal regulatory element (MRE), enhancer core sequences and NF1 binding sites. Of these only that for the glucocorticoid regulatory element occurs more frequently than expected by chance allowing for sequence length and base composition (Ghazal, 1986).

The transcription unit of the Mup genes is 3.9 kb long and contains seven exons, the last of which is non-coding (Clark et al., 1984a; see Figure 3.1B). There are alternative splice sites within the non-translated part of exon 6 giving rise to three different splicing configurations. Two different lengths of MUP liver mRNA
can be resolved by gel electrophoresis. The larger message is 10-20 fold more abundant in male mouse liver.

The exon sequences of four group 1 genes and extensive nucleotide sequence of four group 1 cDNAs are known (Clark et al., 1985a; 1985b; Derman, 1981). These encode proteins with an 18 amino acid signal peptide and a 162 amino acid mature protein with an expected molecular weight of about 19,000 Daltons. The nucleotide sequence homology between the different group 1 genes is greater than 99% but they specify different proteins.

Tissue-specificity And Sexual Dimorphism Of Group 1 Genes.

In BALB/c adult male mice, MUP mRNA is the most abundant class of liver mRNA, whilst in the female the most abundant species is serum albumin (Clissold and Bishop, 1981). MUP mRNA constitutes around 8% of the total liver polyadenylated mRNA in males, this being five times higher than that of females and castrated males (Hastie and Held, 1978; Hastie et al., 1979). Female mice show a simpler liver MUP pattern after in vitro translation, although treatment with testosterone induces a male-like pattern and results in the synthesis of normal male-like levels of MUP mRNA (Finlayson et al., 1965; Szoka and Paigen, 1978; Clissold et al., 1984).

Mup genes are expressed not only in male and female liver, but also in small amounts in prelactational mammary gland (Shaw et al., 1983). Shahan and Derman (1984) have
detected MUP mRNA at a level of about 0.02% of total mRNA in the mammary gland. This was confirmed further by a report from Shahan et al (1987b) showing hybridization of mammary mRNA to a group 1 BL1-specific oligonucleotide probe. In contrast McIntosh and Bishop (1989), using either a universal group 1 probe or two group 1 gene specific probes (BL1 and BS1), failed to detect any hybridization with RNA from prelactational mammary gland. A possible explanation for this observation is that there may well be expression of a mRNA that could react with these probes but which is below the level of detection (this is discussed in more detail in Chapter 5).

Developmental Expression.

In the liver, MUP mRNA is first detected in 3 week old male mice, full expression being reached only at 6-7 weeks after birth, when animals are sexually mature (Barth et al., 1982). Derman (1981) showed that the different levels of MUP mRNA in the livers of mice of different ages and sex, are reflected in differences in the rate of transcription. The lachrymal gland has adult MUP mRNA levels at 2 weeks of age, the earliest time at which the lachrymal gland can be dissected. The submaxillary gland shows detectable levels of MUP mRNA at one week of age, maximal levels between 4-7 weeks and then a decrease. MUP mRNA in the mammary glands is first detected during the first pregnancy.
Proteins Related To MUP.

In the rat, a homologous gene family codes for the alpha-2u globulins (Kurtz, 1981a; Dolan et al., 1982). The alpha-2u globulins are encoded by a multigene family consisting of about 20 genes (Kurtz, 1981b). Comparison of a rat alpha-2u globulin gene (207) and a mouse Mup group 1 gene (BS6) showed that the transcription units are similar in structure and that the exonic sequences are about 80% homologous. Furthermore, alpha-2u globulin has a similar distribution of tissue-specific expression except that it is expressed at high levels in the preputial gland, whereas MUP is not (MacInnes et al., 1986). In addition the rat genes are not expressed in the female liver whereas Mup genes are expressed in both male and female liver. Hepatic alpha-2u globulins are regulated by thyroxine, testosterone, glucocorticoid, growth hormone, insulin and oestrogen, unlike submaxillary gland alpha-2u globulin which does not appear to be under hormonal regulation (Motwani et al., 1980; Lynch et al., 1982; Roy et al., 1983; Laperche et al., 1983; Kulkarni et al., 1985).

It seems likely that the MUPs have an important role, as indicated by the large amount of urinary protein loss (at great expense to the animal); the highly amplified Mup gene region; and the selective pressure to conserve the amino acid sequence. For some time, no biological function
had been assigned to the Mup gene products, although there was strong circumstantial evidence which suggested they may be involved in behavioural communications (Vandenberghe et al., 1976; Shaw et al., 1983). Only recently (Bacchini et al., 1992) has direct evidence shown that MUPs selectively bind the male pheromones 2-(sec-butyl)thiazoline and dehydro-exo-brevicomin, and concentrate them in the urine. This characterises the MUPs as sex pheromone-binding proteins.
Chapter 4: EXPRESSION OF THE HERPES SIMPLEX VIRUS TYPE 1 THYMIDINE KINASE GENE.

The coding region of the herpes simplex type 1 thymidine kinase gene (HSV1-tk) has been used extensively as a reporter sequence. In normal cells endogenous thymidine kinase catalyses the phosphorylation of thymidine to thymidine monophosphate in the salvage pathway of pyrimidine synthesis. The HSV1-tk gene encodes a novel thymidine kinase which is distinguishable from the cellular enzyme in immunogenicity, molecular weight, substrate specificity and electrophoretic mobility. The HSV gene is a delayed early, or beta viral gene (Garfinkle and McAuslan, 1974) and as such, its full expression during lytic viral infections requires the prior synthesis of the immediate early, or alpha, protein ICP4 (Leung et al., 1980; Post et al., 1981; Watson and Clements, 1978; 1980).

Transcription And Translation.

The promoter region of HSV1-tk has been extensively analysed and shown to contain the TATA and CCAAT consensus signals and two GC rich repeated sequences within 105 bp upstream from the normal cap site (McKnight and Kingsbury, 1982; McKnight and Tjian, 1986). The transcribed portion of the gene is approximately 1300 nucleotides in length and contains no intervening sequences (Wagner, M.J. et al., 1981). A second reverse transcript has previously been observed in cells infected with HSV1 (Gompels and Minson, 1986; Jacobson et al., 1989). This unit overlaps the coding region of TK in the
opposite orientation having a predicted start at position 195 (+1), a potential TATA box "TATATAAA" at position 220 (-25), and a CAT box in opposite orientation at position 271, "GGCCAATGA" (-76).

The 5' end of the TK mRNA contains an untranslated region of 107 nucleotides followed by an open reading frame of 1128 nucleotides which codes for a 376 amino acid full-length polypeptide. The TK mRNA contains three initiation codons which are all used to some extent both in vivo and in vitro (Marsden et al., 1983; Haarr et al., 1985). Three polypeptides of molecular weight 43000, 39000, and 38000 (43K, 39K, and 38K) are thus generated from a single gene such that their C-terminal ends are identical, but the N-terminal ends are different (Marsden et al., 1983). The full-length polypeptide is the predominant product in HSV1-infected BHK cells and smaller amounts of the two truncated forms are also present. Certain drug-resistant mutations of HSV1-tk fail to produce the full-length polypeptide. One of these (twice isolated as KG111 and TK4) is due to an amber mutation at Leu44. Another, delta 1, lacks nucleotides -11 to +189 (deleting the first 45 codons) of the wild-type sequence. This removes the first initiation codon of the TK polypeptide. Delta 1 in particular overproduces the 38K polypeptide.

Properties Of The Enzyme.
HSV1-TK is composed of two identical subunits (Jamieson and Subak-Sharp, 1974) and the native enzyme has binding sites for
both the natural nucleoside substrates thymidine and deoxycytidine, and also for the phosphate donor, ATP. Several lines of evidence indicated that a region close to the N-terminus of the polypeptide is involved in ATP binding. Firstly, a comparison of the predicted amino acid sequences of the TK polypeptide of HSV1 and HSV2 with that of a marmoset herpesvirus (Otsuka and Kit, 1984) revealed a region of homology in all three polypeptides corresponding to the sequence of amino acid residues between positions 49 and 66 in the primary sequences of HSV1-TK. Secondly, the amino acid residues 51 to 63, contained in this sequence, show homology with a sequence implicated in ATP recognition present in other, unrelated, ATP-binding enzymes (Otsuka and Kit, 1984; Walker et al., 1982; Liu and Summers, 1988). Finally, results from site-directed mutagenesis of the predicted ATP-binding site (Liu and Summers, 1988) revealed that this segment is essential for the function of the TK. Characterisation of mutations that alter the affinity of TK for nucleoside substrates has indicated that amino acids 168 to 176 and amino acid 336 are involved in binding of the nucleoside substrate (Darby et al., 1986).

While the substrate specificity of the cellular TK is essentially limited to thymidine, the viral TK is a general deoxypyrimidine kinase (Dubbs and Kit, 1964; Cooper, 1973; Jamieson et al., 1974; Chen and Prusoff, 1978); indeed even the purine analog acycloguanosine seems to be a good substrate for HSV1-TK. The viral enzyme is also more promiscuous in its
choice of phosphoryl donors, and will use CTP as well as ATP (Jamieson et al., 1974, Cheng and Ostrander, 1976). Another unique feature of the viral TK is its significant activity as a TMP kinase (Chen and Prusoff, 1978). Apparently, the enzyme is able to catalyse a second phosphoryl transfer with the formation of TDP as well as TMP.

Use Of HSV1-tk As A Reporter.

A promoter deleted tk gene can be conveniently isolated as a 1759 bp BglII-BstEII fragment containing 57 bp of leader sequence (Wilkie et al., 1979). It is inactive in gene expression studies but can be reactivated by insertion of DNA sequences that contain cis-acting regulatory elements. The TK+ phenotype can be efficiently selected over a TK- background by utilising growth conditions in which the pyrimidine salvage pathway enzyme, thymidine kinase, is necessary for survival (Littlefield, 1964). The growth medium HAT contains Hypoxanthine, Aminopterin and Thymidine. By inhibiting dihydrofolate reductase Aminopterin inhibits the de novo synthesis of dATP, dGTP and dTTP. Hypoxanthine is a substrate for the salvage pathway for dATP and dGTP and when in plentiful supply permits growth to be sustained by that pathway. Similarly, thymidine, which is essential for the synthesis of TTP, allows growth through a salvage pathway which is totally dependent on TK activity.

Not only has the HSV1-TK coding region been used extensively as a reporter sequence in cell transfection
(Maitland and McDougall, 1977; Wigler et al., 1977), but it was also one of the first reporter genes used in work with transgenic animals (Brinster et al., 1981; Wagner E.F. et al., 1981; Wagner T.E. et al., 1981). More recently it was adopted as a component of a system for destroying (abrating) specific cell types in transgenic animals (Borrelli et al., 1988; Borrelli et al., 1989; Heyman et al., 1989). The basis of the procedure is to direct the expression of viral HSV1-tk to the target tissue in a transgenic animal by coupling it to a tissue-specific promoter sequence. At this time, target cells may be killed by administering an antiherpetic agent, such as Acyclovir or Ganciclovir, to the transgenic mouse. These compounds are phosphorylated by the promiscuous HSV1-TK but not by cellular TK, and bring about cell death when incorporated into DNA.
Chapter 5: EXPRESSION OF A MUP BS6/HSVtk HYBRID GENE IN TRANSGENIC MICE.

Systems available for studying the regulation of liver-specific genes include livers from intact animals, liver slices, and primary hepatocytes. Work has been carried out on the expression of Mup genes in hepatocytes (Clayton and Darnell, 1983). However, when hepatocytes are plated transcription of the Mup genes is rapidly switched off. This results in a dramatic fall in the level of MUP mRNA to virtually zero in about 4 days (Spiegelberg and Bishop, 1988). During the same period the level of transferrin mRNA rises by about two fold and alpha-fetoprotein mRNA appears de novo, showing that the pattern of gene expression seems to mimic liver regeneration.

Liver derived cell lines, including hepatoma cells and immortalised hepatocytes, have been used to study the expression of some liver specific genes. However, no liver cell line has been identified which expresses the endogenous Mup genes.

In an attempt to study the behaviour of a Mup promoter region in tissue culture, a plasmid containing 2.2 kb of 5' flanking region from the group 1 Mup gene BS6 (including the cap site), linked to the coding region of the Herpes simplex virus type 1 thymidine kinase gene (HSV1-tk) (Figure 5.1A), was introduced into rodent fibroblast cells (BHKtk- cells) by calcium phosphate co-
Schematic representation of hybrid genes.

The four major components of the plasmid constructs are shown. These include:

(i) the entire Simian virus 40 (SV40) transcriptional control fragment containing the SV40 enhancer, early promoter and TATA sequence.

(ii) A fragment of major urinary protein (Mup) gene BS6 containing TATA and cap consensus, with approximately 2.2 kb of 5' flanking region.

(iii) The Herpes simplex virus thymidine kinase type 1 gene as a reporter.

(iv) The bacterial SupF gene for the isolation of recombinant clones.

The restriction site map shows key sites as follows: E, EcoRI; P, PvuII; Ps, PstI; H, HindIII; K, KpnI. The BS6-tk-M fragment was separated from SV40 and vector sequences by digestion with HindIII, and BS6-tk-SupF fragment was separated by digestion with HindIII and KpnI. For other details see Material and Methods. The numbers refer to the length in base pairs of each particular segment.
A) pSVBS6tksupF

B) BS6-tk-SupF

C) BS6-tk-M

D) BS6Sau2-tk-SupF
precipitation. The Mup promoter was inactive in the cell line in this configuration. However, activity was observed when the SV40 enhancer sequence was introduced upstream of the gene. S1 nuclease and primer extension assays showed that transcription was faithfully initiated at the Mup cap site (Ghazal, 1986). This establishes BS6 as an active promoter, when coupled to the SV40 enhancer.

To investigate the regulation of the complex Mup gene family, in an environment which is more compatible to that in which the endogenous gene is normally expressed, transgenic animals were used. Transgenic mice have been useful in defining the cis-acting regulatory elements of a number of tissue-specific and developmentally regulated genes, including beta-globin, elastase, immunoglobulin, alpha-fetoprotein, and insulin (as discussed in Chapter 1 and reviewed by Palmiter and Brinster, 1986; Cuthbertson and Klintworth, 1988; Gordon, 1989; Westphal, 1989). In this study, the tissue specificity, developmental control and hormonal regulation of a Mup/HSV1-tk hybrid gene in transgenic mice is described. Expression of transgenic mice carrying various other constructs, including a promoterless HSV1-tk gene, are also analysed.

My contribution to the study of Mup gene expression was part of a broad team effort. Construction of the hybrid genes was performed by Melville Richardson, Raya Al-Shawi introduced the hybrid genes, by embryo pronuclear
microinjection, into the genome of mice and generated all the Go mice. Once these Go transgenics were produced I scored and maintained the trangenic lines, carried out the expression analysis on the various lines and interpreted the data that resulted from this analysis.

Briefly, the fragment to be injected was isolated from the plasmid pSV-BS6-tk-SupF by digestion with HindIII and KpnI (Figure 5.1B; Al-Shawi et al., 1988; see the Appendix for details of construction of the hybrid gene). This removes the SV40 enhancer and plasmid sequences. The bacterial amber suppressor tRNA gene SupF (Goldfarb et al., 1982) was added to the 3' region, to facilitate the rescue of the hybrid gene (BS6-tk-SupF) from the genomic DNA of transgenic mice. For the generation of the same fragment which lacks the SupF gene (BS6-tk-M) digestion was performed with HindIII only (Figure 5.1C).

Results.

Derivation of transgenic lines carrying the BS6-tk hybrid gene. Of 40 pups born, 11 were identified by Southern blot analysis as transgenic: 6 females and 5 males. One of the females (62) was mosaic and did not transmit the transgene to her offspring. However, the other five transgenic Go females transmitted the transgene to their progeny, and from these transgenic lines were established (Table 5.1). Go males 66 and 79 were mosaic and fertile but did not transmit the transgene to their offspring, and
<table>
<thead>
<tr>
<th>Go No.</th>
<th>Sex</th>
<th>Fertility Status</th>
<th>Transgenic line established</th>
<th>Copy Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>F</td>
<td>Fertile</td>
<td>Yes</td>
<td>10</td>
</tr>
<tr>
<td>46</td>
<td>F</td>
<td>Fertile</td>
<td>Yes</td>
<td>80</td>
</tr>
<tr>
<td>58</td>
<td>F</td>
<td>Fertile</td>
<td>Yes</td>
<td>3</td>
</tr>
<tr>
<td>62</td>
<td>F</td>
<td>Fertile</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>64</td>
<td>F</td>
<td>Fertile</td>
<td>Yes</td>
<td>2</td>
</tr>
<tr>
<td>78</td>
<td>F</td>
<td>Fertile</td>
<td>Yes</td>
<td>1</td>
</tr>
<tr>
<td>66</td>
<td>M</td>
<td>Fertile</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>79</td>
<td>M</td>
<td>Fertile</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>48</td>
<td>M</td>
<td>Sterile</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>49</td>
<td>M</td>
<td>Sterile</td>
<td>No</td>
<td>ND</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>Sterile</td>
<td>No</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND - Not determined. Copy-numbers were determined in individuals from the third generation of transgenic lines.
the other three Go males (48, 49 and 51) were all sterile. Therefore no transgenic lines could be established from the males. The approximate number of copies of the transgene per diploid genome in each transgenic line is listed in Table 5.1. Keeping to the usage of Al-Shawi et al. (1988), here a line is a group of male and female mice descended from the same Go female and containing the same transgene at the same position in the genome.

Tissue-specific expression of the Mup BS6-tk hybrid gene.

To examine the tissue-specificity of expression of the hybrid gene HSV1-TK assays were performed on tissue homogenates. Consistent with the classification of group 1 genes as hepatic type genes, expression of the reporter function was observed in the liver of transgenic offspring. However, expression was also observed in the testis, preputial glands (modified sebaceous glands; Hummel et al., 1975) and in small amounts in the lacrymal and mammary glands.

Protein homogenates were prepared from a variety of tissues of adult transgenic and control mice. HSV1 thymidine kinase from the transgene was specifically detected by using a thymidine kinase assay (see Material and Methods) capable of distinguishing HSV1-TK from endogenous mammalian thymidine kinase. This involved the addition of 0.4 mM TTP to the assay (Jamieson and Subak-Sharp., 1974) which in my hands, inhibited endogenous
thymidine kinase activities in different tissues by 93 to 97%. The same amount of TTP inhibited HSV1-TK by close to 50%, so that even relatively low levels could be measured quite accurately.

Animals in three of the five lines (lines 46, 58, and 78), showed similar patterns of expression, male mice expressing HSV1-TK activities at relatively high levels in the testis and preputial gland but at lower levels in the liver. In these lines the level of expression in the liver ranged from 1.9 to 14.2 U/mg protein. In comparison, the range of expression in the testis and preputial gland was 41.6 to 141.3 U/mg protein and 10.4 to 83.5 U/mg protein, respectively (Table 5.2).

In the fourth line (line 40) HSV1-TK activity was detected in the testis and preputial gland but not in the liver. In contrast, the fifth line (line 64) displayed activity in the testis and liver and not in the preputial gland. Expression was below the limit of detection in the kidney, muscle, submaxillary gland and brain (Table 5.3). Lines 58 and 64 showed a low but significant level of expression in the lachrymal gland. Expression was also observed in the mammary gland (see below).

Northern blots of RNA from liver, preputial gland and testis of line 58 were probed with a HSV1-tk specific DNA fragment from pTK1, which is directed against the 5' part of HSV1-tk gene (Probe A, Figure 5.1A). Transcripts were
<table>
<thead>
<tr>
<th>Line</th>
<th>Copy no.</th>
<th>Enzyme activity (a) in tissues</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>0.018 ± 0.007 (20)</td>
</tr>
<tr>
<td>Lines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>0.05 ± 0.04 (4)</td>
</tr>
<tr>
<td>46</td>
<td>80</td>
<td>1.90 ± 0.34 (8)</td>
</tr>
<tr>
<td>58</td>
<td>3</td>
<td>6.6 ± 0.66 (7)</td>
</tr>
<tr>
<td>64</td>
<td>2</td>
<td>1.46 ± 0.24 (9)</td>
</tr>
<tr>
<td>78a</td>
<td>1</td>
<td>14.2 ± 1.2 (4)</td>
</tr>
<tr>
<td>Go</td>
<td>51</td>
<td>0.74</td>
</tr>
<tr>
<td>66</td>
<td>1.0</td>
<td>9.5</td>
</tr>
<tr>
<td>79</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. n, the numbers of animals assayed, is shown in parentheses.
Table 5.3.

Expression of BS6-tk-SupF hybrid gene in tissues of male transgenic and control mice.

Enzyme activity (a) in tissues

<table>
<thead>
<tr>
<th>Line</th>
<th>Brain</th>
<th>Kidney</th>
<th>Muscle</th>
<th>Lachrymal gland</th>
<th>Submaxillary gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.19 ± 0.06 (8)</td>
<td>0.05 ± 0.02 (13)</td>
<td>0.002 ± 0.001(5)</td>
<td>0.014 ± 0.003 (7)</td>
<td>0.006 ± 0.004 (6)</td>
</tr>
<tr>
<td>40</td>
<td>0.14 ± 0.01 (2)</td>
<td>0.06 ± 0.012 (4)</td>
<td>0.002 (1)</td>
<td>0.002 (1)</td>
<td>0.0 (1)</td>
</tr>
<tr>
<td>46</td>
<td>0.18 ± 0.037 (3)</td>
<td>0.04 ± 0.037 (3)</td>
<td>0.003 (1)</td>
<td>0.01 (1)</td>
<td>0.005 (1)</td>
</tr>
<tr>
<td>58</td>
<td>0.50 (1)</td>
<td>0.03 ± 0.02 (4)</td>
<td>0.0 (1)</td>
<td>2.1 ± 1.2 (2)</td>
<td>0.02 (1)</td>
</tr>
<tr>
<td>64</td>
<td>0.3 ± 0.09 (3)</td>
<td>0.01 ± 0.003 (6)</td>
<td>0.016 ± 0.01 (4)</td>
<td>0.23 ± 0.13 (4)</td>
<td>0.04 ± 0.009 (4)</td>
</tr>
<tr>
<td>78</td>
<td>0.08 ± 0.01 (3)</td>
<td>0.04 ± 0.01 (4)</td>
<td>0.012 ± 0.01 (3)</td>
<td>0.04 ± 0.04 (3)</td>
<td>0.004 ± 0.004 (3)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors.

n, the number of animals assayed, is shown in parentheses.
observed in each case (Figure 5.2). The expected size of a transcript running from the BS6 cap site to the tk polyadenylation site is approximately 1.35 kb. An RNA transcript of approximately this size was detected in male liver and preputial gland RNA. A shorter transcript approximately 1.2 kb in length was identified in the testis. Each tissue contains a second smaller RNA, which in the liver and testis RNA is about 0.9 kb and in the preputial gland RNA is about 1 kb. In each case the smaller RNA transcript reacted with the tk probe to a lesser extent than the larger transcript.

Mup genes are expressed not only in male and female liver, but also in small amounts in the prelactational mammary gland (Shaw et al., 1983). Shahan and Derman (1984) have detected MUP mRNA at a level of about 0.02% of total mRNA in the mammary gland. It was therefore of interest to examine transgenic mammary tissue using thymidine kinase assays, to determine whether the transgene is expressed in this tissue. Both virgin and prelactating mammary tissue from line 58 female and control female mice were analysed. As expected, all non-transgenic virgin and prelactating female mammarys revealed no detectable levels of HSV1-TK activity. In contrast, both virgin and prelactating line 58 mammary tissue exhibited small amounts (approximately 1.4 U/ mg of protein) of HSV1-TK activity (Table 5.4).
Figure 5.2.

An autoradiogram of a Northern blot to show the sizes of HSV1-tk RNA in tissues from mice containing the BS6-tk-Sup transgene. Total cellular RNA from the liver, testis and preputial gland from an adult male mouse was applied to a 0.8% formamide gel, and after blotting, the filter was hybridized with an 840 bp PstI DNA fragment from pTK1.

Lane 1 HSV1-tk specific markers,
Lane 2 non-transgenic testis RNA,
Lane 3 30ug of testicular RNA from mouse line 58, a 1 day exposure,
Lane 4 15ug of testicular RNA from mouse line 58, a 1 day exposure,
Lane 5 HSV1-tk specific markers,
Lane 6 30ug of liver RNA from mouse line 58, a 7 day exposure,
Lane 7 30ug of liver RNA from mouse line 58, a 2 day exposure,
Lane 8 non-transgenic liver RNA,
Lane 9 HSV1-tk specific markers,
Lane 10 non-transgenic preputial RNA,
Lane 11 10ug of preputial gland RNA from mouse line 58, a 7 day exposure.
is 1816.

Testicular RNA

Liver RNA

Preputial RNA
Table 5.4.

HSV1-TK activities in virgin and prelactating mammary glands.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Enzyme activity (a)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Line 58 females</td>
</tr>
<tr>
<td>Virgin mammary gland</td>
<td>1.45 ± 0.35 (2)</td>
</tr>
<tr>
<td>Prelactating mammary gland</td>
<td>1.39 ± 0.08 (3)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. 

n, the numbers of animals assayed, is shown in parentheses.
Shahan et al (1987b) have proposed that the BL1 gene is the most abundantly expressed group 1 gene in the prelactational mammary tissue. This is inconsistent with the findings of McIntosh and Bishop (1989), who failed to detect any hybridization with RNA from prelactational mammary tissue, using either a universal group 1 probe or the BL1 and BS1 gene-specific probes. Several hypotheses may explain this observation, it is possible that there is no expression of MUP mRNA in the mammary gland. Alternatively, there is expression but it is not a group 1 mRNA or it is a group 1 mRNA but one that is sufficiently different from the MUP11 probe and as such the gene-specific oligonucleotides could be washed off the filter during the high stringency washes. Finally, there may be expression of a mRNA that could react with these probes but which is below the level of detection.

The results presented here demonstrate that the hybrid BS6-\(tk\) gene is expressed at a low level in the mammary gland of at least one line of mice, and that there was no significant difference in expression between virgin and prelactating mammary tissue.

**Developmental regulation of the BS6-\(tk\) hybrid gene in the liver and testis.** Barth et al (1982) showed that MUP mRNA is first detected in male liver at about 3 weeks of age and rises to adult levels by approximately 6 weeks. To determine whether the transgene exhibited a similar
developmental pattern, I determined the levels of HSV1-TK present in livers from line 78 male transgenic animals and control littermates, prior to determining their transgenic status, at approximately 16, 21 and between 50-180 days of age, the latter animals being sexually mature. HSV1-TK activity just above background was observed in the liver of line 78 at just over 2 weeks of age (Table 5.5, and Figure 5.3). At 21-22 days a specific activity of 0.71 U/mg of protein was observed, significantly above background. At this time an increase in the level of testosterone occurs (McKinney and Desjardins., 1973) leading eventually to sexual maturity. Maximum levels of expression were reached when the animals were sexually mature approximately 50-180 days after birth (Figure 5.3). The time of the onset of expression in the preputial gland could not be determined because the gland could not be located in prepuberal animals. It appears that at least some if not all the elements responsible for developmental control of liver expression are present within the hybrid gene, since the time period of expression of the hybrid gene corresponds approximately to the time that MUP mRNA is first detected in the liver (Barth et al., 1982).

Sexually dimorphic expression of the hybrid gene in the liver. HSV1-TK levels in the liver of transgenic male and female mice reveal sex-specific differences. In three of five transgenic lines, male liver showed higher levels of
Table 5.5.

Time course of HSV1-TK expression in line 78 and control livers during sexual maturation.

Male transgenic mice and non-transgenic littermates were sacrificed at the indicated ages, and protein extracts were isolated from livers and analysed by thymidine kinase assays.

<table>
<thead>
<tr>
<th>AGE (Days)</th>
<th>Line 78 males</th>
<th>Control males</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.22 ± 0.02 (2)</td>
<td>0.02 ± 0.008 (3)</td>
</tr>
<tr>
<td>21-22</td>
<td>0.71 (1)</td>
<td>0.01 ± 0.01 (7)</td>
</tr>
<tr>
<td>50-180</td>
<td>14.2 ± 1.2 (4)</td>
<td>0 (1)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. n, the numbers of animals assayed, is shown in parentheses.

Figure 5.3.

Time course of HSV1-TK expression in livers during sexual maturation.

The bars show the average HSV1-TK activity for cases in which more than one mouse was measured. The numbers of transgenic (□) and control littermates are shown in Table 5.5.
activity than female liver (Table 5.6). The two exceptions
were line 40, which shows no activity in liver of either
sex, and line 64, in which male and female liver have
similar activity levels. Of the three lines with sexually
dimorphic activity, the male to female ratio of line 46
was about 15, while the ratios in lines 58 and 78 were
about 2 and 5, respectively. For comparison the average
male/female ratio of liver group 1 MUP mRNA is about 13
(McIntosh and Bishop, 1989). McIntosh and Bishop (1989)
used synthetic oligonucleotide probes to determine the
relative levels of expression of three group 1 Mup genes
(BL1, BS1 and BS6). They showed that all three are
expressed in male liver, that two (BL1 and BS1) are
expressed in female liver at quite different levels, and
that all three are expressed at male-like levels in the
livers of testosterone-treated females. RNA homologous to
the BL1-specific probe is the most abundant MUP RNA in
both male and female liver and the average male/female
ratio was 11 for BL1 mRNA and 250 for BS1 mRNA. However,
BS6 is a member of a small sub-group of Mup genes the RNA
products of which are indistinguishable. Consequently, the
levels of expression of BS6 mRNA cannot be determined.
This prevents any conclusions from being drawn between the
levels of HSV1-TK in the livers of male and female
transgenics and the rates of Mup transcription in the two
sexes.
Table 5.6.

**Sexually dimorphic expression of HSV1-TK in the liver of transgenic mouse lines.**

<table>
<thead>
<tr>
<th>Line</th>
<th>Enzyme activity (a) in livers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
</tr>
<tr>
<td>Control</td>
<td>0.018 ± 0.007 (22)</td>
</tr>
<tr>
<td>40</td>
<td>0.05 ± 0.04 (4)</td>
</tr>
<tr>
<td>46</td>
<td>1.90 ± 0.34 (8)</td>
</tr>
<tr>
<td>58</td>
<td>6.6 ± 0.66 (7)</td>
</tr>
<tr>
<td>64</td>
<td>1.46 ± 0.24 (9)</td>
</tr>
<tr>
<td>78a</td>
<td>14.2 ± 1.22 (4)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. 

n, the numbers of animals assayed, is shown in parentheses.
Testosterone treatment induces HSV1-TK activity in the liver of transgenic females. The stable expression of the transgene allowed experiments to be carried out to determine whether the signals required for testosterone regulation were present within the 2.2 kb of 5' BS6 sequence. The induction experiments were performed with females of line 46 which has the highest male/female HSV1-TK expression ratio (Table 5.6).

Seven transgenic and five non-transgenic female mice were implanted with testosterone pellets, and a further seven transgenics were implanted with placebo pellets (Samantha Martin demonstrated and supervised the implantation of these pellets). After 14 days the animals were sacrificed and the level of HSV1-TK in liver and (in some cases) kidney and preputial gland was measured (Table 5.7). As expected the non-transgenic female mice treated with testosterone did not show any detectable levels of HSV1-TK in any of the tissues analysed. In all seven transgenic mice given a placebo instead of testosterone, the expression of the transgene was also undetectable. In contrast, in the seven transgenic mice given testosterone, HSV1-TK activities in the liver were induced, although to only 32% of male levels.

No induction was detected in the kidney, which is not surprising since transgene expression has never been detected in this tissue. In contrast the female preputial
Table 5.7.
Induction of the expression of the BS6-tk hybrid gene in line 46 female liver.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Female mice</th>
<th>Male mice</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Non-Transgenic + Testosterone</td>
<td>Transgenic + Placebo</td>
</tr>
<tr>
<td>Liver</td>
<td>0.0 ± 0.0 (5)</td>
<td>0.0 ± 0.0 (7)</td>
</tr>
<tr>
<td>Preputial gland</td>
<td>0.04 (2)</td>
<td>0.0 (2)</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.03 ± 0.0 (3)</td>
<td>0.04 ± 0.19 (3)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. The numbers of animals assayed, is shown in parentheses. Values for male mice are taken from Tables 5.2 and 5.3.
gland showed increased HSV1-TK activity, to levels comparable to those of normal males. In these treated females the preputial gland increased substantially in size. Presumably this increase reflects sexual differentiation of the tissue.

These results demonstrate that the level of liver expression could be modulated from undetectable levels to low levels by testosterone administration to female mice. Therefore, all or most of the elements required for testosterone responsiveness are included within the 2.2kb 5'flanking region of the hybrid gene. Removal of the bacterial SupF gene does not influence expression of the hybrid gene. The consistent expression of the BS6-tk-SupF gene in preputial gland and testis is unexpected, since the Mup genes are not expressed in either tissue. For some genes inappropriate expression has been reported to be due to prokaryotic vector sequences or a new combination of sequences which activate its transcription in these tissues (Swanson et al., 1985). If the bacterial SupF gene which is incorporated at the 3' end of the hybrid gene were contributing to the expression pattern of the gene, its usefulness would be seriously reduced. To examine this possibility the BS6-tk-M hybrid gene, which lacks the SupF DNA fragment, was introduced into mice (Figure 5.1C). Five transgenic Go animals were obtained, two females (M42 and M12) and three males (M1,
M4 and M5). The two transgenic Go females transmitted the foreign gene to their progeny and from these, transgenic lines were established. Go male M4 was mosaic and fertile but did not transmit the transgene to its offspring, and the other two males, M1 and M5 were sterile. Therefore no transgenic lines could be established from the Go males. Animals from the two transgenic lines and two of the Go males showed a similar pattern of expression. Male mice expressed HSV1-TK activities at high levels in both the testis and preputial gland, but at a lower level in the liver (Table 5.8). In these mice the level of HSV1-TK expression in the liver ranged from 0.3 to 1.45 U/mg protein. In comparison, the range of expression in the testis and preputial gland was 9.1 to 210 U/mg protein and 21.8 to 128 U/mg protein, respectively. No HSV1-TK activity was detected in male kidneys (Table 5.8). When the expression of the BS6-tk-M and BS6-tk-SupF constructs was averaged over all transgenic lines for which data were available, the tissue-specific pattern was found to be the similar (Table 5.9). The similarity of these results indicates that the SupF gene is not responsible for the testis and preputial gland expression.

Aberrant expression in the preputial gland of line 64 is due to a chromosomal position effect. Animals in four of the five BS6-tk-SupF lines show relatively high levels of HSV1-TK activity in the preputial gland (Table 5.2), the
Table 5.8. 

HSV1-TK activities of the BS6-tk-M hybrid gene in tissues of male transgenic and control mice.

<table>
<thead>
<tr>
<th>Male</th>
<th>Liver</th>
<th>Preputial gland</th>
<th>Testis</th>
<th>Kidney</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.03±0.03(3)</td>
<td>0.04 ± 0.03(3)</td>
<td>0.34 ± 0.03(3)</td>
<td>0.043</td>
</tr>
<tr>
<td>Go males</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M1</td>
<td>0.82</td>
<td>28.5</td>
<td>132</td>
<td>0.034</td>
</tr>
<tr>
<td>M4</td>
<td>0.3</td>
<td>21.8</td>
<td>9.1</td>
<td>0.031</td>
</tr>
<tr>
<td>Males from transgenic lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M42</td>
<td>0.66</td>
<td>128</td>
<td>210</td>
<td>ND</td>
</tr>
<tr>
<td>N12</td>
<td>1.45</td>
<td>98</td>
<td>106</td>
<td>ND</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. The number of animals assayed are shown in brackets and the number was 1 otherwise.
Table 5.9

Expression of HSV1-TK in tissues of lines of transgenic mice carrying the BS6-tk-SupF and BS6-tk-M constructs.

<table>
<thead>
<tr>
<th>Construct</th>
<th>n(b)</th>
<th>Testis Mean ± SE</th>
<th>Range(d)</th>
<th>Liver Mean ± SE</th>
<th>Range</th>
<th>Preputial gland Mean ± SE</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>BS6-tk-SupF</td>
<td>5</td>
<td>85.12 ± 22.63</td>
<td>41.6-141.3</td>
<td>4.84 ± 2.58</td>
<td>0.05-14.2</td>
<td>31.51 ± 15.59</td>
<td>0.06-83</td>
</tr>
<tr>
<td>BS6-tk-M</td>
<td>2</td>
<td>158 ± 52.0</td>
<td>105-106</td>
<td>1.06 ± 0.395</td>
<td>0.66-1.45</td>
<td>113.0 ± 15.0</td>
<td>98-128</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein.
(b) Number of transgenic lines studied.
(c) Mean of lines, ± standard error.
(d) Range of lines.
exception being line 64. To investigate this observation further, the foreign gene from line 64 was recovered by molecular cloning (performed by Jane Kinnaird) and reintroduced into the genome of mice by embryo pronuclear microinjection (Al-Shawi et al., 1990; see the Appendix). These second cycle animals are referred to as secondary transgenic mice (BS6-tk-SupF TR). Twelve transgenic Go animals were obtained: six females and six males. The six transgenic females transmitted the foreign gene to their progeny and from these, transgenic lines were established. Three of six Go males were sterile and the fertile Go males did not father transgenic offspring. Therefore, no transgenic lines could be established from the Go males. HSV1-TK assays showed expression in the liver and high expression not only in the testis but also in the preputial gland (Table 5.10). Indeed, HSV1-TK activity was present in the preputial gland of all the male mice analysed (Table 5.10). This expression pattern is characteristic of primary Go transgenic males containing the BS6-tk-SupF hybrid gene, thus demonstrating that the lack of preputial gland expression in line 64 is due to chromosomal location and not to the foreign DNA itself. It can therefore be concluded that the expression of the transgene in line 64 is influenced by DNA sequences located on the same chromosome.
Table 5.10.
Expression of HSV1-TK in the tissues of the secondary transgenic mice.

<table>
<thead>
<tr>
<th>Go Line of Go</th>
<th>Sex</th>
<th>Enzyme activity in Testis (a)</th>
<th>Enzyme activity in the preputial gland</th>
<th>Enzyme activity in the liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>0.11 ± 0.044 (16)</td>
<td>0.21 ± 0.11 (9)</td>
<td>0.03 ± 0.005 (10)</td>
</tr>
<tr>
<td>Secondary transgenic mice BS6-tk-SupF(TR).</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A49*</td>
<td>F</td>
<td>77.1 ± 22.0 (3)</td>
<td>47.2 (1)</td>
<td>0.82 (1)</td>
</tr>
<tr>
<td>B65*</td>
<td>F</td>
<td>285.7 ± 50.7 (3)</td>
<td>251.0 (1)</td>
<td>27.1 (1)</td>
</tr>
<tr>
<td>B56*</td>
<td>F</td>
<td>1.77 ± 1.0 (3)</td>
<td>41.2 (1)</td>
<td>0.02 (1)</td>
</tr>
<tr>
<td>B85*</td>
<td>F</td>
<td>180.0 ± 22.0 (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B73*</td>
<td>F</td>
<td>5.9 ± 2.3 (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B83*</td>
<td>F</td>
<td>212.0 ± 105.0 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A6</td>
<td>M</td>
<td>36.7 (1)</td>
<td>17.6 (1)</td>
<td>0.01 (1)</td>
</tr>
<tr>
<td>A17</td>
<td>M</td>
<td>24.9 (1)</td>
<td>47.9 (1)</td>
<td>3.14 (1)</td>
</tr>
<tr>
<td>A45</td>
<td>M</td>
<td>330.0 (1)</td>
<td>196.0 (1)</td>
<td>19.6 (1)</td>
</tr>
<tr>
<td>A60</td>
<td>M</td>
<td>38.8 (1)</td>
<td>80.3 (1)</td>
<td>2.26 (1)</td>
</tr>
<tr>
<td>B27</td>
<td>M</td>
<td>50.6 (1)</td>
<td>7.8 (1)</td>
<td>0.42 (1)</td>
</tr>
<tr>
<td>B50</td>
<td>M</td>
<td>5.6 (1)</td>
<td>1.4 (1)</td>
<td>1.2 (1)</td>
</tr>
</tbody>
</table>

* Founders of transgenic lines.
(a) Units per milligram of protein; means and standard errors.
(n, the numbers of animals assayed, is shown in parentheses.)
Expression of a promoterless HSV1-tk gene in the testis of transgenic mice. Testis expression was high in transgenic mice containing the BS6-tk-SupF, BS6-tk-M and BS6-tk-SupF(TR) constructs. Arguing that expression of HSV1-tk might be independent of the attached tissue-specific promoters, analysis of transgenic lines containing a disabled HSV1-tk gene was undertaken. This hybrid gene is not coupled to any cellular promoter or enhancer and lacks sequences upstream of the TATA box which are required for its normal expression in frog oocytes and fibroblasts (McKnight et al., 1984; Eisenberg et al., 1985). Of the transgenic founder mice generated from the promoterless HSV1-tk hybrid gene (HSV1-tkDS2), 5 were assayed: 4 male and 1 female. 3 Go males were fertile and of these 2 did not transmit the transgene to their offspring, in contrast to the third which did (TK22). Go male TK 22 had no testis HSV1-TK activity, and indeed showed no HSV1-TK activity in any tissue. This male presumably carried transcriptionally inert copies of the hybrid gene. The Go female transmitted the transgene to establish line TK16 and G1 males from line TK16 were all sterile. 3 of the 4 Go males and males of line TK16 expressed HSV1-TK at moderate to high levels in the testis (Table 5.11). Expression in other tissues was sporadic and, other than in the brain and lachrymal glands of TK16 males, at very low levels. These results show that the HSV1-tk coding region itself is important in
Table 5.11.
HSV1-TK activities (pmol/min/mg. protein) in tissues of transgenic mice carrying a promoterless HSV1-tk gene (HSV1-tkDS2).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Non-transgenic control</th>
<th>Go Transgenic Males</th>
<th>Line TK16 male</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TK10</td>
<td>TK22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fertile(a)</td>
<td>Fertile</td>
</tr>
<tr>
<td>Testis</td>
<td>0.11</td>
<td>23.15</td>
<td>0.16</td>
</tr>
<tr>
<td>Liver</td>
<td>0.06</td>
<td>0.46</td>
<td>0.00</td>
</tr>
<tr>
<td>Preputial</td>
<td>0.16</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.08</td>
<td>0.27</td>
<td>0.20</td>
</tr>
<tr>
<td>Brain</td>
<td>0.20</td>
<td>ND(b)</td>
<td>ND</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.00</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lachrymal</td>
<td>0.07</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Submaxillary</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

(a) Fertile nontransmitting.
(b) ND, not determined.
determining testicular misexpression.

Developmental onset of HSV1-TK expression in the testis. HSV1-TK assays were performed on testes extracts of transgenic mice at different ages, to determine at which point in spermatogenesis the transgene is activated. In the developing mouse testis a significant number of secondary spermatogonia begin to advance towards meiosis at about the same time. Consequently, at different times during the first spermiogenic 'wave' the testis contains different mixed populations of germ cells. Importantly, the stage of the most advanced cells changes with time (Nebel et al., 1961). The seminiferous epithelium from day 6 animals contains only primitive type A spermatogonia and Sertoli cells. Type A and type B spermatogonia are present by day 8. At day 10, meiotic prophase is initiated, with the germ cells reaching the early and late pachytene stages by days 14 and 18, respectively. Secondary spermatocytes and haploid spermatids appear in increasing numbers between days 18 and 20 (Figure 5.4). Thus, an indication of the stage at which HSV1-TK is first expressed may be obtained by performing assays on total testis extracts throughout this developmental period. At approximately 8 days after birth, when the testis first becomes large enough to permit the assay, HSV1-TK activity was detected above background levels (Figure 5.5 and Table 5.12). However, a more significant increase in activity
Figure 5.4.
The temporal appearance of spermatogenic cells in the prepuberal mouse testis.

Data are expressed as percentage of total cells in the seminiferous epithelium. The cell counts were determined by classifying nuclei present in 50 cross sections of seminiferous cords chosen at random from the testes of three mice sacrificed at each designated age. Adopted from Bellve et al., 1977. The cell types are as follows: spermatogonia ( ), primary spermatocytes ( ), secondary spermatocytes ( ), and round spermatids ( ).

Figure 5.5.
Developmental analysis of HSV1-TK expression in the testis of line 78 and line 40 mice.

The bars show the average HSV1-TK activity for cases in which more than one mouse was measured. The numbers of transgenic mice from line 40 ( ), line 78 ( ) and control littermates ( ) are shown in Table 5.12.
Table 5.12.

Developmental analysis of HSV1-TK expression in the testis of line 78 and line 40 mice.

Male transgenic mice and non-transgenic littermates were sacrificed at the indicated ages, and protein extracts were isolated from testes and analysed by thymidine kinase assays.

<table>
<thead>
<tr>
<th>AGE (Days)</th>
<th>Enzyme activity (a) in livers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Transgenic males</td>
</tr>
<tr>
<td>Line 40</td>
<td></td>
</tr>
<tr>
<td>6-8</td>
<td>0.48 ± 0.11 (6)</td>
</tr>
<tr>
<td>16</td>
<td>1.37 ± 0.14 (3)</td>
</tr>
<tr>
<td>25</td>
<td>6.33 ± 0.48 (7)</td>
</tr>
<tr>
<td>50-180</td>
<td>141.3 ± 4.9 (4)</td>
</tr>
<tr>
<td>Line 78</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.67 ± 0.32 (2)</td>
</tr>
<tr>
<td>25</td>
<td>0.61 (1)</td>
</tr>
<tr>
<td>50-180</td>
<td>58.2 ± 11.7 (5)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. n, the numbers of animals assayed, is shown in parentheses.
occurred at about 25 days, the time at which haploid spermatids are becoming predominant. These findings show that expression occurs predominantly in post-meiotic spermatids and possibly also at low levels in the spermatogonia.

Discussion.

Relationship of HSV1-TK activities and mRNA levels. The data presented show that the BS6-tk-SupF gene is consistently expressed in the liver, testis and preputial gland of transgenic mice. Mice from the same lines were compared by Northern blot analysis to detect transcripts containing the HSV1-tk sequence (data compiled by Raya Al-Shawi). The correlation between HSV1-TK activities and tk-specific mRNA levels was good, indicating that the HSV1-TK activities directly reflect the mRNA levels. Run-on experiments performed by Cheryl Jones (Al-Shawi et al., 1988; see the Appendix), demonstrated that the different expression levels of HSV1-tk RNA in males and females are due to differential transcription, as is the case with MUP mRNA.

Two main sizes of HSV1-tk specific RNA were present in the liver, testis and preputial gland of transgenic mice carrying BS6-tk-SupF. The longer of these in the testis is about 1.2 kb in length, significantly shorter than the 1.35 kb transcript found in the liver and preputial gland. The shorter transcript in the liver and
testis is around 0.9 kb and in the preputial gland 1.0 kb in length. When Northern blots were hybridized to a probe which corresponds to the 5' end of the HSV1-tk mRNA, the signal strength of the shorter testis RNA was less relative to the longer RNA. This is consistent with transcription initiation downstream of the cap site, a phenomenon previously observed in cell lines carrying integrated HSV1-tk genes (Roberts and Axel, 1982). This smaller transcript may give rise to an HSV1-TK polypeptide, which may contribute to the observed HSV1-TK activity.

However, C. Jones showed that a single stranded riboprobe specific for the sense RNA from the 5' tk coding region failed to hybridize to the 0.9 kb band in testicular RNA (Al-Shawi et al., 1988; see the Appendix). This indicates that the 0.9 kb testis transcript is an antisense transcript, possibly related to the internal HSV1-tk reverse transcript previously reported (Gompels and Minson, 1986; Jacobson et al., 1989). Thus, translation of the 0.9kb transcript, if it occurs at all, cannot generate a HSV1-TK polypeptide or contribute to the HSV1-TK activity. The 0.9 kb liver and 1.0 kb preputial gland transcripts have not been further characterized.

Tissue specificity of expression. HSV1-TK activity was not detected in kidney, muscle, submaxillary gland or brain. Low enzyme levels were observed in the lachrymal gland
(Table 5.3) of two of five lines and in the mammary gland (Table 5.4) of the only line tested. HSV1-TK activities significantly above background were consistently detected in line 58 and line 64 lachrymal glands. Since expression in the lachrymal gland is line-specific it most probably relates to the different chromosomal integration events which characterize the different lines. However, the fact that expression was observed in two different transgenic lines raises the alternative possibility that the foreign gene is consistently expressed in the lachrymal gland but that in some lines the level of expression is below the limit of detection.

Previous work indicated that MUP mRNA is present in mammary tissue (Shaw et al., 1983; Shahan and Derman, 1984; Shahan et al., 1987b). The presence of low amounts of HSV1-TK activity in mammary tissue in one of the transgenic lines (58) is therefore not unexpected. However, mammary tissue from only one line was examined, and further analysis will be needed to determine whether this phenomenon is a general one.

Barth et al (1982) showed that MUP mRNA is first detected in male liver at about 3 weeks of age and rises to adult levels by approximately 6 weeks. Consistent with these observations, Held et al (1989) reported that a Mup/SV40 T-antigen transgene exhibited tissue specific and developmental regulation in transgenic mice. The Mup/SV40
T-antigen transgene, which contained about 2.5 kb of 5' flanking region, exhibited expression in the liver of three different transgenic lines. The first line expressed the transgene at higher levels in male than in female liver, while the second line exhibited equal levels of liver expression in both sexes. The third line generated only two F1 females, which again expressed the transgene in the liver, but which did not breed so this line was lost. A developmental analysis of the expression of the Mup/SV40 T-antigen transgene showed that expression in the liver was first detectable at approximately three weeks of age. Similarly, the results presented here indicate that the BS6-tk SupF transgene is expressed in the liver between 21 and 22 days of age. This corresponds to the time of onset of sexual maturity and increased levels of testosterone (McKinney and Desjardins, 1973).

Sexually dimorphic expression and hormonal control. The expression of Mup genes in the liver is strongly dependent on testosterone, thyroxine and growth hormone (Clissold et al., 1984; Knopf et al., 1983; Norstedt and Palmiter, 1984; Shaw et al., 1983; Spiegelberg and Bishop, 1988). The dependence on testosterone leads to sexual dimorphism in Mup expression and female expression can be induced to male levels by testosterone induction (Clissold et al., 1984). The difference in expression levels reflects different rates of transcription in the two sexes (Derman,
1981). My result show that in 3 of 4 transgenic lines, expression was lower in female than in male liver. When females of one of the sexually dimorphic lines was treated with testosterone, elevated levels of HSV1-TK activity and HSV1-tk RNA resulted. Northern blot analysis of mRNA from induced and noninduced female liver also showed induction of HSV1-tk RNA levels. Furthermore, run-on experiments performed by Cheryl Jones demonstrated that testosterone induction of HSV1-TK activities is controlled through transcriptional regulation.

The results presented here indicate that the hybrid gene exhibits many of the characteristics of the endogenous group 1 Mup gene. Cis-acting elements responsible for hormonal regulation, as well as those responsible for liver expression and developmental regulation of the Mup gene appear to be localized within about 2.2 kb of 5' flanking BS6 DNA. This region(s) responsible for liver expression has been defined further by analysing transgenic mice harbouring a construct containing only the proximal part (to nucleotide -314) of the BS6 promoter (BS6Sau2-tk-SupF, Figure 5.1D). (Al-Shawi et al., 1991; see the Appendix). Measurement of mRNA and HSV1-TK levels showed that, although expression in the testis was high, there was no expression in the liver in any of the transgenic lines analysed. This indicates that cis-acting sequences responsible for liver expression are
located more than 314 bp away from the BS6 cap site.

Inappropriate expression in the testis and preputial gland. Consistent misexpression of foreign genes has been observed before and may be explained in several ways (Behringer et al., 1988; Botteri et al., 1987; Low et al., 1986). It is possible that the Mup genes are transcribed in the preputial gland and testis but the mRNA is not stable, whereas the hybrid transgene mRNA may be sufficiently stable to allow the production of HSV1-TK. This explanation is unlikely however, since a rat alpha 2u-globulin transgene is expressed at a high level in the mouse preputial gland: it is improbable that there could be an extremely large difference in the stability of MUP mRNA and a highly homologous alpha 2u-globulin mRNA. Alternatively, the transgene might contain newly created sequences which activate expression in inappropriate tissues (Swanson et al., 1985). This explanation also seems unlikely since a Mup/SV40 T-antigen transgene was also found to be expressed in the preputial gland (Held et al., 1989).

Another more plausible explanation for misexpression is that it is attributable to silencer sequences which normally inhibit the expression of the endogenous Mup gene in the preputial gland and testis, but which are located outside the promoter regions included in the hybrid constructs. A BS6 gene with the same promoter and an
artificial mutation in exon 1 is expressed correctly (John Bishop, personal communication). This observation suggests that the control element is located downstream of the cap site, as is the case with many other genes (Franklin et al., 1991; Mauxion et al., 1990; Killen et al., 1988). Silencers might be sites for covalent modifications (e.g. methylation), they might initiate condensation into an inactive chromatin conformation or they might phase nucleosomes in an inappropriate manner.

The homologous rat alpha 2u-globulins are expressed in the rat preputial gland and a rat alpha 2u-globulin transgene is consistently expressed at high levels in mouse preputial glands (da Costa Soares et al., 1987). One possible interpretation is that the natural rat alpha 2u-globulin gene lacks a silencer sequence which is present in the mouse genes, resulting in its expression in the mouse preputial glands despite the presence in the mouse tissue of the corresponding trans-acting repressor protein (da Costa Soares et al., 1987)

Misexpression of the BS6-tk hybrid gene in the testis and preputial gland are due to different causes. Testicular misexpression could be due to the same cause as preputial misexpression. If this were the case, the expression of alpha 2u-globulin in the rat testis and of the alpha 2u-globulin transgene in the mouse testis would be anticipated. However, no alpha 2u-globulin specific mRNA
is detected in this tissue (Al Shawi, personal communication). This observation suggests that misexpression of the hybrid Mup gene in the preputial gland and testis have different causes. Two further observations support this conclusion. First, expression in the testis is promoter-independent while preputial gland expression is not. Secondly, expression in the preputial gland and not the testis occurs in transgenic mice containing a hybrid Mup/SV40 T-antigen transgene (Held et al., 1989).

HSV1-tk contains a cryptic promoter which directs its expression to the testis. As shown above, 5 of 5 BS6-tk-SupF transgenic lines and 2 of 2 BS6-tk-M transgenic lines had a high level of HSV1-TK activity in the testis. Analysis of the secondary transgenic mice (BS6-tk-SupF TR) revealed 6 of 6 transgenic lines had expression in the testis. In all cases HSV1-TK was expressed in the testis. These results in addition to analysis of other constructs in our laboratory gave rise to the suggestion that the HSV1-tk coding region is important in determining testicular expression. One of these constructs, containing only 314 bp of BS6 5' flanking sequence (Figure 5. 1D BS6Sau2-tk-SupF), had low preputial gland expression and no expression in the liver (Al-Shawi et al., 1991; see the Appendix). However, expression in the testis was at a level similar to that observed with the 2.2 kb BS6
promoter and 5' flanking region. More significantly, a thyroglobulin promoter and 5' flanking region directed expression of HSV1-tk at high levels in the testis of transgenic mice (Wallace et al., 1991). Some lines of mice transgenic for HSV1-tk linked to the mouse mammary tumour virus long terminal repeat also expressed HSV1-tk in the testis (Ross and Solter, 1985). In another case, a cosmid vector bearing the MHC class IIE alpha gene and a HSV1-tk gene, were used to generate transgenic mice and the resulting mice were also found to express HSV1-tk in the testis. (Braun et al., 1990).

Expression in the testis of transgenic mice which carry HSV1-tk coupled to different tissue-specific promoters suggested that the HSV1-tk reporter gene itself acts as a tissue-specific promoter. To test this directly transgenic mice carrying a promoterless HSV1-tk gene were generated and shown to express the transgene in the testis and to be sterile.

From these results it may be concluded that ectopic expression of HSV1-tk in the testis is a common feature of transgenic mice carrying the HSV1-tk reporter. In all cases HSV1-TK was expressed at a similar level in the testis. The results therefore suggest that expression in the testis is a function of an internal promoter within the HSV1-tk structural gene or its associated 3' flanking region, which is active in the testis but not elsewhere.
This is discussed further in chapter 8.

**Line-specific effects and copy number.** The relative expression levels in the liver, preputial gland and testis are line specific. Line-specific effects may be due to the foreign DNA itself, for example to mutation or rearrangement, or to the abnormal insertion of multiple copies of the foreign gene at a single site. Alternatively, expression might be modified by the chromosomal environment into which the DNA has been inserted. Gene expression is believed to depend partly on the organisation of chromosomal domains. A foreign gene inserted into a domain may in some cases take on the expression properties of the domain. Genes brought close to heterochromatic DNA are inactivated in *Drosophila* and mammals (Spradling and Rubin, 1983; Cattanach, 1974). Any of these changes may affect expression in different lines. The absence of preputial gland expression in line 64 (see Table 5.2) seems to be due to chromosomal location such that the expression of the gene is under the influence of flanking DNA sequences at the site of integration. This was demonstrated by cloning out the transgene from line 64 and reintroducing it into recipient mice. The resulting transgenic offspring show expression of the transgene in the preputial gland (Al Shawi *et al.*, 1989; see the Appendix). This is direct evidence of the effect of chromosomal position on transgene expression.
The quantitative analysis of transgene expression is complicated because multiple copies of the genes are usually integrated in a tandem array, and there is no means of determining how many of these are functional templates for transcription. No correlation was found between the number of copies of BS6-tk integrated and the level of transcription (Table 5.2). This suggests that only a few of the genes are expressed or alternatively that expression level of each copy falls as the copy number increases (Palmiter and Brinster, 1986). The absence of gene dosage effects in transgenic mice has often been observed (Palmiter et al., 1982; McKnight et al., 1983; Overbeek et al., 1986; Morello et al., 1986; Davis and MacDonald, 1988) and in one study in which the measure of expression was transcription rate the same lack of correlation was observed (Davis and MacDonald, 1988).
Chapter 6: INITIATION OF TRANSCRIPTION IN THE HYBRID GENE.

The Northern blot data showed that the longest HSV1-tk-specific transcript in the testis is about 1.2 kb in length, shorter than the 1.35 kb transcript expected from transcription initiated at the cap site of the BS6 gene, and observed in the liver and preputial gland. It was therefore of interest to determine the cap site of the 1200 bp testis transcript. Here, I have determined the points of initiation of the tk transcripts in the male mouse testis by primer extension.

Results.

Primer extension experiments. The BS6 cap site was mapped by Clark et al (1985b), by primer extension analysis and S1 mapping, at 30 + 1 bp downstream of the TATA box. To determine the start sites of transcription of the tk-specific mRNA, primer extension assays were carried out, with two synthetic oligonucleotides. Primer A is 22 nucleotides long and corresponds to nucleotides +107 to +125 from the BS6 cap site. Primer B is 23 nucleotides long and corresponds to nucleotides +347 to +369 (Figure 6.1). The primers were end-labelled using T4 kinase and annealed with total RNA. The DNA primer was then extended upstream to the 5' end of the RNA using AMV reverse transcriptase. The lengths of the products of the reaction were determined using a 6% polacrylamide/urea denaturing gel (see Materials and Methods). RNA transcribed from a
Figure 6.1.

Restriction map of the 5' region of the hybrid gene showing its relationship to the probes used for RNase protection and oligonucleotides used in primer extension. Open and closed boxes show the untranslated region and translated regions. Also shown are the MUP TATA box and the proposed BS6 cap site as previously mapped in the liver by Clark et al (1985b).

EcoRV  △ AccI  ● Sau3A

5' mRNA
tata cap

100bp

PE A

PE B

RNase probe
1009 bp EcoRI-BanI fragment of pTK1, inserted downstream of the T7 promoter was used as a control template. The extension of this RNA from primer A is expected to yield a product which is 256 bp long. Primer B should yield a 498 bp extension product. The results observed are in agreement with the sizes expected.

In these experiments, liver and testis RNA from transgenic lines 46 and 78 was examined.

Although a wide variety of conditions was employed, no extension products were observed when total RNA or purified liver poly(A)+ RNA from transgenic mice was used together with either primer. Under all of these conditions the control template generated the expected extension products. The conclusions to be drawn are that either the primer extension assay is not sufficiently sensitive to detect the small amounts of HSV1-tk mRNA present in the liver or that the sequences corresponding to primer A is not present in the transcript.

When primer B was employed together with total testis RNA from transgenic mice, extension products of 240 bp, 225 bp, 210 bp, 200 bp, 161 bp and 128 bp were observed, the most prominent being 210 bp and 225 bp, (Figure 6.2, lanes 3 and 4). Additional extension products of 282 and 139 nucleotides were observed with both wild-type and transgenic testis RNA templates and as such are presumably artefactual. These results show that none of the HSV1-tk-
Figure 6.2.

Electrophoretic analysis of the products of primer extension assays.

Lane A: is a sequence ladder of M13 used to provide molecular weight markers.

Lane 1 and 2: Primer extension of 20ug total RNA from non-transgenic testis.

Lane 3: Primer extension of 20ug total RNA from line 78 testis.

Lane 4: Primer extension of 20ug total RNA from line 46 testis.

Lane 5 and 6: Primer extension of 20ug total RNA from line non-transgenic liver.

Lane 7: Primer extension of 20ug total RNA from line 78 liver.

Lane 8: Primer extension of 20ug total RNA from line 46 liver.

Lane 9: Primer extension control. Extension product of the 1009 bp EcoRI-BanI fragment of pTK1.
specific transcripts present in transgenic testis are initiated at the previously-defined BS6 cap site. The longest transcript observed in the testis is initiated 127 nucleotides downstream. The initiation sites of five of the six testis transcripts map to positions between the first and second ATG codons of the HSV1-tk reading frame (Figure 6.3). No extension products were observed from transgenic testis RNA when primer A was employed. This is understandable since primer A overlaps the initiation site of the longest transcript detected with primer B.

**Discussion.**

The primer extension results obtained using testis transcripts were confirmed using the more sensitive technique of RNase protection, using the method described by Krieg and Melton, 1987. The RNase protection data was provided by Stephen Harrison and Paul Smith.

Table 6.1 lists the products obtained from primer extension and RNase protection data, respectively, and the distance of each of these fragments from the previously defined cap site (Clark *et al.*, 1985b). The primer extension results for male testis map the start of transcription to sites that are approximately the same as those indicated in the RNase protection assays. The slight discrepancy in size is probably due to the slightly different way RNA migrates in comparison to the sequencing DNA ladder (DNA and RNA molecules of the same chain length
Figure 6.3.
The mapped transcription initiation sites of the six testis HSV1-tk-specific transcripts.
The nucleotide sequence of the hybrid gene and part of its flanking region. The polylinker fusion region between the BS6 promoter and the HSV1-tk sequence and the location of the cap site is shown. The first two ATG codons are underlined and the mapped transcription initiation sites of all the six testis tk-specific RNAs are shown in boxes. The numbers indicate the distance, in base pairs, of each product from the previously mapped cap site. The predicted amino acid sequence of the TK protein is also given.

Cap site  Polylinker  HSV1-tk sequence
GGAGTGTAGCCAC/GATCCCC/GATCTTGTTGGCGTGAAA ACTCCC GCACCTCTTTTGCAAGCG

CCTTG T AAGGG C GGTATGGC T TCGTACC CCTGCCATCAACACGC GTCTGGTCACCAGCG
Met Ala Ser Tyr Pro Cys His Gln His Ala Ser Ala Phe Asp Glu

127 142 157 167

TGCGTTCTC GCCGGCGCATAGCAACCGACGTACC CGGTTGCCGCTCGCCC GGCAGC AAGA GAC
a Ala Arg Ser Arg Gly His Ser Asn Arg Arg Thr Ala Leu Arg Pro Arg Arg Glu Glu Ala

205 228

CACGAAAGTCCG C CCGCA AATG C CACGCTACTC GGGTTTATATA GACGGTCCT
a Thr Glu Val Arg Leu Glu Glu Lys Met Pro Thr Leu Leu Arg Val Tyr Ile Asp Gly Pro
Table 6.1.

The table shows the sizes of the products obtained from primer extension and RNase protection assays. Also shown are the distances of each product from the previously mapped BS6 liver cap site (Clark et al., 1985b).

<table>
<thead>
<tr>
<th>RNA species</th>
<th>Size of PE extension products (bp)</th>
<th>Distance from defined cap site (bp)</th>
<th>Size of RNase protection products (bp)</th>
<th>Distance from defined cap site (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver 78</td>
<td>/</td>
<td>/</td>
<td>307</td>
<td>-1</td>
</tr>
<tr>
<td></td>
<td>/</td>
<td>/</td>
<td>235</td>
<td>72</td>
</tr>
<tr>
<td>Testis 78</td>
<td>240</td>
<td>127</td>
<td>175</td>
<td>134</td>
</tr>
<tr>
<td></td>
<td>225</td>
<td>142</td>
<td>155</td>
<td>154</td>
</tr>
<tr>
<td></td>
<td>210</td>
<td>157</td>
<td>148</td>
<td>161</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>167</td>
<td>140</td>
<td>169</td>
</tr>
<tr>
<td></td>
<td>161</td>
<td>205</td>
<td>105</td>
<td>206</td>
</tr>
</tbody>
</table>
do not co-migrate precisely at the same position).

The HSV1-\textit{tk} RNA molecules synthesized in the testis are truncated at their 5' ends and most lie between the normal ATG codon (Met1) and the second ATG codon (Met46). This adequately explains the fact that no full-length polypeptides are produced in the testis (see Chapter 7). Using the RNase protection method, Stephen Harrison showed that the same transcription products are present in transgenic mice carrying the HSV1-\textit{tk} coding sequence in association with a number of different promoters. These include a truncated BS6 promoter which is inactive in the liver, the bovine thyroglobulin promoter which is active only in thyrocytes and a short inactive segment of the viral HSV1-\textit{tk} promoter (Al-Shawi et al., 1991; see Appendix). This observation leads to the conclusion that transcription of HSV1-\textit{tk} in the testis is not directed by any of the tissue-specific promoters to which it has been coupled, but rather is due to an otherwise cryptic promoter which forms part of the HSV1-\textit{tk} reporter sequence and which is active in the testis, in postmeiotic germline cells.

The putative promoter within the HSV1-\textit{tk} coding region, assumed to be located upstream of the sites of transcription, possesses properties associated with promoters of housekeeping genes. These properties include the initiation of transcription at multiple sites, the
absence of a conventional TATA box, and a relatively high GC content with the characteristics of a CpG island (Bird, 1986; Gardiner-Garden and Frommer, 1987). However, it is clearly not a true housekeeping promoter since it does not exhibit widespread cellular expression. It is consistently expressed at a detectable level only in the testis. The location of this promoter is presently being investigated.

RNase protection analysis of liver RNA of transgenic mice from line 78 and 46 (using the same RNA as was used in primer extension analysis) revealed protected fragments of length 307 and 235 nucleotides in each line (Al-Shawi et al., 1991; see Appendix). The larger and most prominent protected fragment, obtained with liver RNA of transgenic mice, map the start of transcription at a site within one nucleotide of the predicted Mup gene cap site. This maps the 5' end of the transcript to 100 bp upstream from the initiation codon and 31 bp downstream from the TATA box.
Chapter 7: TRANSLATION OF HSV1-tk mRNA IN THE PREPUTIAL GLAND AND TESTIS OF TRANSGENIC MICE.

In chapter 5 it was reported that two main sizes of HSV1-tk specific RNA were present in the liver, testis and preputial gland of transgenic mice carrying BS6-tk-SupF. The longer of these in the testis is about 1.2 kb in length, shorter than the 1.35 kb transcript predicted if transcription was initiated at the cap site of the BS6 gene, and present in the liver and preputial gland. The second smaller transcript in the liver and testis is around 0.9 kb and in the preputial gland is 1.0 kb in length. The 0.9kb transcript in the testis is an antisense transcript as shown by its failure to hybridize to a sense RNA probe (Al-Shawi et al., 1988; see the Appendix). The smaller transcripts in the liver and preputial gland have not been characterised. Analysis of HSV1-TK activities revealed similar levels in the preputial gland and testis extracts, despite the fact that the preputial gland contains full-length HSV1-tk transcripts and the testis a smaller transcript length. The longest transcript in each tissue is the only one which could be expected to act as template for the synthesis of active thymidine kinase.

The different transcript sizes, together with the similarities in enzyme activity, made it of interest to determine the nature of the polypeptides which are synthesised in the different tissues.
Results.

Sizes of polypeptides related to HSV1-TK. To determine which polypeptides were immunologically related to HSV1-TK, tissue extracts from transgenic and control mice were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The filters were incubated with a polyclonal rabbit antiserum raised against HSV1-TK (which was a generous gift of William Summers) and bound antibody was detected using biotinylated anti-rabbit IgG followed by treatment with a streptavidin-alkaline phosphate conjugate (See Materials and Methods for a detailed protocol).

As a positive control and to provide size markers, BHKtk- cells were infected with HSV1- strains 17 and CL101, and also the HSV1- mutant delta 1 (generous gift from Howard Marsden). Mutant delta 1, which was derived from CL 101, carries a deletion between -11 and +189 from the cap site. This region contains the first 45 codons of the reading frame including the initiation codon (M1). The infected cell extracts were subjected to SDS-PAGE, electroblotted and incubated with antiserum (Figure 7.1).

In cells infected with HSV1 strain 17 five major polypeptides reacted with HSV1-TK-specific antiserum: the full-length protein, Mr = 43,000, and smaller amounts of shorter polypeptides with Mr = 41,000, 39,000, 38,000, and 35,000 (Figure 7.1, lanes 1 and 6) (Marsden et al., 1983). The 39K and 38K forms were present in greater
Figure 7.1.

HSV1-TK-specific polypeptides produced by mutant and wild-type
HSV1-infected cells. BHKtk- cells were infected with the
indicated viruses and proteins were subjected to SDS PAGE,
transferred to a filter and developed with anti-HSV1-TK
antibody as described in materials and methods. Molecular
weights (X 1000) are indicated in the left margin. Positions of
the 43K, 39K and 38K polypeptides are designated.

Lane 1 Cells infected with HSV1-strain 17,
Lane 2 Cells infected with the TK mutant delta 1,
Lane 3 Cells infected with HSV1-strain CL 101,
Lane 4 Mock-infected cells,
Lane 5 Line 46 preputial gland extracts (50ug).
Lanes 6-8, loaded twice as much cell extract from:
Lane 6, Cells infected with HSV1-strain 17;
Lane 7, Cells infected with the TK mutant delta 1;
Lane 8, Cells infected with HSV1- strain CL 101.
amounts in the HSV1 mutant delta 1, which did not produce the full-length polypeptides (Figure 7.1, lane 2 and 7). HSV1 strain CL101 produced the full-length polypeptide and smaller amounts of the shorter polypeptides (Figure 7.1, lanes 3 and 4). Previous reports have demonstrated that the 39K and 38K polypeptides arise from translational initiation at the second and third AUG codons, as indicated by the results of elegant hybrid-arrest experiments; comparisons of the HSV1-TK proteins produced by cells infected with wild-type virus and the deletion mutant delta 1; and the relative sizes and charges of the various HSV1-TK proteins (Preston and McGeoch, 1981; Marsden et al., 1983; Haarr et al. 1985). Sizing of the related HSV1-TK polypeptides obtained from the HSV1-strains 17 and CL101, and also the HSV1- mutant delta 1, allowed the accurate sizing of the polypeptides obtained from the testis and preputial gland.

*Polypeptides present in extracts prepared with protease inhibitors.* It has been reported that some HSV1 proteins made in certain cells have undergone limited proteolytic cleavage (Pereira et al., 1982; Zezulak and Spear, 1984). More recently, it was demonstrated that HSV1-TK undergoes limited proteolytic cleavage in extracts of HSV1-infected Vero cells (Irmiere et al., 1989). To investigate the effect of cellular proteases on HSV1-TK protein products in tissue extracts, protease inhibitors were added to the
homogenisation buffer at the time of extraction.

Table 7.1 shows the 4 major classes of proteases which may be encountered. A routine approach to inhibition of proteolysis, often quoted in the literature, is to add PMSF. As a strategy to inhibit all cellular protease this is inadequate as PMSF is only effective against serine proteases and has no effect on other classes of protease. A relatively new inhibitor, 3,4 DCI, inhibits serine proteases more rapidly. E64 and leupeptin are very effective inhibitors of cysteine proteases. Leupeptin, a peptide aldehyde derivative binds tightly to the active site of various serine and cysteine proteases. In contrast E64, a peptide analogue which reacts irreversibly with the thiol group at the active site, has a strong affinity only for cysteine proteases. Metallo-proteinases are best inhibited by chelating agents such as EDTA or 1,10-phenanthroline. Aspartic proteinases are less frequently encountered but can be inhibited effectively by Pepstatin.

Line 46 (BS6-HSVtk-SupF) was used in the investigation of proteolysis since the hybrid gene is highly expressed in both the testis and the male preputial glands. A large number of polypeptides in the standard preputial gland extract reacted with anti-HSV1-TK antiserum (Figure 7.2, lane 3, Figure 7.3, lane 8-12). In the absence of the appropriate protease inhibitors the most prominent component in the preputial gland was a
## Table 7.1.

### Classes of endopeptidases.

<table>
<thead>
<tr>
<th>Protease class</th>
<th>Example Members</th>
<th>Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serine</td>
<td>Trypsin, elastase</td>
<td>PMSF (Phenylmethane sulphonylfluoride)</td>
</tr>
<tr>
<td></td>
<td>chymotrypsin, subtilisin</td>
<td>3,4 DCI (3,4 Dichloroisocoumarin)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TLCK (tosyl-lysine chloromethyl ketone)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leupeptin</td>
</tr>
<tr>
<td>Cysteine</td>
<td>Papain, cathepsins</td>
<td>E-64 (L-trans-Epoxysuccinyl-leucylamido (4-guanidino) butane)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>pHMB (para-hydroxymercuribenzoate)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Leupeptin</td>
</tr>
<tr>
<td>Metallo-</td>
<td>Thermolysin, meprin</td>
<td>Chelators: EDTA, 1,10-phenanthroline</td>
</tr>
<tr>
<td>Aspartic</td>
<td>Pepsin, cathepsin D</td>
<td>Pepstatin</td>
</tr>
</tbody>
</table>
polypeptide of 41K. Similar amounts of the full-length and 38K polypeptides and smaller amounts of the 39K polypeptides were also present (Figure 7.2 lane 3). In addition a further polypeptide with Mr of about 35,000 and smaller polypeptides with apparent molecular weights ranging from 30,000 to 20,000 which were related to HSV1-TK were present. Adding to the homogenisation buffer either Leupeptin or a general purpose mixture of protease inhibitors, containing 0.05mM E64, 0.1mM 3,4 DCI, and 0.1mM 1,10 phenanthroline (Salvesen and Nagase, 1989) had no effect on the relative amounts and sizes of the HSV1-TK related polypeptides in the preputial gland extracts (data not shown). However, when preputial gland tissue was homogenised in the presence of 10mM or 20mM of each of the inhibitors TLCK and pHMB or of 20mM 3,4 DCI, the truncated polypeptide with Mr=41,000 virtually disappeared (Figure 7.2, lanes 4-6). At the same time some of the smaller polypeptides in the preputial gland in the range Mr=30,000 to 20,000 either disappeared or decreased in amount. These treatments increased the intensity of the full-length form of HSV1-TK at the expense of the smaller molecular weight polypeptides, demonstrating that the 41K polypeptide and several smaller polypeptides are proteolytic products. Similarly, Irmiere et al (1989) showed that the 41K polypeptide present in HSV1-TK immunoprecipitates from infected Vero cells was also due to proteolysis. The
Figure 7.2.
Effect of protease inhibitors on HSV1-TK polypeptides in extracts of transgenic preputial glands and testes. 50ug of protein in the presence and absence of protease inhibitors, was loaded on each track. Proteins were transferred to a filter and developed with anti-HSV1-TK antibody as described in material and methods. Positions of the 43K, 39K and 38K polypeptides are designated.
Lane 1 and 13, HSV1-infected cells.
Lanes 2-6, preputial gland extracts:
lane 2, from non-transgenic mouse;
lanes 3-6 from line 46 mice,
lane 3 without additional protease inhibitors,
lane 4 with 20mM DCI,
lane 5 with 10mM TLCK + 10mM pHMB,
lane 6 with 20mM TLCK +20mM pHMB.
Lane 7, prestained markers.
Lanes 8-12, testis extracts:
lane 12, from non-transgenic control mouse;
lanes 8-11, from line 46 mice, lane 8 with 20mM TLCK + 20mM pHMB,
lane 9 with 10mM TLCK + 10mM pHMB,
lane 10 with 20mM DCI,
lane 11 without additional protease inhibitor.
nature of the protease activity or activities present in mouse preputial gland extracts can be explored by examining the inhibitors which prevent such activities. 3,4 DCI and TLCK, serine protease inhibitors seemed to prevent most of the protease activity, but so did pHMB, a cysteine protease inhibitor. Since pHMB is known to be a general protease inhibitor, it is possible that serine proteases are responsible for the phenomena reported here and elsewhere.

The same pattern of HSV1-TK polypeptides was observed in the preputial gland extracts from the four transgenic lines that had high HSV1-TK activities (Figure 7.3, lanes 8-13). In line 64 preputial gland extracts, which have low levels of HSV1-TK activity, the pattern was essentially the same, but the amounts of the polypeptides were low. The pattern is similar to that observed in HSV1-infected cells (Figure 7.2, lanes 1 and 6). This is strong circumstantial evidence that the mode of translation of the HSV1-tk mRNA is the same in the preputial gland as in HSV1-infected cells, i.e. that translational initiation occurs at each of the first three methionine codons.

Testis extracts were similar to preputial extracts except that they essentially lacked the full-length 43K polypeptide and its 41K proteolytic product (Figure 7.2). Testis extracts were unchanged when prepared in the presence of proteolysis inhibitors, indicating that the
Figure 7.3.

HSV1-TK-specific polypeptides in testis and preputial gland extracts from different BS6-\textit{tk}-SupF lines of transgenic mice. 50ug of tissue extract were subjected to SDS-PAGE, transferred to a filter and developed with anti-HSV1-TK antibody as described in materials and methods. Positions of the 43K, 39K and 38K polypeptides are designated.

Lanes 1-6, testis extracts from:
lane 1, non-transgenic control;
lane 2, Line 40 (BS6-\textit{tk}-SupF);
lane 3, Line 46 (BS6-\textit{tk}-SupF);
lane 4, Line 58 (BS6-\textit{tk}-SupF);
lane 5, Line 64 (BS6-\textit{tk}-SupF);
lane 6, Line 78 (BS6-\textit{tk}-SupF).
Lane 7 contains prestained markers.

Lanes 8-13, contain preputial gland extracts from:
lane 8, Line 40 (BS6-\textit{tk}-SupF);
lane 9, Line 46 (BS6-\textit{tk}-SupF);
lane 10, Line 58 (BS6-\textit{tk}-SupF);
lane 11, Line 64 (BS6-\textit{tk}-SupF);
lane 12, Line 78 (BS6-\textit{tk}-SupF);
lane 13, non-transgenic control.
lack of the 43K polypeptide is not due to proteolysis. The pattern of HSV1-TK polypeptides was the same in testis from each of the five transgenic lines, all of which showed high levels of testis HSV1-TK activity (Figure 7.3, lanes 2-6). In some Western blots (Figure 7.2, lanes 8-11), a trace of full-length polypeptide was detected. These results show that the pattern of polypeptides produced in the testis of the transgenic mice is very sharply skewed in favour of the 39K and 38K truncated forms, relative to translation in HSV1-infected cells or in transgenic preputial glands.

The same translational patterns were also observed on Western immunoblot analysis with testis RNA from transgenic mice carrying BS6Sau2-tk-SupF, TG-tk-SupF and HSV1-tkDS2 constructs (Figure 7.4). These constructs contain respectively an ineffectual MUP promoter, a promoter that directs expression to the thyrocytes, and no promoter. Transgenic mice carrying each of these produced the same truncated transcripts in the testis (Chapter 5). It was therefore expected that they would generate the same pattern of HSV1-TK polypeptides. These observations demonstrate that the production in the testis of a truncated transcript correlates with a lack of synthesis of the full-length HSV1-TK polypeptide, and substantial synthesis of the 39K and 38K forms, previously shown to be due to initiation of translation at the second and third
Figure 7.4.

**HSV1-TK-specific polypeptides in testis extracts from different lines of transgenic mice.**

The positions of the Mr 39,000 and 38,000 polypeptides are marked. 50ug of protein was loaded onto each track.

Lane 1, HSV1-infected cells.

Testis extracts from:

- Lane 2, non-transgenic control;
- Lane 3, Line 64 (BS6-tk-SupF);
- Lane 4, Line 78 (BS6-tk-SupF);
- Lane 5, Line 46 (BS6-tk-SupF);
- Lane 6, Line TK16 (HSV1-tkDS2-);
- Lane 7, Line TG66 (TG-tk-SupF);
- Lane 8, Line S87 (BS6Sau2-tk-SupF).
methionine codons of the reading frame (Preston and McGeoch, 1981; Marsden et al., 1983; Haarr et al. 1985).

Discussion.
The complete nucleotide sequence of the thymidine kinase gene is known (Wagner, M.J. et al., 1981). The first ATG codon occurs 78 nucleotides from the BS6 cap site in BS6-HSV1-tk (Figure 7.5). Twenty-nine nucleotides prior to this site is a region of the HSV1-tk gene partially complementary to the 3' end of the eukaryotic 18s ribosomal RNA (Figure 7.5). Such regions have been proposed as possible ribosome binding sites (Hagenbuchle et al., 1978). From the first ATG there is an open reading frame of 1128 nucleotides terminated by TGA. The gene codes for a protein of 376 amino acids, which based on the known composition of the amino acid sequence, has a molecular weight of 40,866. This compares with estimated molecular weights of 40,000 to 44,000 obtained from NaDodSo4/polyacrylamide gel electrophoresis (Preston, 1979; Cremer et al., 1979). The second and third ATG codons of the sequence are located 135 bases and 177 bases downstream from and in phase with the first ATG codon. By initiation at these codons proteins of molecular weights 35,656 and 34,107 (Table 7.2) would be generated. These sizes are compatible with the 39K and 38K polypeptide molecular weights estimated by SDS-PAGE. A further 75 bases downstream is a fourth in phase codon which, if used
The nucleotide sequence of the hybrid gene and part of its flanking region. The polylinker fusion region between the BS6 promoter and the HSV1-tk sequence and the location of the cap site are shown. The possible ribosome binding site, the ATG codons, and polyadenylation signals are underlined. The predicted amino acid sequence of the TK protein is also given.

Cap site Polylinker HSV1-tk sequence

GGAGTGTAGC/GATCCCG/GATCTTGGGCGTGAACATCCCAGCACCTTTGGCAGGCG

CTTGTAGAAGCGCGTGATATGCTTACCCGTGACATACGCGTCTGCGGAGGCG

The predicted amino acid sequence of the TK protein is also given.
Table 7.2.
The number of amino acids used for full-length and truncated HSV1-TK polypeptides, initiating at each of the AUG codons. Also shown is the molecular weight of each polypeptide, based on the known amino acid composition.

<table>
<thead>
<tr>
<th>No. of amino acids per molecules initiating at each of the AUG codons</th>
<th>First AUG</th>
<th>Second AUG</th>
<th>Third AUG</th>
<th>Fourth AUG</th>
<th>Fifth AUG</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of amino acids.</td>
<td>376</td>
<td>331</td>
<td>317</td>
<td>292</td>
<td>256</td>
</tr>
<tr>
<td>mol.wt.</td>
<td>40,866</td>
<td>35,656</td>
<td>34,107</td>
<td>31,463</td>
<td>27,575</td>
</tr>
<tr>
<td>Estimated mol.wt. from SDS PAGE</td>
<td>43K</td>
<td>39K</td>
<td>38K</td>
<td>35K</td>
<td>31K</td>
</tr>
</tbody>
</table>
as an initiation codon, would give a polypeptide of molecular weight 31,463.

Since the TK gene contains only one long open reading frame, the 43K, 41K, 39K, 38K and small molecular weight proteins could be related to proteolytic cleavage and/or secondary initiation of translation downstream from the first AUG codon. The addition of protease inhibitors during protein extraction showed that the 41K protein and also several small polypeptides present in preputial gland extracts are proteolytic products (Figure 7.2). In contrast, the testis extract was unaffected by the addition of protease inhibitors, showing that it does not proteolyse HSV1-TK (Figure 7.2). From this it can be argued that the truncated 39K and 38K polypeptides, which are the only HSV1-TK polypeptides present in significant amounts in testis extracts, are the primary translation products of the mRNA produced in the testis of transgenic mice carrying any of a number of different HSV1-tk constructs. The common features of these constructs are that they contain the coding region of the HSV1-tk gene, in the testis they generate a class of 5'-truncated mRNAs and also in the testis they express only the 39 and 38K HSV1-TK polypeptides.

The evidence that the two truncated polypeptides (39K and 38K) in transgenic extracts result from initiation at the second and third AUG codons, is that these
polypeptides co-migrate with the truncated polypeptides found in HSV1-infected cells. Previous studies have demonstrated that the 39K and 38K polypeptides in HSV1-infected cells arise by translational initiation at the second and third AUG codons (Preston and McGeoch, 1981; Marsden et al., 1983). Haarr et al (1985) obtained direct evidence from elegant hybrid arrest experiments and from a comparison of the TK proteins produced by cells infected with wild-type virus and HSV1-TK mutants. Mutant TK4 has an amber mutation between the first and second AUG codon, whereas mutant delta 1 has a deletion which removes the first AUG codon but leaves other AUG codons intact. Both mutants synthesise only the two truncated polypeptides.

The synthesis of these polypeptides was examined more closely by means of hybrid-arrest-translation experiments employing a DNA oligonucleotide complementary to 50 nucleotides at the 5' end of the HSV1-tk mRNA. When annealed with mRNA from cells infected with either wild-type HSV1 or the TK4 mutant, the oligonucleotide prevented the synthesis not only of the 43K full-length polypeptide but also of the 39K and 38K polypeptides. This demonstrated that, in these cells, both of the truncated polypeptides arise from translation complexes initiated at the 5' end of the mRNA. This evidence suggests that the truncated HSV1-TK polypeptides observed in the preputial gland result from translation complexes initiated in the
same way.

Translation of the 39K and 38K polypeptides was unaffected by the oligonucleotide, showing that a truncated transcript can support polypeptide chain initiation at the second and third ATG codons of the reading frame. The published data supports the conclusion that the testis HSV1-tk mRNA transcript, shown to be truncated at the 5'-end, is translated by polypeptide chain initiation at whatever 5'-terminal codons are available.

It has been proposed that translational initiation in eukaryotes depends on a scanning mechanism whereby the initiation complex settles upon the first AUG triplet that it encounters. Recognition of this triplet depends to some extent upon the sequence immediately surrounding it, the consensus of which, from a comparison of several hundred eukaryotic translation initiation sequences, is CCRCCAUGG (Kozak, 1981, 1984). If the AUG sequence first encountered lies within an unfavourable context, some initiation complexes will form at the site but scanning will continue downstream.

The sequences surrounding the first three AUG codons of the HSV1-tk genes are CGUAUGC, AAAAUGGC, and GGGAUUGG. Thus the flanking sequences of the first and second codons conform poorly with the consensus. The third codon conforms better to the consensus. Thus it is not unlikely,
when a full-length transcript is present, that some initiation complexes will bypass the first and/or the second AUG codons and initiate on the third. This is the most likely explanation of the synthesis of smaller amounts of the 39K and 38K HSV1-TK polypeptides both in virus-infected cells and in the preputial glands of transgenic mice.

When the first AUG codon is not present, translational initiation is expected to occur at the second and third AUG codons at a level correspondingly higher in relation to the absence of initiation at the first AUG. This is observed in the case of the delta 1 deletional mutation of HSV1, which lacks the normal 5'-end of the mRNA. Similarly, the testis transcripts found in transgenic mice, carrying several different constructs that have as their only common factor the HSV1-tk coding region, lack the 5'-end of the full-length mRNA, and also express mainly the 39K and 38K proteins.

Properties of the truncated TK polypeptides. TK activity is similar in the preputial gland and testis extracts, despite the fact that the preputial glands contain mainly full-length TK and the testes mainly the truncated 38K and 39K forms. Halpern and Smiley (1984) and Irmire et al (1989) found that the mutant delta 1, which overproduces the 39K and 38K polypeptides, but fails to produce the 43K polypeptide, produces about 40% of the wild type TK
activity, showing that one or both of the 39K and 38K polypeptides has enzyme activity. These results demonstrate that the 38K and/or the 39K polypeptides retain part of the catalytic activity. Therefore, it appears that the amino terminus of the TK polypeptide is dispensable for catalytic activity.

Although viruses that synthesize TK polypeptide lacking the first 45 amino acids appear to retain about 40% of wild-type activity and sensitivity to most antiviral nucleoside analogs, delta 1 is resistant to bromovinyldeoxyuridine (BVDU) (Coen et al., 1989). An explanation for this result is that delta 1 is defective in the TMP kinase activity of HSV1-TK. Such a defect could account for BVDU-resistance, because TMP kinase activity is required for efficient conversion of BVDU-monophosphate to BVDU-diphosphate (Fyfe, 1982; Ayisi et al., 1984). In agreement with this is a report that the TMP kinase activity of TK partially purified from cells infected with mutant delta 1 was greatly reduced relative to that of cells infected with wild-type HSV1 (Haarr and Flatmark, 1987). These findings suggest that the first 45 amino acids of TK are necessary for full TMP-kinase activity. It has been suggested that the polypeptide initiated at the third AUG codon (38K), which lacks part of the the nucleoside binding consensus sequence (Liu and Summers, 1988), is likely to have no TK or TMP activity but this
has not been demonstrated.

The full functional significance of the 39K and 38K polypeptides remains unclear, although it should be noted that HSV1-TK is a multimer (Jamieson and Subak-Sharp, 1974). The presence of the three polypeptide monomers (43K, 39K and 38K) could extend the range of protein-protein complexes which might be formed.
Chapter 8: STERILITY IN MALE TRANSGENIC MICE IS CAUSED BY THE EXPRESSION OF HSV1-TK IN THE TESTIS.

As previously described (Chapter 5), the HSV1-tk reporter sequence was expressed in the testis. In this chapter I describe an analysis of the fertility of transgenic animals in relation to the expression of HSV1-TK.

Results.

As noted in the previous chapters, 3 of 5 BS6-tk-F Go males (48, 49 and 51) and 2 of 3 BS6-tk-M Go males (M1 and M5) were sterile (Table 8.1). None of the fertile Go males transmitted the hybrid gene to their offspring. In addition, in 7 of 7 transgenic lines derived from Go females the males were invariably sterile (Table 8.1). Two exceptional mice, one from line 58 and one from a BS6-tk-M line, each sired a single offspring of unknown transgenic status. No further offspring were sired despite repeated mating. These mice, and also all the completely sterile mice, were housed with two female mice each for a minimum of 7 weeks and plugged females in the normal way. The fertility of an equal number of non-transgenic male siblings was tested in the same way and found to be fertile.

All of these established lines (BS6-tk-SupF and BS6-tk-M) which show consistent male sterility have a high level of HSV1-TK activity in the testis (Table 8.1). In contrast, line 40 shows no liver HSV1-TK activity and line
### Table 8.1.

Transmission from Go to G1 transgenics and expression of HSV1-TK in the testis of transgenic mice carrying the 2.2 kb 5’ BS6 promoter linked to the HSV1-tk reporter gene.

<table>
<thead>
<tr>
<th>Go Line of Go</th>
<th>Sex</th>
<th>Fertility Status (a)</th>
<th>G1 Transgenics</th>
<th>Enzyme activity in Testis (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>0.11 ± 0.044 (16)</td>
</tr>
<tr>
<td>Transgenic mice BS6-tk-SupF</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40* F</td>
<td>Sterile</td>
<td>/</td>
<td>141.3 ± 4.9</td>
<td>(4)</td>
</tr>
<tr>
<td>46* F</td>
<td>Sterile</td>
<td>/</td>
<td>139.0 ± 17.6</td>
<td>(3)</td>
</tr>
<tr>
<td>58* F</td>
<td>Sterile</td>
<td>/</td>
<td>41.6 ± 5.8</td>
<td>(7)</td>
</tr>
<tr>
<td>62 F</td>
<td>Fertile</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>64* F</td>
<td>Sterile</td>
<td>/</td>
<td>45.5 ± 4.3</td>
<td>(9)</td>
</tr>
<tr>
<td>78* F</td>
<td>Sterile</td>
<td>/</td>
<td>58.2 ± 11.7</td>
<td>(5)</td>
</tr>
<tr>
<td>66 M</td>
<td>Fertile</td>
<td>X</td>
<td>1.5</td>
<td>(1)</td>
</tr>
<tr>
<td>79 M</td>
<td>Fertile</td>
<td>X</td>
<td>0.0</td>
<td>(1)</td>
</tr>
<tr>
<td>48 M</td>
<td>Sterile</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>49 M</td>
<td>Sterile</td>
<td>X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>51 M</td>
<td>Sterile</td>
<td>X</td>
<td>25.0</td>
<td>(1)</td>
</tr>
<tr>
<td>Transgenic mice BS6-tk-M</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12* F</td>
<td>Sterile</td>
<td>/</td>
<td>106.0</td>
<td>(1)</td>
</tr>
<tr>
<td>42* F</td>
<td>Sterile</td>
<td>/</td>
<td>210.0</td>
<td>(1)</td>
</tr>
<tr>
<td>M1 M</td>
<td>Sterile</td>
<td>X</td>
<td>132.0</td>
<td>(1)</td>
</tr>
<tr>
<td>M4 M</td>
<td>Fertile</td>
<td>X</td>
<td>9.1</td>
<td>(1)</td>
</tr>
<tr>
<td>M5 M</td>
<td>Sterile</td>
<td>X</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Founders of transgenic lines.

(a) Sterile or fertile males.

(b) Units per milligram of protein; means and standard errors. n, the numbers of animals assayed, is shown in parentheses.
show no preputial gland HSV1-TK activity, although both lines are consistently male sterile. Thus testis expression of HSV1-TK is the likely cause of male sterility, and both liver and preputial gland expression can be ruled out as the sole cause.

A similar fertility pattern was observed in the secondary transgenic mice (BS6-tk-SupF TR). Three of 6 Go males were sterile and the fertile Go males did not father transgenic offspring (Table 8.2). Furthermore, 4 of 6 transgenic lines showed male sterility which correlated with high levels of HSV1-TK in the testis (Table 8.2). Transgenic mice harbouring the disabled HSV1-tk reporter gene also exhibited sterility in most cases. Of the 5 Go animals (4 males and 1 female) that were assayed, 3 Go males were fertile and of these 2 did not transmit the transgene to their offspring (Table 8.2). The Go female established line TK16 and G1 males from line TK16 were all sterile. 3 of the 4 Go males and males of line TK16 expressed HSV1-TK at moderate to high levels in the testis (Table 8.2).

Effects of HSV1-TK expression. Exceptions to the sterility of transgenic males were observed. Two of 6 secondary transgenic (BS6-tk-SupF) mice lines were fertile and transmitted the transgene to their offspring (Table 8.2, mice B56 and B73). These fertile lines had a relatively low level of HSV1-TK activity. One possible explanation is
Table 8.2.
Transmission from Go to G1 transgenics and expression of HSV1-TK in the testis of transgenic mice containing the BS6-tk-SupF and HSV1-tkDS2 constructs.

<table>
<thead>
<tr>
<th>Go Line</th>
<th>Sex of Go</th>
<th>Fertility Status (a)</th>
<th>G1 Transgenics</th>
<th>Enzyme activity in Testis (b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td>$0.11 \pm 0.044$ (16)</td>
</tr>
</tbody>
</table>

Secondary transgenic mice BS6-tk-SupF(TR).

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>A49*</td>
<td>F</td>
<td>Sterile</td>
<td></td>
<td>$77.1 \pm 22.0$ (3)</td>
</tr>
<tr>
<td>B65*</td>
<td>F</td>
<td>Sterile</td>
<td></td>
<td>$285.7 \pm 50.7$ (3)</td>
</tr>
<tr>
<td>B56*</td>
<td>F</td>
<td>Fertile</td>
<td></td>
<td>$1.77 \pm 1.0$ (3)</td>
</tr>
<tr>
<td>B85*</td>
<td>F</td>
<td>Sterile</td>
<td></td>
<td>$180.0 \pm 22.0$ (4)</td>
</tr>
<tr>
<td>B73*</td>
<td>F</td>
<td>Fertile</td>
<td></td>
<td>$5.9 \pm 2.3$ (3)</td>
</tr>
<tr>
<td>B83*</td>
<td>F</td>
<td>Sterile</td>
<td></td>
<td>$212.0 \pm 105.0$ (2)</td>
</tr>
<tr>
<td>A6</td>
<td>M</td>
<td>Fertile</td>
<td>X</td>
<td>$36.7$ (1)</td>
</tr>
<tr>
<td>A17</td>
<td>M</td>
<td>Fertile</td>
<td>X</td>
<td>$24.9$ (1)</td>
</tr>
<tr>
<td>A45</td>
<td>M</td>
<td>Sterile</td>
<td>X</td>
<td>$330.0$ (1)</td>
</tr>
<tr>
<td>A60</td>
<td>M</td>
<td>Sterile</td>
<td>X</td>
<td>$38.8$ (1)</td>
</tr>
<tr>
<td>B27</td>
<td>M</td>
<td>Sterile</td>
<td>X</td>
<td>$50.6$ (1)</td>
</tr>
<tr>
<td>B50</td>
<td>M</td>
<td>Fertile</td>
<td>X</td>
<td>$5.6$ (1)</td>
</tr>
</tbody>
</table>

Transgenic mice HSV1-tkDS2

<p>| | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>TK16*</td>
<td>F</td>
<td>Sterile</td>
<td></td>
<td>$97.23$ (1)</td>
</tr>
<tr>
<td>TK10</td>
<td>M</td>
<td>Fertile</td>
<td>X</td>
<td>$23.15$ (1)</td>
</tr>
<tr>
<td>TK22</td>
<td>M</td>
<td>Fertile</td>
<td>X</td>
<td>$0.16$ (1)</td>
</tr>
<tr>
<td>TK39</td>
<td>M</td>
<td>Sterile</td>
<td>X</td>
<td>$37.75$ (1)</td>
</tr>
<tr>
<td>TK40</td>
<td>M</td>
<td>Fertile</td>
<td>X</td>
<td>$4.23$ (1)</td>
</tr>
</tbody>
</table>

* Founders of transgenic lines.
(a) Sterile or fertile males.
(b) Units per milligram of protein; means and standard errors.
n, the numbers of animals assayed, is shown in parentheses.
that lower expression levels do not cause sterility and that there is a threshold level of HSV1-TK above which sterility occurs. In line 78 and 64 which exhibit a moderate level of expression in the testis (58.2 and 45.4U/mg of protein, respectively), sperm counts were normal but sperm motility was reduced and males were sterile. In transgenic male mice from line 40 or 46 which have high levels of TK expression in the testis (141.3 and 139U/mg of protein, respectively), sperm counts were very low, malformed non-motile sperm were present and males were again sterile. Therefore, the severity of the lesion is greater when TK expression in the testis is at its highest. All the female transgenics exhibited normal fertility and no HSV1-TK activity was detected in ovaries of BS6-tk-SupF lines 40, 46, 58, and 64 (Table 8.3).

Discussion.

**Male sterility and expression of the HSV1-tk reporter in the testis.** The constructs employed in this work and that of other investigators utilize two promoters with entirely different tissue-specificity. One directs expression to the liver under the control of growth hormone. The other directs expression to the thyroid under the control of TSH. In both cases HSV1-TK was expressed at a similar high level in the testis and caused male sterility. Furthermore, the HSV1-tk reporter gene itself, when disabled by removal of its distal regulatory element,
Table 8.3.

HSV1-TK activities in the female ovaries of transgenic mouse lines.

<table>
<thead>
<tr>
<th>Line</th>
<th>Enzyme activity (a) in the ovaries</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0 (4)</td>
</tr>
<tr>
<td>40</td>
<td>0.0 (1)</td>
</tr>
<tr>
<td>46</td>
<td>0.0 (1)</td>
</tr>
<tr>
<td>58</td>
<td>0.027 (1)</td>
</tr>
<tr>
<td>64</td>
<td>0.0 (1)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein. 

n, the numbers of animals assayed, is shown in parentheses.
caused male sterility. From these results it may be concluded that irrespective of properties of the promoter, ectopic expression of HSV1-tk frequently occurs in the testis of transgenic mice and results in male sterility.

A number of transgenic mice bearing HSV1-tk alone or driven by the metallothionein promoter showed normal fertility (Palmiter et al., 1982; Palmiter and Brinster, 1986). However, the level of expression in testis was not monitored in these animals, and may have been below the threshold necessary for infertility. Several other examples have been reported. Some lines of mice transgenic for HSV1-tk linked to the mouse mammary tumour virus long terminal repeat expresses HSV1-tk in the testis (Ross and Solter., 1985) and are sterile. In another case, a cosmId vector bearing the MHC class II alpha gene and a HSV1-tk gene was used to generate transgenic mice and the resulting mice were sterile (Braun et al., 1990). To ascertain the cause of the sterility, various subfragments of the cosmId were tested in transgenic mice and only those pieces of DNA that include some of the E alpha flanking chromosomal DNA and the HSV1-tk gene resulted in male sterility. Transgenic male mice bearing an IFN-gamma transgene driven by the mouse metallothionein promoter were also sterile (Iwakura et al., 1988). Here, too, HSV1-tk gene expression (from the plasmid vector) was found in the testis. Finally reduced male fertility was also
reported in transgenic mice bearing HSV1-tk driven by an immunoglobulin gene enhancer and promoter (Heyman et al., 1989), although the researchers did not assess the expression of HSV1-tk in the testis.

Braun et al (1990) also showed that the complete HSV1-tk gene alone did not effect fertility, when introduced into transgenic mice, despite the presence of HSV1-tk mRNA and protein in germ cells. These mice exhibit a lower level of expression, in comparison to the Ealpha-HSV1-tk construct, and it may be that this level is below the threshold necessary for sterility. Since the truncated HSV1-tkDS2- gene caused sterility, the possibility arises the HSV1-tk promoter prevents this.

Causes of male sterility. Available evidence suggests that a high level of HSV1-TK activity in the haploid spermatids is the likely cause of male sterility. The enzyme could exert an effect in a number of ways which disrupt the cellular metabolism of nucleic acid precursors. First, its substrate specificity is different from that of the cellular enzyme, and it efficiently phosphorylates mononucleotides as well as nucleosides (Chen and Prusoff, 1978; Veerisetty and Gentry, 1985), a fact used to advantage for the killing of cells with ganciclovir, an analog that is incorporated into DNA to arrest replication (Heyman et al., 1989). Second, the high levels of phosphorylated products produced by the enzyme could
inhibit the normal pathway of pyrimidine metabolism (Kelley, 1983). Third, HSV1-TK may be active at times in the cell cycle when the cellular enzyme is normally repressed (Hofbauer et al., 1987; Merrill et al., 1984) and if so could interfere with some vital cellular function. Finally, since spermatids have completed DNA replication, it is possible that enzymes are induced that degrade deoxynucleotide monophosphates. Consequently, the expression of HSV1-tk might establish a futile cycle of thymidine phosphorylation and dephosphorylation at the expense of ATP. Indeed sterile mice have non-motile spermatozoa and this may suggest a simple depletion of ATP.
Chapter 9: SYNTHESIS AND ANALYSIS OF ETHYNYLTHYMIDINE, A
COMPETITIVE INHIBITOR OF HSV1-TK.

In most cases the HSV1-TK activity in the testis rendered the
transgenic male mice sterile. In an attempt to restore
fertility in transgenic male mice or at least reduce HSV1-TK
activity, synthesis of a thymidine analogue which is a non
toxic competitive inhibitor of HSV1-TK was undertaken. A report
by Nutter et al (1987) described in detail the use of such an
inhibitor. In this chapter I describe the effects of 5'-
ethynyl-thymidine (5'-Et-dThd) as a selective inhibitor of
HSV1-TK (in vitro and in vivo).

Results.

Synthesis of 5'-Et-dThd. 5'-Et-dThd (1-(2,5,6-trideoxy-B-D-
erthro-hex-5-enofuranosyl)-5-methyluracil) was synthesized, as
described in Materials and Methods, by the protocol employed by
Sharma and Bobek, 1978.

Activity of 5'-Et-dThd against viral and cellular thymidine
kinase activities in vitro. To investigate the usefulness of
the compound, its effects on viral and cellular thymidine
kinases were examined. Extracts of BHK fibroblasts, which
possess an active cellular tk gene (BHKTK), and extracts of BHK
cells, which stably express a Mup/HSV1-tk gene (BHKHSVTK) but
which lack the cellular kinase, were utilized for this purpose.
Extracts of transgenic testis which actively expressed the
Mup/HSV1-tk transgene were also utilized. The activity of
cellular TK is regulated by end-product inhibition to a much
greater extent than HSV1-TK, and endogenous cellular activity can be selectively inhibited by the addition of 0.4 mM dTTP (Jamieson and Subak-Sharp, 1974). In my hands, the addition of 0.4 mM dTTP inhibited cellular TK activities by approximately 92% and HSV1-TK by approximately 50%. This property was utilized in the assays for tissue-specific expression of the transgene in mice and is employed in this investigation.

BHKTK and BHKHSVTK cell extracts were prepared from sub-confluent monolayers and thymidine kinase assays were performed in the presence of different concentrations of 5'-Et-dThd (see Materials and Methods). Incubations were conducted at 37°C for 60 minutes. 5'-Et-dThd inhibited cellular thymidine kinase only slightly (Figure 9.1, BHKTK). In contrast, both BHKHSVTK and testis extract were inhibited by 5'-Et-dThd. 50% inhibition of HSV1-TK was observed at a concentrations of about 40uM in each case. These data confirm the report by Nutter et al (1987) who observed selective inhibition of HSV1-TK relative to human thymidine kinase in the presence of 5'-Et-dThd.

Inhibition of HSV1-TK and lack of inhibition of cellular thymidine kinase in cells in culture. When cells are cultured in the growth medium HAT, which contains Hypoxanthine, Aminopterin and Thymidine, survival is dependent on the pyrimidine salvage pathway enzyme, thymidine kinase (Littlefield, 1964). Aminopterin inhibits the de novo synthesis of dATP, dGTP and dTTP from dihydrofolate. Hypoxanthine is a substrate for the salvage pathway for dATP and dGTP and
Figure 9.1.
The effect of 5'-Et-dThd on in vitro thymidine kinase activity.

Extracts from BHKTK (○), containing no dTTP, BHKHSVTK (□), containing dTTP, and testis (●), containing dTTP, were examined by thymidine kinase assays, for their sensitivity to 5'-Et-dThd as described in the text.
therefore allows these nucleotides to be synthesised. Thymidine is essential for the synthesis of dTTP and is totally dependent on an active tk gene. BHKHSVTK cells are totally dependent on the viral thymidine kinase for survival and proliferation in HAT medium. To determine whether 5'-Et-dThd treatment would inhibit the proliferation of BHKHSVTK cells in HAT medium, exponentially growing cells were exposed to various concentrations of 5'-Et-dThd, (see Materials and Methods).

When BHKHSVTK cells were exposed to 5'-Et-dThd for 5 days, cell proliferation was strongly inhibited (Figure 9.2). In contrast the exposure to 5'-Et-dThd of BHKTk cells, which are totally dependent on cellular thymidine kinase, had no effect on cell proliferation (Figure 9.2). These experiments show that 5'-Et-dThd is not excluded from fibroblasts and exhibits no toxicity over the range of concentrations tested. Furthermore, 5'-Et-dThd specifically inhibits the viral TK. Figure 9.2 shows that 50% growth inhibition was observed at a 5'-Et-dThd concentration of 80μM (Figure 9.1).

Effects of 5'-Et-dThd on antiviral activity of antitherpetic agents. The pathogenic effects of HSV infection have led to the development of nucleoside analogs that inhibit virus multiplication. Both Acyclovir (ACV) and Ganciclovir (GCV) are phosphorylated by HSV1-TK, but not by the cellular kinase. The phosphorylated compound is lethal to cells. Consequently, virus infected cells are killed, but uninfected cells are not. If 5'-Et-dThd competes effectively with ACV or GCV in the HSV1-TK
Figure 9.2.
The effect of 5'-Et-dThd on cells in culture in the presence of HAT medium.

BHKTK ( ) and BHKHSVTK ( ) cells were exposed to various concentrations of 5'-Et-dThd for 5 days in the presence of HAT medium.
phosphorylation reaction it should increase cell survival in the presence of the antiherpetic agent.

The effect of 5'-Et-dThd on the survival of BHKHSVTK cells in the presence of ACV and GCV was determined using medium not supplemented with HAT. The experiment was similar to the one just previously described. Cells were plated at low density and approximately 15 hours later 5'-Et-dThd was added. After a further 24 hours either a 3uM ACV or 1uM GCV was added and the cultures were incubated for an additional 96 hours. Viable cells were again identified by trypan blue exclusion.

Figure 9.3 shows that in the presence of 5'-Et-dThd cell survival increased with increasing 5'-Et-dThd concentration. At a 5'-Et-dThd concentration of 500uM 5'-Et-dThd survival was 90% (ACV) and 80% (GCV). This is in agreement with the observation of Nutter et al (1987) who showed using the virus yield assay that 5'-Et-dThd reversed the antiviral activity of five nucleoside analogs, including ACV and GCV, which require viral kinase for their action. The concentration of 5'-Et-dThd which gave 50% survival was 175uM for cells exposed to 3uM ACV and 265uM for those exposed to 1uM GCV.

Effect of 5'-Et-dThd on transgenic mice. The amount of 5'-Et-dThd available was limiting, so that only a few experiments could be performed on transgenic and control animals. Transgenic male mice from line 58 were employed, since this line expressed the lowest amount of HSV1-TK in its testis. Adult transgenic and control male mice were exposed daily to
Figure 9.3.
The effect of 5'-Et-dThd on antiviral activity of antiviral agents.

The antiviral activities of 3μM acyclovir (●) and 1μM ganciclovir (○) in the presence of 5'-Et-dThd were determined by carrying out cell growth analysis on BHKHSVTK cells.
5'-Et-dThd by intraperitoneal injection and sacrificed as detailed in Table 9.1. Drug treatment was at 0.47mg/g, to give an overall concentration of about 100μM. I showed previously that this concentration is effective in tissue culture cells.

Seven transgenic and three non-transgenic mice were injected with 5'-Et-dThd, and a further three transgenics were injected with PBS. At various times, the animals were sacrificed and the levels of TK in testis, liver and preputial gland was measured (Table 9.1). As expected the non-transgenic mice treated with 5'-Et-dThd did not show any detectable levels of TK, in the tissues analysed. In all transgenic mice given PBS, the expression of the transgene was as expected. In all animals tested the organs removed appeared normal, with no apparent toxic effects, due to the presence of 5'-Et-dThd.

Since only one mouse was analysed at most time points it was difficult to assess the significance of these results. Tentatively, the mouse from line 58 which had been exposed to 5'-Et-dThd for 5 days appears to have a significant decrease in activity in comparison to the control mice. This appears to be the case in all of the three expressing tissues examined. In the testis, liver and preputial gland tissues, there was a 48%, 29% and 36% decrease in enzyme activity at 5 days, respectively, in comparison to the untreated transgenic animals. These preliminary experiments appears to indicate that 5'-Et-dThd has an affect on TK activity in transgenic animals. Further analysis is necessary to confirm these findings.
Table 9.1.

HSV1-TK activity in tissues of control and transgenic male mice from line 58, which had been exposed to 5'-Et-dThd for different time durations.

<table>
<thead>
<tr>
<th>Exposure time to 5'-Et-dThd</th>
<th>Testis</th>
<th>Liver</th>
<th>Preputial gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.07 ± 0.1 (3)</td>
<td>0.0 (3)</td>
<td>0.0 (3)</td>
</tr>
<tr>
<td>Line 58 PBS only</td>
<td>35.1 ± 10.2 (3)</td>
<td>7.2 ± 1.4 (3)</td>
<td>10.8 ± 0.7 (3)</td>
</tr>
<tr>
<td>Line 58 15min</td>
<td>29.3 (1)</td>
<td>7.7 (1)</td>
<td>9.4 (1)</td>
</tr>
<tr>
<td>Line 58 1hr</td>
<td>34.1 (1)</td>
<td>9.5 (1)</td>
<td>6.2 (1)</td>
</tr>
<tr>
<td>Line 58 2hr</td>
<td>40.6 ± 2.1 (2)</td>
<td>7.2 ± 0.1 (2)</td>
<td>8.5 ± 2.4 (2)</td>
</tr>
<tr>
<td>Line 58 7hr</td>
<td>25.2 (1)</td>
<td>7.8 (1)</td>
<td>9.0 (1)</td>
</tr>
<tr>
<td>Line 58 24hr</td>
<td>25.1 (1)</td>
<td>7.4 (1)</td>
<td>9.4 (1)</td>
</tr>
<tr>
<td>Line 58 5days</td>
<td>20.5 (1)</td>
<td>5.1 (1)</td>
<td>6.9 (1)</td>
</tr>
</tbody>
</table>

(a) Units per milligram of protein: means and standard errors. n, the numbers of animals assayed, is shown in parentheses.
Discussion.
The thymidine analog 5'-ethynylthymidine was a potent inhibitor of HSV1-induced thymidine kinase and showed no inhibition against mammalian thymidine kinase under the conditions tested. When tested alone, 5'-ethynylthymidine exhibited no toxicity, but antagonized the toxic effects of two compounds (ACV and GCV) which require HSV1-TK for their action. Therefore, it appears that 5'-Et-dThd not only competes with thymidine but also with ACV and GCV. Furthermore, preliminary experiments performed on transgenic animals indicate that 5'-Et-dThd may cause a decrease in HSV1-TK activity, although further analysis is necessary to determine whether this phenomenon is a general one.
Chapter 10: MATERIALS AND METHODS.

Enzymes.
Restriction and DNA/RNA modifying enzymes were purchased from either Amersham International or Pharmacia, and were used with the buffers supplied or as directed by the supplier.

Northern Blotting and DNA Probes.
Total RNA was extracted from homogenised mouse tissues in guanidinium thiocyanate and pelleted through a caesium chloride gradient as described by Chirgwin et al (1979). Once the tissue is removed and after a phosphate buffered saline (PBS) wash, a solution of 4M guanidinium thiocyanate, 0.5% w/v sodium N-lauryl sacosinate, 25mM sodium citrate, 0.1% w/v Sigma antifoam A and 0.1M beta-mercaptoethanol, was added to the appropriate tissue. The resulting solution was homogenised for a few minutes, followed by layering over 1.2mls of 5.7M caesium chloride buffered with 25mM sodium acetate (pH 5) and centrifuged at 36K, 20°C for 12 hours. The pelleted RNA was resuspended in 1ml of 7.5M guanidine hydrochloride, 25mM sodium citrate (pH 7), 5mM DTT and precipitated twice, once with 0.025 volumes of 1M acetic acid, 0.5 volumes of ethanol and once with 2mls double distilled water, 0.1 volumes of 2M sodium acetate and 2 volumes of ethanol. The RNA was finally resuspended in double distilled water.

Northern blot analysis was carried out as described
by Church and Gilbert (1984), using hybond-N (Amersham) nylon membranes and the double-stranded DNA probes were labelled by a variation of the method of Feinberg and Vogelstein (1984). The tk probe was an 840 bp PstI fragment of pTK1 which overlaps 806 bp between the point of fusion with the Mup promoter region and a PstI site internal to the TK coding region (Wagner, M.J. et al., 1981; McKnight et al., 1981).

Protocol For Primer Extension.
The synthetic oligonucleotides were purchased from the Oswell DNA Service, Chemistry Department, Edinburgh University. Concentrations of oligonucleotide in water were determined by measuring the A260 of the solution. The probes (oligonucleotides) were labelled to a specific activity of $5 \times 10^8$ to $2 \times 10^9$, using gamma-$^{32}P$ ATP and T4 polynucleotide kinase as described by Maxam and Gilbert (1980). Primer extension assays were carried out according to Ghosh et al (1980). In a microcentrifuge tube 0.2pmols of end labelled primer was mixed with 20ug of TK total RNA, 2ul of 5 x hybridisation buffer (50mM PIPES pH 6.5, 2M NaCl) and water to a final volume of 10ul. After sealing with paraffin oil, the eppendorf tube was denatured at 90°C for 5 minutes and left at the annealing temperature of 55°C for 6 hours to overnight. After annealing, 90ul of extension reaction buffer (50mM Tris-HCl pH 8.2, 10mM DTT, 6mM MgCl2, 0.5mM each of dATP, dCTP,
dGTP, dTTP, 2.5 g of actinomycin D and 15 units of AMV reverse transcriptase), was added to the sample tube. The extension reaction was carried out at 42°C for 60 minutes and terminated by extraction with phenol/chloroform twice. This is followed by precipitation with 7.5M ammonium acetate and 2.5 volumes of ethanol. After an overnight precipitation the samples were centrifuged, rinsed twice with 70% ethanol, dried and loaded on to a 6% polyacrylamide/ 8M urea gel. Gels were fixed for 20 minutes in 10% methanol, 10% acetic acid, dried and exposed directly to X-ray film.

Tissue Culture Methods.
The cells used in the experiments were baby hamster kidney fibroblasts (Macpherson and Stoker, 1962) which were grown at 37°C in CO₂ in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 3.7g/litre sodium hydrogen carbonate, 10% foetal calf serum, penicillin (100μg/ml) and streptomycin (100μg/ml). Cultured vessels were purchased from NUNC. Cells were detached by trypsinisation with 0.25% trypsin, 0.02% EDTA.

Cell Growth Inhibition.
Cells, BHKTK and BHKHSVTK, were seeded at low density in DMEM containing 10% foetal calf serum in the presence of HAT. Approximately 15 hours later various concentrations of 5'-Et-dThd were administered to the cultures. After a total of 5 days cells were detached by trypsin treatment
and resuspended in medium. Viable cells were recognized by trypan blue exclusion, cell numbers were determined by an average of at least two cultures for each concentration.

**Infection Of BHKtk- Cells.**

A monolayer of BHKtk- cells was infected at 10 PFU/cell with HSV strain 17, Cl 101 or the TK mutant delta 1, which were kindly provided by Howard Marsden. After 1 hour the cells were washed once with phosphate buffered saline, replaced with DMEM and incubated overnight. Next day the cells were harvested in a denaturing buffer, which containing 0.05M Tris HCl, pH 6.8, 2% SDS, 5% beta-mercaptoethanol, 10% glycerol and bromophenol blue. The cells were heated at 100°C for 2 minutes and stored at -70°C until needed.

**Herpes Simplex Thymidine Kinase Assay.**

Thymidine kinase assays were carried out as described by Brinster et al (1981), but 0.4mM dTTP was added to inhibit endogenous thymidine kinase (Jamieson and Subak-Sharp., 1974).

Mice were sacrificed and organs were rinsed in cold PBS. Tissues were homogenised (Polytron homogenizer) in homogenisation buffer containing 10mM potassium chloride, 2mM magnesium chloride, 10mM Tris (pH 7.5), 1mM ATP, 10mM sodium fluoride and 50mM aminocaproic acid, (pH 7.4). The extracts were pelleted by centrifugation (10000g, 4°C for 10 minutes). The enzyme assay is described by Brinster et
al (1981) and is based on the conversion of radiolabelled thymidine to thymidine phosphate. A 15ul aliquot of tissue supernatant was added to 75ul of reaction mixture containing 10mM magnesium chloride, 150mM Tris (pH 7.5), 10mM ATP, 25mM sodium fluoride, 10mM beta-mercaptoethanol and 15uCi of tritiated thymidine (90 Ci/mmol, Amersham). The mixture was incubated at 37°C. The ³H-TMP produced was measured by spotting 25ul of mixture onto DEAE-81 paper (Whatman) discs at time intervals 30, 60 and 120 minutes. The discs were washed 4 times, 10 minutes each, in 10mM Tris (pH 7.5) with shaking and dried under vacuum at 80°C. The radioactivity on the filters was determined by liquid scintillation counting (scintillant was 5g/l PPO, 0.3g/l dimethyl POPOP in toluene). The tissue extracts were stored at -70°C. The assaying of thymidine kinase in tissue culture cell extracts was as above, but with the following refinements. The cells were harvested by washing twice in ice cold PBS before they were scraped off into 5mls of PBS and pelleted by centrifugation. A further washing with 5mls of PBS and centrifugation followed, before the cells were taken up in 200ul of homogenisation mix. The cells were pelleted by centrifugation after being lysed by sonication at 4°C for 5 hours. The unit of thymidine kinase activity is the amount of enzyme which catalyses the formation of 1 pmol of TMP in 1 minute.

The soluble protein content of the cell extracts were
estimated by the Bradford method (Bradford, 1976), using the Bio-Rad assay system. This is a dye-binding assay based on the differential colour change of a dye in response to various concentrations of protein.

Protein Analysis.

Preparation and use of tissue extracts in SDS-PAGE. Tissue extracts were homogenized in the same homogenization buffer as was used in thymidine kinase assays, and where indicated the addition of further protease inhibitors were added. Following homogenization tissue extracts were centrifuged at 10K, 4°C for 10 minutes, the supernatant removed and protein assays were carried out to determine the concentration. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12% separating gel with a 4% stacking gel, according to Laemmli (1970).

Electroblotting and immunostaining of blotted proteins. After electrophoresis, the proteins were transferred to nitrocellulose by using a homemade semi-dry Trans-blotting apparatus. The blotting assembly was prepared in a sandwich configuration, with the nitrocellulose membrane, which had previously been soaked in distilled water, placed on the side of the gel facing the anode in the blotting unit. Whatman type 3MM chromatography paper was soaked in blotting buffers, details of the buffers used and the sandwich set up are shown in Figure 10.1. Electroelutions were performed at 200mA for 1-2 hours at
Figure 10.1. Assembly for electrophoretic blotting procedure.

Electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose filters. The apparatus is assembled as follows: on top of the anode plate are successively placed, 2 sheets of 3MM chromatography paper soaked in Anode 1 buffer (0.3M Tris, 20% Methanol), 1 sheet of 3MM chromatography paper soaked in Anode 2 buffer (25mM Tris, 20% Methanol), a sheet of nitrocellulose filter paper soaked in distilled water (DH2O), the gel, 2 sheets of 3MM chromatography paper soaked in Cathode buffer (25mM Tris, 20% Methanol, 40mM Hexanoic acid) and finally the Cathode plate is placed on top.
4°C. After transfer was complete, the nitrocellulose was blocked by incubating in BLOTTO (5% w/v nonfat dry milk, 0.01% Antifoam in PBS) for 4 hours at room temperature with gentle agitation (Johnson et al., 1984). The same reagent used for blocking was utilized as a carrier for primary and secondary antibodies. The blots were reacted with primary antibody, 1:250 of anti-rabbit HSV1-TK, (which was a kind gift of William Summers), overnight at room temperature with agitation. This was followed by three 15 minute washes with 0.5% Tween 80 in PBS, before incubation with the second antibody, 1:400 anti-rabbit Ig-biotinylated (Amersham), for 2-3 hours at room temperature. Blots were then washed as described above, followed by treatment with a 1:1000 streptavidin-alkaline phosphatase conjugate (Amersham), for 45 minutes at room temperature. The membrane was then washed a third time and suspended in its substrate system to develop colour. Which in the case of alkaline phosphatase conjugate was 7mM 5-bromo-4-chloro-3-indolyphosphate (BCIP), 6mM nitroblue tertrazolium (NBT) in 0.1M Tris/HCl pH 9.5, at room temperature for about 20 minutes. Development results in brown protein bands that indicate specific reactivity, which were immediately photographed.

Staining and destaining of SDS-polyacrylamide gels and membrane blots. The gels were stained with Coomassie brilliant blue R-250 (0.2% in 1% TCA, 7.4% acetic acid,
50% methanol) for approximately 2 hours followed by destaining in 7% acetic acid/23% ethanol. Membrane blots were stained in 0.2% (w/v) Coomassie brilliant blue R-250, 50% methanol, 10% acetic acid for 10-15 minutes. The blots were then destained in 45% methanol, 7% acetic acid for 5-10 minutes.

**Synthesis of 5'-Et-dThd.**

5'-Et-dThd (1-(2,5,6-trideoxy-D-erythro-hex-5-enofuranosyl)-5-methyluracil) was synthesized as described by the method employed by Sharma and Bobek, 1978. The synthesis consists of five steps. Briefly 3'-O-Acetylthymidine (Figure 10.2A) was dissolved in dimethyl sulfoxide containing dicyclohexylcarbodiimide and phosphoric acid. The solvent was evaporated to dryness and the residue was partitioned between water and ether. After removal of dicyclohexyl-urea by filtration, a further ether extraction was performed and the aqueous solution was added to an excess of 2,4-dinitrophenylhydrazine in 1N sulphuric acid. After chilling the dinitrophenylhydrazone of 3'-O-acetylthymidine-5'-aldehyde was removed by filtration (Figure 10.2B).

3'-O-Acetylthymidine-5'-aldehyde (Figure 10.2B) was condensed with (dibromomethylene)triphenylphosphorane in methylene chloride. The mixture was evaporated to a small volume and chromatographed twice on a dry silica gel column, eluting with benzene/ethyl acetate. The combined
Figure 10.2.

The five major compounds involved in the synthesis of 5'-ethynyl-thymidine (5'-Et-dThd).
fractions were dissolved in methanol, where 1-(2,5,6-
trideoxy-6,6-dibromo-3-O-acetyl-\(\beta\)-D-erythro-hex-5-
enofuranosyl)-5-methyluracil) (Figure 10.2C) crystallized within 30 minutes.

A solution of the product in Figure 10.2C, in excess of sodium methoxide in methanol was neutralized with Dowex 50 (H+) resin and filtered. The resin was washed with methanol, combined, and evaporated to a small volume where 1-(2,5,5-trideoxy-6,6-dibromo-\(\beta\)-D-erythro-hex-5-
enofuranosyl)-5-methyluracil (Figure 10.2D) crystallized.

A solution of the product in Figure 10.2D, in dry tetrahydrofuran was treated with n-butyl-lithium. The mixture was stirred, neutralized with acetic acid, evaporated and co-evaporated with ethanol. The crude material was chromatographed on a dry silica gel column using ethyl acetate as eluant. After evaporation and crystallization from ethanol, the desired product was 1-(2,5,6-trideoxy-\(\beta\)-D-erythro-hex-5-enofuranosyl)-5-methyluracil (Figure 10.2E), which is abbreviated to 5'-ethynyl-thymidine (5'-Et-dThd).
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ACV</td>
<td>acyclovir</td>
</tr>
<tr>
<td>AFP</td>
<td>alpha-fetoprotein gene</td>
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<td>AMV</td>
<td>avian myeloblastosis virus</td>
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<td>APRT</td>
<td>adenosylphosphoribosyltransferase</td>
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<td>ATP</td>
<td>adenosine 5' triphosphate</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolylphosphoate</td>
</tr>
<tr>
<td>BHK</td>
<td>baby hamster kidney cells</td>
</tr>
<tr>
<td>BLOTTO</td>
<td>bovine lacto transfer technique optimizer</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
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<tr>
<td>BPV</td>
<td>bovine papilloma virus</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>BVdU</td>
<td>5-bromovinyldeoxyuridine</td>
</tr>
<tr>
<td>C</td>
<td>cytidine</td>
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<td>CAT</td>
<td>chloramphenicol acetyl-transferase</td>
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<td>cDNA</td>
<td>DNA copy of RNA</td>
</tr>
<tr>
<td>CTF</td>
<td>cellular transcription factor</td>
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<td>d</td>
<td>deoxyribo-</td>
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<tr>
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<td>dichloroisocoumarin</td>
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<tr>
<td>dCTP</td>
<td>deoxycytidine triphosphate</td>
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<tr>
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<td>dGTP</td>
<td>deoxyguanosine triphosphate</td>
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<tr>
<td>DHFR</td>
<td>dihydrofolate reductase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
<td>------------</td>
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<tr>
<td>DMEM</td>
<td>dulbecco's modified Eagles medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNase</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>dpm</td>
<td>depositions per minute</td>
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<tr>
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<td>diaminoethanetetra-acetic acid</td>
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<tr>
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<td>ganciclovir</td>
</tr>
<tr>
<td>GH</td>
<td>growth hormone</td>
</tr>
<tr>
<td>GRE</td>
<td>glucocorticoid responsive element</td>
</tr>
<tr>
<td>HAT</td>
<td>hypoxanthine, aminopterin, thymidine</td>
</tr>
<tr>
<td>HNF-1</td>
<td>hepatocyte nuclear factor-1</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyl transferase</td>
</tr>
<tr>
<td>hsp</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>HSTF</td>
<td>heat shock transcription factor</td>
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<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IGF-I</td>
<td>insulin-like growth factor I</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
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<td>kb</td>
<td>kilobase pair</td>
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<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>Met</td>
<td>methionine</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
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<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>MMTV</td>
<td>mouse mammary tumour virus</td>
</tr>
<tr>
<td>MRE</td>
<td>metal regulatory element</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MT</td>
<td>metallothionein</td>
</tr>
<tr>
<td>MUP</td>
<td>major urinary protein</td>
</tr>
<tr>
<td>Mup</td>
<td>major urinary protein gene</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
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<td>NBT</td>
<td>nitroblue tetrazolium</td>
</tr>
<tr>
<td>NF1</td>
<td>nuclear factor 1</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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| pH           | -log [H+]
| pHMB         | para-hydroxymercuribenzoate |
| PIPES        | Piperazine-N,N'-bis[2-ethanesulfonic acid] |
| PMSF         | phenylmethanesulphonylfluoride |
| polII        | polymerase II |
| poly(A) RNA  | polyadenylated RNA |
| POPOP        | 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzine |
| PPO          | 2,5-diphenyloxazole |
| r            | ribo- |
| RNA          | ribonucleic acid |
| RNase        | ribonuclease |
| rRNA         | ribosomal RNA |
| S1           | single strand specific nuclease |
SDS  sodium dodecyl sulphate
SP1  specific transcription factor 1
SV40 simian virus 40
T thymidine
TCA  tris-[hydroxymethyl]-aminomethane
tk thymidine kinase (gene)
TK thymidine kinase (enzyme)
TLCK tosyl-lysine chloromethyl ketone
TMP thymidine monophosphate
Tris tris-[hydroxymethyl]-aminomethane
tRNA transfer RNA
TSH thyroid stimulating hormone
TTP thymidine triphosphate
U uridine
UASs upstream activator sequences
UPEs upstream promoter elements
UTP uridine triphosphate
w/v weight per volume
REFERENCES


Clark, A.J., J. Hickman, and J.O. Bishop. 1984b. A 45kb DNA domain with two divergently orientated genes is the unit of organisation of the murine major urinary protein genes. EMBO J. 3: 2055-2064.

Clark, A.J., A. Chave-Cox, X. Ma, and J.O. Bishop. 1985a. Analysis of mouse major urinary protein genes: variation between the exonic sequences of group 1 genes and comparison with an active gene outwith group 1 both suggests that gene conversion has occurred between the Mup genes. EMBO J. 4: 3167-3171.


with the fibrinogen and alpha 1-antitrypsin promoters. Science 238: 688-692.


Preston, C.M. 1979. Control of herpes simplex virus type 1 mRNA synthesis in cells infected with wild-type virus or the temperature-sensitive mutant tsK. J. Virol. 29: 275-284.


Shahan, K., M. Denaro, M. Gilmartin, Y. Shi, and E. Derman. 1987b. Expression of six mouse major urinary protein genes in


Thompson, S., A.R. Clarke, A.M. Pow, M.L. Hooper, and D.W. Melton. 1989. Germ line transmission and correct expression of


A Mup Promoter-Thymidine Kinase Reporter Gene Shows Relaxed Tissue-Specific Expression and Confers Male Sterility upon Transgenic Mice

RAYA AL-SHAWI,1 JOANNE BURKE,3 CHERYL T. JONES,1 J. PAUL SIMONS,2 AND JOHN O. BISHOP1*

Department of Genetics, University of Edinburgh, Edinburgh EH9 3JN, 1 and AFRC IAPGR-ERS, Edinburgh EH9 3JQ, 2 Scotland

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A hybrid gene was made by fusing the 2.2-kilobase 5' promoter region of a mouse group 1 major urinary protein (Mup) gene to the coding region of the herpes simplex virus type 1 thymidine kinase gene (HSV tk) and introduced into the genomes of mice by microinjection. Transgenic G0 males were sterile, or when fertile did not transmit the foreign gene, and the transgenic male descendants of G0 females were also sterile. Seven “lines” were established by breeding from G0 females and their transgenic female descendants. Six lines expressed HSV thymidine kinase activity in the liver, and activity correlated perfectly with the presence of HSV tk RNA. In three of four lines examined, expression was lower in female than in male liver, and in these lines the same sex difference was observed in the rate of run-on transcription of the foreign genes in liver nuclei. When females of one of the sexually dimorphic lines were treated with testosterone, the levels of HSV tk RNA and thymidine kinase activity were increased, although not to male levels. In these aspects of liver expression, and also in a lack of expression in seven other tissues, the hybrid gene exhibits many of the characteristics of an endogenous group 1 Mup gene. However, the gene was also expressed (at high levels) in the preputial gland and testis, two tissues in which Mup genes are not expressed. The gene, when introduced into five of the seven lines, carried a copy of the Escherichia coli supF gene attached beyond the 3' end of the HSV tk gene, but this did not affect the overall expression pattern. All of the lines were male sterile and expressed HSV thymidine kinase in the testis, but one line showed no activity in the liver, and another showed none in the preputial gland. Testicular expression is therefore the likely cause of sterility. Data are described which suggest that the causes of misexpression in the preputial gland and testis are different. Since expression in each tissue occurred in several lines, the structure of the hybrid gene must be responsible in each case. Five intensively studied lines showed at least four consistently different patterns of relative expression in preputial gland, testis, male liver, and female liver. These differences do not correlate in any way with the copy number of the foreign gene in the different lines and must be due to some other aspect of line-specific integration.

The mouse major urinary proteins (MUPs) are coded for by a family of about 35 genes clustered on chromosome 4 (3, 12, 23). Four groups of Mup genes have been identified, the largest of which are the group 1 genes and the probably inert group 2 pseudogenes (3, 12, 25, 35, 51; R. Al-Shawi, P. Ghazal, A. J. Clark, and J. O. Bishop, submitted for publication). Most group 1 genes are expressed exclusively in the liver, while a small subset are also expressed in the mammary glands of pregnant females (50, 52). Of the two smaller groups, the group 3 genes are also expressed in the liver, but under hormonal control different from the group 1 genes (35), and the group 4 genes are expressed in the lachrymal and salivary glands (50, 51).

Female mouse liver contains at least five times less MUP mRNA than male liver (23), although expression in females may be rather variable (I. McIntosh and J. O. Bishop, unpublished data). The difference in mRNA levels reflects different rates of transcription in the two sexes (18), and male levels of expression can be induced in females by administration of testosterone (14, 58). Relevant to the work presented here, the group 1 genes are regulated by circulating testosterone (14, 58). We are attempting to identify the regions in Mup genes which lead to the liver expression, and in particular the hormone responses. Because liver cell lines which synthesize MUP are unknown, we are employing transgenic mice for this purpose. In the first instance we have studied the expression of a hybrid gene which contains as a reporter the herpes simplex virus type 1 thymidine kinase gene (HSV tk) attached to the putative promoter region of the group 1 Mup gene BS6 (11, 13). The hybrid gene was expressed in the liver of transgenics, and in three of five lines it displayed a level of sexual dimorphism comparable to that of the Mup genes. Unexpectedly, it was also expressed consistently and at high levels in both preputial gland and testis. In all other tissues examined, RNA and protein products of the hybrid gene were absent, except that in one line there was expression in the lachrymal gland. Transgenic males were sterile, probably because of the presence of HSV thymidine kinase activity in the testis.

MATERIALS AND METHODS

Construction of hybrid genes. Plasmids were based on pSV2gpt (43), retaining the 341-base-pair (bp) PvuI-HindIII simian virus 40 (SV40) enhancer-early promoter region and the 2,293-bp PvuI-EcoRI portion of pBR322 containing the origin of replication and the β-lactamase gene. The SV40 enhancer in cis configuration is known to activate the BS6 promoter in transfected rodent fibroblasts, allowing us to confirm before microinjection that the construct directs the synthesis of thymidine kinase. Between the HindIII site and the EcoRI site the following fragments were inserted (an asterisk shows that the site was blunt-ended with DNA

* Corresponding author.
polymerase I: an approximately 2.2-kilobase (kb) HindIII-Sau3A1 fragment of *Mup* gene *BS6* (13); seven nucleotides of polylinker (GATCCCC); and a 1,759-bp BglII*-BsrEII* fragment of *HSV tk* from plasmid pTK1 (59). The plasmid pSV-BS6-tk-supF was completed with a 10-bp *SalI*-BamHI* fragment of the M13tg130 polylinker (31), a 400-bp AluI-XbaI fragment of PiVX containing the *Escherichia coli* *supF* gene (49), and a 36-bp XbaI-EcoRI fragment of the M13tg130 polylinker. Plasmid pSV-BS6-tk-M was completed with a 57-bp *SalI*-EcoRI fragment of the polylinker. The fragments for microinjection were prepared by digestion with HindIII and KpnI (*BS6-tk-supF*) or HindIII alone (*BS6-tk-M*), preparative agarose electrophoresis, electrophoresis, purification with Elutips (Schleicher and Schuell), phenol extraction, and repeated ethanol precipitation, and were dissolved in double-distilled water at a final concentration of 1.5 μg/ml.

**Transgenic animals and lines.** C57BL/6J × CBA F1 females were superovulated by treatment with pregnant mare’s serum and human chorionic gonadotropin (27) and mated with F1 males. About 2 pl of DNA solution was injected into the most convenient pronucleus of each egg, and eggs were incubated in culture medium overnight. Two-cell embryos were introduced into pseudopregnant MF1 foster mothers. Transgenic mice were identified by Southern blot analysis of CBA F1 males. Copy number estimates of the transgene were determined from Southern blot data.

**Assessment of fertility.** Male mice were housed with two female mice each, for a minimum of 7 weeks. Females were checked daily until vaginal plugs were seen. The fertility of an equal number of nontransgenic male siblings was tested in the same way, and all were found to be fertile.

**Thymidine kinase assay.** Thymidine kinase assays were carried out as described (6), but 0.4 mM TTP was added to inhibit endogenous thymidine kinase (29). Each determination involved equal portions of duplicate assays taken at three time intervals. The unit of thymidine kinase activity is the amount of enzyme which catalyzes the formation of 1 pmol of TMP in 1 min. Protein was determined by the Bradford method (5).

**Northern blots and run-on transcription.** RNA was prepared by a guanidinium thiocyanate method (9), and Northern blot analysis was performed as described (54) using Hybond-N (Amersham) and random-primed probe synthesis to a specific activity of approximately 10⁶ cpm/μg. Probe A, the BamHI-to-BamHI *HSV* fragment of pTK1 (59), overlaps the *HSV* portion of the two constructs completely. Probe B, an 840-bp *PstI* fragment of pTK1, overlaps 806 bp between the point of fusion with the *BS6* promoter region and a *PstI* site internal to the *tk* coding region. Marker RNA was made by run-off transcription of plasmid DNA containing a 1,922-bp EcoRI-PvuII fragment of pTK1 inserted in the polylinker region of pT7.1 (United States Biochemicals) 10 bp downstream of the T7 RNA polymerase transcription initiation site. The plasmid was digested separately with *Acel*, *Bali*, *Smal*, and *Narl*, and each fragment was transcribed in the presence of low-specific-activity [3H]UTP to generate RNA fragments 434, 1,009, 1,306, and 1,816 nucleotides long. After recovery, these were combined to make a set of markers. Run-on transcription assays were performed as described (54). The plasmid target sequences were pTK1, MUP11 (10), and LV6 (54).

**RESULTS**

We linked 2.2 kb of *5'tk* sequence to *HSV tk*, with the junction point between the *BS6* cap site and the *HSV tk* initiation codon (Fig. 1). To facilitate the rescue of the hybrid gene from the genomic DNA of transgenic mice, the *BS6-tk-M*

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**FIG. 1.** Structures of hybrid genes. The restriction site map shows key sites as follows: E, *EcoRI*; P, *PvuII*; Ps, *PstI*; H, HindIII; K, KpnI; 1, the *Sau3A1*-polylinker-*BglII* fusion between the *BS6* promoter and the *HSV tk* sequence, shown in the nucleotide sequence above; 2, the *BsrEII*-SalI fusion between the *HSV* sequence and the M13tg130 polylinker. The *BS6-tk-M* fragment was separated from SV40 and vector sequences by digestion with HindIII, and the *BS6-tk-supF* fragment was separated by digestion with HindIII and KpnI. For other details see the text.
endogenous thymidine kinase activities in different tissues
the addition of 0.4 mM TTP to the assay (29) inhibited
three G
by means of thymidine kinase assays (Table 2). Tissues of
was detected in male lachrymal gland, salivary gland.
transgenic line. No RNA that reacted with the HSV
downstream of the cap site, a phenomenon previously ob-
(0.9 kb, and the preputial gland RNA is about 1 kb in size.
Expression of the hybrid gene in liver, testis, and preputial
gland. Expression of the hybrid gene was studied by probing
Northern blots with probes specific for HSV tk and by
assaying HSV thymidine kinase activity in tissue homoge-
nates. Group 1 MUP mRNA is present in male and female
and also in small amounts in the prelactation mammary
gland, but not in other tissues (25, 50). The foreign HSV tk
mRNA and thymidine kinase activity, however, were con-
sistently present not only in male and female liver but also
in testis and in preputial glands.
The expected size of a transcript running from the BS6 cap
site to the tk polyadenylation site is 1.3 kb. An RNA of this
size was detected in male liver RNA from four of five lines,
in testis RNA of five of five lines, and in male preputial gland
RNA of four of five lines. The amount of this RNA is lowest
in liver and highest in testis (Fig. 2). Each tissue contains a
second smaller RNA that reacts with the tk probe to the
same (testis) or a lesser (liver, preputial gland) extent than
the 1.3-kb RNA. The smaller liver and testis RNAs are about
0.9 kb, and the preputial gland RNA is about 1 kb in size.
Figure 2 was obtained after hybridization with probe tk-A
(Fig. 1), which spans the entire HSV tk mRNA and the
3'-flanking region. When blots were hybridized with the
shorter probe tk-B, which corresponds to the 5' end of the
HSV tk mRNA, the signal strength of the shorter liver and
testis RNAs was less relative to the 1.3-kb RNA. This is
consistent with transcriptional initiation of these RNAs
downstream of the cap site, a phenomenon previously ob-
served in cell lines carrying integrated HSV tk genes (47).
The lengths of the smaller RNAs and their signal intensities,
relative to the 1.3-kb RNA, are consistent with each
transgenic line. No RNA that reacted with the HSV tk probe
was detected in male lachrymal gland, salivary gland,
spleen, heart, kidney, muscle, or brain.
The five transgenic lines were compared more intensively
by means of thymidine kinase assays (Table 2). Tissues of
three G0 males were also examined in this way. In our hands
the addition of 0.4 mM TTP to the assay (29) inhibited
endogenous thymidine kinase activities in different tissues

supF construct contains the bacterial amber suppressor
tRNA gene supF (19). This hybrid gene, freed from the SV40
and vector sequences, was microinjected into the pronuclei
of fertilized mouse eggs. Of 40 live-born pups, 11 were
identified as transgenic. From these, five "lines" were
established, all from G0 females (Table 1). Here a line is a
group of male and female mice descended from the same G0
female and containing the same transgenic insertion of
foreign DNA.

Expression of the hybrid gene in liver, testis, and preputial
gland. Expression of the hybrid gene was studied by probing
Northern blots with probes specific for HSV tk and by
assaying HSV thymidine kinase activity in tissue homoge-
nates. Group 1 MUP mRNA is present in male and female
and also in small amounts in the prelactation mammary
gland, but not in other tissues (25, 50). The foreign HSV tk
mRNA and thymidine kinase activity, however, were con-
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The expected size of a transcript running from the BS6 cap
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size was detected in male liver RNA from four of five lines,
in testis RNA of five of five lines, and in male preputial gland
RNA of four of five lines. The amount of this RNA is lowest
in liver and highest in testis (Fig. 2). Each tissue contains a
second smaller RNA that reacts with the tk probe to the
same (testis) or a lesser (liver, preputial gland) extent than
the 1.3-kb RNA. The smaller liver and testis RNAs are about
0.9 kb, and the preputial gland RNA is about 1 kb in size.
Figure 2 was obtained after hybridization with probe tk-A
(Fig. 1), which spans the entire HSV tk mRNA and the
3'-flanking region. When blots were hybridized with the
shorter probe tk-B, which corresponds to the 5' end of the
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The lengths of the smaller RNAs and their signal intensities,
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was detected in male lachrymal gland, salivary gland,
spleen, heart, kidney, muscle, or brain.
The five transgenic lines were compared more intensively
by means of thymidine kinase assays (Table 2). Tissues of
three G0 males were also examined in this way. In our hands
the addition of 0.4 mM TTP to the assay (29) inhibited
endogenous thymidine kinase activities in different tissues

by 93 to 97%, and the residual endogenous activity in each
tissue was quite consistent. The same amount of TTP
inhibited HSV thymidine kinase by about 50%, so that even
relatively low levels could be measured quite accurately. In
most cases in which both mRNA determinations and enzyme
activity measurements were made, the two were in broad
agreement. Exceptions were the detection of low enzyme
levels but no mRNA in line 64 female liver (see Table 4) and
line 58 lachrymal gland (Table 2). In particular, the enzyme
assays confirmed the expression of the foreign gene in the
liver of four of five lines and, at higher levels, in the preputial
glands of four of five lines and testes of all five lines (Table 3).

If the supF gene which is incorporated at the 3' end of the
hybrid gene were contributing to the expression pattern of
the gene, its usefulness would be seriously reduced. To
examine this possibility, the BS6-tk-M hybrid gene, which
lacks the supF DNA fragment (Fig. 1), was also introduced
into mice. Five transgenic G0 animals were obtained, and
two transgenic lines were established from G0 females.
Thymidine kinase assays again showed expression in liver
and higher expression in testis and preputial gland (Table 2).
The similarity of these results to those obtained with the
construct which contains supF indicates that the supF gene
does not influence the expression of this hybrid gene.

Two features of the expression of the foreign gene are line
specific. (i) Sporadic or occasional expression: we observed
thymidine kinase expression in the lachrymal gland of one of
four lines tested, and the absence of preputial gland expres-
sion in one of five lines and of liver expression in one of five
lines (Table 2). (ii) Relative levels of expression: the relative
levels of expression in different tissues are also line specific
(Table 3). With the exception of lines 58 and 78, each line
shows a different expression pattern. Line-specific features of
expression in transgenics most probably relate to the
different chromosomal integration events which characterize
the different lines.

Sexually dimorphic expression of the BS6-tk hybrid gene. In
three of five transgenic lines, male liver showed higher levels

![FIG. 2. HSV tk RNA in tissues of a transgenic mouse. Total cellular RNA from tissues of an adult male mouse of line 46 was
applied to the gel, 30 µg to each track except for 10 µg of preputial
gland RNA. The filter was hybridized with probe A and exposed for
(A) 24 h and (B and C) 3 days. B and C are two prints of the 3-day
exposure. SVEPtk, RNA from BHKtk- cells stably transformed
with a plasmid containing HSV tk linked directly to the SV40
enhancer-early promoter region; -ve liver, nontransgenic liver
RNA.]

TABLE 1. Transmission of foreign genes from
G0 to G1 transgenics

<table>
<thead>
<tr>
<th>G0 line</th>
<th>Sex</th>
<th>G0 offspring</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total</td>
</tr>
<tr>
<td>40</td>
<td>F</td>
<td>44</td>
</tr>
<tr>
<td>46</td>
<td>F</td>
<td>58</td>
</tr>
<tr>
<td>58</td>
<td>F</td>
<td>31</td>
</tr>
<tr>
<td>62</td>
<td>F</td>
<td>18</td>
</tr>
<tr>
<td>64</td>
<td>F</td>
<td>21</td>
</tr>
<tr>
<td>78</td>
<td>F</td>
<td>26</td>
</tr>
<tr>
<td>66</td>
<td>M</td>
<td>49</td>
</tr>
<tr>
<td>79</td>
<td>M</td>
<td>31</td>
</tr>
<tr>
<td>48</td>
<td>M</td>
<td>Sterile</td>
</tr>
<tr>
<td>49</td>
<td>M</td>
<td>Sterile</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>Sterile</td>
</tr>
</tbody>
</table>

* Founders of transgenic lines.
of HSV thymidine kinase activity than female liver (Table 4). The two exceptions were line 40, which shows no activity in liver of either sex, and line 64, in which male and female liver have similar activity levels. Of the three lines with sexually dimorphic HSV tk activity, the male-to-female ratio of line 46 was about 10, while the ratio in lines 58 and 78 was about 4. These ratios compare with a ratio of 5 to 10 in liver MUP mRNA levels (23; McIntosh and Bishop, in preparation).

As noted above, HSV tk RNA was present in male liver RNA of four of five lines (lines 46, 58, 64, and 78). HSV tk RNA was detected in liver of line 58 females but not in liver of 46 or 64 females, presumably because of the low levels of RNA present. (Line 78 females have not been examined.)

The different levels of MUP mRNA in male and female liver reflect different transcription rates (18). It was therefore of interest to determine by run-on transcription measurements whether the different expression levels of the transgene in males and females are also due to differential transcription. The experiments were controlled by simultaneously measuring the run-on transcription of the group 1 Mup genes and also of the apolipoprotein A1 genes, which do not show sexually dimorphic expression (T. Spiegelberg and J. O. Bishop, unpublished data). The results (Fig. 3 and Table 5) support the hypothesis that the levels of HSV thymidine kinase activity in liver of male and female transgenics reflect different rates of transcription in the two sexes. In line 40, which has no thymidine kinase activity in the liver of either sex, no HSV tk transcription was detected. In line 46, which has thymidine kinase activity in the livers of both sexes, but at a much lower level in females, transcription was detected in male but not female liver nuclei. In lines 58 and 78, which have a male/female liver thymidine kinase activity ratio of about 4, the male/female run-on transcription ratios were 6.5 and 2.5, respectively. Line 46 contains about 80 tandemly arranged copies of the hybrid gene per cell, while lines 58 and 78 contain about 3 and 1 copies, respectively (Table 3). Transcription rate, in common with enzymatic activity, is clearly independent of copy number.

The Mup gene target sequence used in these experiments is expected to react with all of the group 1 Mup gene transcripts. Our best estimate of the level of expression of BS6 is that it accounts for 25% or less of total Mup gene mRNA in male liver (McIntosh and Bishop, in preparation). By an independent estimate based on cDNA cloning frequency, an mRNA identical to BS6 mRNA has been shown to be very abundant in male liver mRNA (51). In the male transgenics the rate of run-on transcription of HSV tk is about 15% as high as that of all of the Mup genes taken together (Table 5). We recall that a proportion of the liver HSV tk mRNA is unexpectedly small in size (Fig. 2). How much of the primary transcript corresponds to this smaller mRNA is not known, and consequently a rigorous quantitative interpretation of the run-on data is not possible at this time. However, they do suggest that the rate of transcription of the hybrid gene in line 58 and line 78 liver is comparable to that of the endogenous BS6 gene.

**Testosterone induction of HSV thymidine kinase activity.** We have observed considerable variation in expression between individuals within each transgenic line (see stan-

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**TABLE 2. HSV thymidine kinase activities in tissues of male transgenic and control mice**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Transgenic (BS6-tk-supF)</th>
<th>Transgenic (BS6-tk-M)</th>
<th>Controls*: mean activity ± S.E. (no. of mice assayed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Linesb</td>
<td>G0c</td>
<td>Totald</td>
</tr>
<tr>
<td>Liver</td>
<td>4/5</td>
<td>2/3</td>
<td>6/8</td>
</tr>
<tr>
<td>Preputial gland</td>
<td>4/5</td>
<td>2/3</td>
<td>6/8</td>
</tr>
<tr>
<td>Testis</td>
<td>5/5</td>
<td>2/3</td>
<td>7/8</td>
</tr>
<tr>
<td>Lachrymal gland</td>
<td>1/3</td>
<td>0/1</td>
<td>1/4</td>
</tr>
<tr>
<td>Salivary gland</td>
<td>0/3</td>
<td>0/1</td>
<td>0/4</td>
</tr>
<tr>
<td>Brain</td>
<td>0/3</td>
<td>0/3</td>
<td>0/6</td>
</tr>
<tr>
<td>Kidney</td>
<td>0/5</td>
<td>0/2</td>
<td>0/7</td>
</tr>
<tr>
<td>Muscle</td>
<td>0/3</td>
<td>ND</td>
<td>0/3</td>
</tr>
</tbody>
</table>

*Nontransgenic littermates.
b Lines founded from G0 females; lines positive/lines tested.
c G0 males, individuals positive/individuals tested. ND, Not determined.
d One G0 male showed no activity in any tissue.
e Thymidine kinase activity, units per milligram of protein, mean of line means and G0 values (positive lines and G0 only), not weighted for numbers of determinations per line mean. Among the G0 males there are potential mosaics, which will depress the average values. Zero values were not significantly different from endogenous values measured under the same conditions.

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**TABLE 3. HSV thymidine kinase activities in male tissues of transgenic mouse lines**

<table>
<thead>
<tr>
<th>Line no.</th>
<th>Copy no.</th>
<th>Thymidine kinasea in tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Liver</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>0.05 ± 0.04 (4)</td>
</tr>
<tr>
<td>46</td>
<td>80</td>
<td>1.9 ± 0.32 (8)</td>
</tr>
<tr>
<td>58</td>
<td>3</td>
<td>5.82 ± 1.15 (3)</td>
</tr>
<tr>
<td>64</td>
<td>2</td>
<td>1.52 ± 0.71 (2)</td>
</tr>
<tr>
<td>78</td>
<td>1</td>
<td>12.73 ± 1.85 (3)</td>
</tr>
</tbody>
</table>

*a* Approximate.

**TABLE 4. Sexually dimorphic expression of HSV thymidine kinase in liver of transgenic mouse lines**

<table>
<thead>
<tr>
<th>Line</th>
<th>Enzyme activitya</th>
<th>t testb (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>40</td>
<td>0.05 ± 0.04 (4)</td>
<td>0 (3)</td>
</tr>
<tr>
<td>46</td>
<td>1.9 ± 0.32 (8)</td>
<td>0.17 ± 0.16 (8)</td>
</tr>
<tr>
<td>58</td>
<td>5.82 ± 0.15 (3)</td>
<td>1.49 ± 0.1 (2)</td>
</tr>
<tr>
<td>64</td>
<td>1.52 ± 0.71 (2)</td>
<td>2.35 ± 0.32 (2)</td>
</tr>
<tr>
<td>78</td>
<td>12.73 ± 1.85 (3)</td>
<td>2.80 ± 0.59 (3)</td>
</tr>
</tbody>
</table>

*a* Units per milligram of protein; means and standard errors. Parentheses indicate n.

b Probability that male and female samples are drawn from the same population.
dard errors in Tables 3 and 4), which could complicate the interpretation of testosterone induction experiments. For this reason the induction experiments were carried out with females of line 46, which consistently show very low HSV thymidine kinase activity in the liver (Table 4). Testosterone pellets were implanted subcutaneously 12 to 14 days before the livers were analyzed. Control transgenic litters were sham-operated at the same time. Testosterone treatment did not elicit any change in thymidine kinase activity in nontransgenic female liver. The results (Fig. 4A) clearly show that HSV thymidine kinase activity is induced by testosterone. Northern blot analysis of mRNA from induced and noninduced female liver showed induction of HSV tk RNA levels (Fig. 4B).

Liver HSV thymidine kinase activity in the induced females was on average 37% of the level in noninduced males, whereas in normal females MUP is induced to male levels (14, 58). This difference may relate to the very low HSV thymidine kinase levels in livers of noninduced line 46 females. For example, if, like the alpha2 globulin genes (36), Mup genes are repressed by estrogen, the hybrid genes in line 46 may be more sensitive to estrogen repression. Other explanations are of course possible. The results, however, do show clearly that in line 46 the expression of the hybrid gene is induced by testosterone.

Sterility of male transgenic mice that carry the BS6-tk hybrid gene. Three of five BS6-tk-supF G0 males and two of three BS6-tk-M G0 males were sterile. None of the fertile G0 males transmitted the hybrid gene to their offspring. In addition, in seven of seven transgenic lines the males were invariably sterile. Two exceptional mice, one from line 58 and one from a BS6-tk-M line, each sired a single offspring of unknown transgenic status. No further offspring were sired despite repeated mating. These mice, and also all the completely sterile mice tested, plugged females in the normal way. The female transgenics exhibited normal fertility. While no HSV thymidine kinase was detected in ovaries (data not shown), expression in the follicles themselves would be obscured by the large excess of nongerminal tissue. The transgenic males have reduced sperm counts, and most of the spermatozoa are structurally abnormal to varying degrees. Thus sperm maturation seems to be impaired rather than absolutely blocked, and this is confirmed by the two offspring sired.

DISCUSSION

Consistent tissue-specific expression and sexual dimorphism. We have shown that a 2.2-kb group 1 Mup gene promoter region and cap site consistently direct synthesis of the HSV tk reporter gene to the liver, preputial gland, and testis. Expression in the liver mirrors the expression of the endogenous group 1 Mup genes; three of four lines showed differential expression in males and females, and when females of one of the lines were treated with testosterone, elevated levels of both HSV tk RNA and thymidine kinase activity resulted. Thus many or all of the cis-acting signals necessary for correct Mup expression in the liver appear to be present within the 2.2-kb BS6 promoter region. Run-on experiments showed that the different levels of HSV tk RNA and thymidine kinase activity in males and females are due at least in part to different rates of transcription. By analogy with the mechanism of action of other steroid hormones (61), the data suggest that testosterone, through its receptor, is acting on sequences within the 2.2-kb BS6 promoter region to increase transcription. As yet it is not known whether the cis-acting signals responsible for the effects of thyroxine and growth hormone on MUP RNA synthesis in the liver (33, 54) are also present.

In the livers of lines 58 and 78, both of which carry a small number of foreign genes, the rate of HSV tk transcription

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**TABLE 5. Run-on transcription of BS6-tk and Mup genes in transgenic liver**

<table>
<thead>
<tr>
<th>Line</th>
<th>Transcription ratea</th>
<th>Thymidine kinase activityb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male (cpm/10^5 cpm)</td>
<td>Female (cpm/10^5 cpm)</td>
</tr>
<tr>
<td>40</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>46</td>
<td>0.15 (0.08)</td>
<td>0.17 (1.0)</td>
</tr>
<tr>
<td>58</td>
<td>0.13 (0.02)</td>
<td>0.08 (0.02)</td>
</tr>
<tr>
<td>78</td>
<td>0.2 (0.08)</td>
<td>2.5 (12.7)</td>
</tr>
</tbody>
</table>

a The data are based on densitometer readings of autoradiographs. Each value is the ratio of the HSV tk measurement to the Mup measurement obtained from male liver nuclei in the same experiment. Variation within and between experiments was compensated by normalizing the other measurements to the run-on value observed for apolipoprotein A1 with RNA from the same incubation of nuclei.

b Average thymidine kinase activity measurements from Table 4.

---

**FIG. 3.** Sex difference in run-on transcription rate of the BS6-tk hybrid gene in transgenic liver. M1, Transgenic male; F1, transgenic female; M2, nontransgenic male. Immobilized target sequences: tk, HSV tk; MUP, MUP11 cDNA; LPA1, apolipoprotein A1 cDNA.
seems to be comparable to that of a Mup gene. The steady-state level of HSV tk RNA, although not measured with accuracy, is known to be very much less than that of MUP RNA, implying that the stability of the HSV tk RNA is less or that its processing is defective. The low HSV tk mRNA content of liver is reflected in low levels of HSV thymidine kinase protein. In line 78 liver, the most highly expressing, the thymidine kinase (having allowed for TTP inhibition) forms about 25 pmol of TMP per min per mg of soluble liver protein. Taking the soluble protein content of a liver to be 100 mg and the specific activity of purified HSV thymidine kinase as 67 nmol of TMP formed per min per mg (8), the total amount of HSV thymidine kinase protein in the liver is about 0.04 mg. This may be compared with a MUP production rate of 10 mg per male mouse per day (58).

**Consistent misexpression.** It is frequently found that a foreign gene is correctly expressed in transgenic mice, sometimes to the extent that the timing of expression is correct (for example, see references 1, 22, 34, 40, 46, and 57). Misexpression is most often observed with hybrid genes like the BS6-tk fusion gene described here. So long as misexpression is consistent, its study can lead to insights into mechanisms of gene regulation. Also, insight into misexpression is important in relation to attempts to direct the synthesis of proteins to novel tissues for commercial purposes (21, 53).

The consistent expression of the BS6-tk gene in preputial gland and testis is unexpected, since the Mup genes are not expressed in either tissue. While the level of expression both of RNA and of thymidine kinase activity in both tissues is higher, sometimes much higher, than in liver, we have not measured transcription rates in these tissues, and the differences may be due to RNA processing or stability differences rather than to transcription rates themselves.

Consistent misexpression of foreign genes has been observed before (2, 4, 38, 42, 45, 56). If, as seems likely, it is due to changes in transcriptional behavior rather than increased RNA stability, it may be explained in one of two ways. Either (i) the hybrid gene lacks cis-acting “silencer” sequences which are required for repression of the endogenous gene in preputial gland and testis and which are located outside the 2.2-kb promoter region, or (ii) it contains newly created sequences or a new combination of sequences which activate its transcription in these tissues (56). We are presently attempting to distinguish between these alternatives.

The α2 μ-globulins, the rat homologs of the Mup genes, show a similar pattern of gene expression (37, 39). A clear difference in the expression of the rat and mouse genes, however, is that α2 μ-globulin is expressed at a high level in male and female preputial gland, while Mup is not expressed in the preputial gland (24, 39). However, an α2 μ-globulin gene is expressed as a foreign gene in the preputial glands of transgenic mice (16). One interpretation of this observation is that the natural rat gene lacks a silencer sequence which is present in the mouse genes, resulting in its expression in mouse preputial glands despite the presence in the mouse tissue of the corresponding trans-acting repressor protein (16). The BS6-tk hybrid gene was consistently expressed not only in male and female preputial glands but also in testis. If misexpression in preputial gland and testis were due to the same cause, we might expect there to be α2 μ-globulin expression in rat testis. However, no α2 μ-globulin mRNA could be detected by Northern blotting (data not shown). Appropriate controls showed that the testis RNA used in this experiment was not degraded. This observation suggests either that the misexpression of the hybrid Mup gene in preputial gland and testis has different causes, or else, if there is a common cause, that it is independent of the causes of α2 μ-globulin expression in rat and mouse preputial glands. Two observations suggest that different causes bring about the misexpression of the hybrid gene in the preputial gland and testis. First, expression in the testis occurs in a transgenic line with no preputial gland expression. Second, the smaller preputial gland and testis RNAs differ from each other in size and in their hybridization to different probes.

Despite some variability between mice within each transgenic line, the pattern of expression levels in liver, preputial gland, and testis is line specific. Line-specific effects are assumed to be due to the uniqueness of different chromosomal integration events. These events differ in several ways. Different sites may contain different numbers and different arrangements (head-to-head, head-to-tail, tail-to-tail) and rearrangements of the integrated foreign genes. In addition, flanking DNA sequences at different chromosomal sites of integration are thought possibly to influence transcription of the foreign genes through “position effects.” In the present case no correlation has been detected between copy number and expression in any tissue (Table 3), a common observation in transgenics (17, 44). The question of whether the different patterns of expression are due to arrangements, rearrangements, or position effects has not been addressed. Lines 58, 64, and 78, all of which contain a small number of integrated gene copies, provide convenient models with which to distinguish these causes.

**Transgenic male sterility is due to HSV thymidine kinase activity in the testis.** All of the established lines show both consistent male sterility and a high level of HSV thymidine kinase in the testis. In contrast, line 40 shows no liver HSV thymidine kinase activity and line 64 shows no preputial gland HSV thymidine kinase activity, although both lines are consistently male sterile. Thus testis expression of HSV thymidine kinase is the likely cause of male sterility, and both liver and preputial gland expression can be ruled out as the sole cause.

Minor exceptions to the sterility of transgenic males were observed. First, three G0 males (two carrying BS6-tk-supF and one with BS6-tk-M) sired offspring, none of which were transgenic. G1 male 79 had no testis HSV thymidine kinase activity, and indeed showed no thymidine kinase activity in any tissue. This male presumably carried transcriptionally inert copies of the hybrid gene and in addition was a mosaic with nontransgenic germ cells. G0 male 66 had a very low level of testis HSV thymidine kinase activity (60% over the endogenous level, after discounting TTP inhibition). This male may be explained in either of two ways. He may have had a mosaic germ line, with the implication that germ cells carrying the transgene were ineffectual or possibly very few in number. Alternatively, he may have had a nontransgenic germ line, in which case the gene is inactive in the somatic tissue. Expression in the third G1 male, which carries BS6-tk-M, has not yet been examined. Secondly, 2 G1 transgenic males, out of 21 G1 and G2 males tested, each sired a single offspring, but although repeatedly mated thereafter failed to sire more. This shows that the sterile males form some functional sperm. The mice are oligospermic rather than aspermic.

Some lines of mice transgenic for HSV tk linked to the mouse mammary tumor virus long terminal repeat express HSV thymidine kinase in the testis (48) and are also sterile (S. Ross and D. Solter, personal communication). However, at least one fertile mouse line which expresses HSV thymidine kinase in the testis has been reported (32). In this case
the HSV tk gene was under the control of its natural promoter. Thus expression of HSV thymidine kinase in the testis does not invariably lead to sterility. One possible explanation is that lower expression levels do not cause sterility; the HSV thymidine kinase activity level in the fertile line is about 3 times the endogenous level (32), whereas the lowest level among our sterile lines (line 58) is about 12 times the endogenous level. However, since the testis contains several cell types with different functions and since sperm differentiation is a slow process accompanied by changes in both the germ cells and accessory cells (60), an alternative explanation is that sterility is linked to expression in particular cell types or at particular developmental stages. This explanation is especially plausible because HSV tk expression is under the control of different promoters in the different experiments. Furthermore, the expression of the mouse mammary tumor virus tk hybrid gene which confers sterility may, like that of BS6-tk, be induced by testosterone (7, 15).

Given that sterility is due to HSV thymidine kinase expression in one or more testis cell types, there are several ways in which the enzyme could have its effect. First, its substrate specificity is different from that of the cellular enzyme, and it efficiently phosphorylates deoxyctydine (28). Second, the high levels of phosphorylated deoxynucleosides produced by high levels of the enzyme will inhibit the normal pathway of pyrimidine phosphate metabolism (30). Third, HSV thymidine kinase may be active at times in the cell cycle when the cellular enzyme is normally repressed (26, 41, 55). Any of these could perturb cellular metabolism, primarily of nucleic acid precursors, but with secondary effects on related pathways. Transgenic mice expressing a mutant form of dihydrofolate reductase, which is also involved in pyrimidine metabolism, show reduced growth, reduced female fertility, and morphological defects (20).

ACKNOWLEDGMENTS

We thank Neil Wilkie for providing pTK1 and BHK cells; David Firtel for PIVX; Sam Martin for implanting female mice with testosterone pellets; Sheila Barton and Azim Surani for advice on oviduct transfers; and Anne Walker, Roberta Wallace, Melville Richardson, Ann Duncan, Helen McIlroy, and Garry Brown for their assistance.

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LITERATURE CITED


10. Clark, A. J., A. Chave-Cox, X. Ma, and J. O. Bishop. 1985. Analysis of mouse major urinary protein genes: variation between the exonic sequences of Group I genes and a comparison with an active gene outwith Group I both suggest that gene conversion has occurred between MUP genes. EMBO J. 4:3167-3171.


Expression of a Foreign Gene in a Line of Transgenic Mice Is Modulated by a Chromosomal Position Effect

RAYA AL-SHAWL, JANE KINNAIRD, JOANNE BURKE, AND JOHN O. BISHOP

Department of Genetics and Animal Genome Research, University of Edinburgh, Edinburgh EH9 3JN, Scotland

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Unusual aberrant expression of a foreign gene in a particular transgenic mouse line is often attributed to chromosomal position effect, although proof of this is lacking. An alternative explanation is that expression has been modified by the arrangement of multiple copies of the foreign gene at the insertion site or by mutation or gene rearrangement. We have distinguished between these explanations in the case of one particular transgenic line by recovering the aberrantly expressed foreign DNA and reintroducing it into the mouse genome to produce secondary transgenic mice. The expression pattern of the gene in the secondary transgenic mice was normal, showing that this case of aberrant expression is due to a chromosomal position effect.

To date, most transgenic mice have been produced by embryo pronuclear microinjection. As far as is known, the foreign DNA is integrated into the chromosomes at random. Integration usually occurs at only one chromosomal site in a given embryo, but the number of copies of the input DNA that become integrated varies from one to hundreds (see, for example, Lacy et al. [2]). When integration occurs at more than one site, the different sites can be segregated from each other by mating the founder (G0) animal with nontransgenic mice. A set of related mice all of which carry the same configuration of foreign DNA at the same integration site is a transgenic line.

Several studies have demonstrated that in transgenic mice derived from different microinjected embryos, the same foreign gene is expressed to different degrees. Although the number of copies of the foreign gene varies greatly from one integration site to another, there is no general relationship between copy number and level of expression (9, 17, 23). When the foreign gene is expressed in more than one tissue, variation in expression may confer on the mice different patterns of tissue-specific expression.

Because there is considerable variation in expression between individual mice, comparisons are most convincing when transgenic lines are established. By measuring expression levels in several individuals of a line, environmental variation and variation due to genetic background is canceled out. We previously reported results obtained in this way (1). The DNA fragment that we introduced into the mouse genome consists of a 2.2-kilobase-pair (kb) major urinary protein (MUP) gene promoter and 5'-flanking region linked to the herpesvirus 1 (HSV) thymidine kinase (TK) gene coding region. At its 3' end, beyond the HSV TK gene polyadenylation site, the DNA fragment carries a copy of the bacterial SupF gene. In four of five transgenic lines, HSV TK activity and HSV TK mRNA are present in the liver, the tissue in which the endogenous MUP gene promoter is active. However, four lines have high levels of HSV TK activity in the preputial gland, and all five have high levels in the testis. Three of the lines are of particular value because each has a small number of foreign DNA sequences at the insertion site (1, 2, and 3 copies respectively). One of these lines (line 64) has negligible HSV TK activity in the preputial gland. The expression pattern of line 64 mice has been the same for four generations of outcrossing. Here we report experiments exploring the cause of the aberrant preputial gland expression in line 64.

The foreign DNA inserted into the chromosome of a transgenic line is the result of a unique set of molecular events that occur in the pronucleus after microinjection. A small set of foreign DNA molecules (effectively two in the case of line 64) is joined together and becomes integrated into a site on a single chromosome. In principle, unusual expression of the foreign DNA may be due to either of two sorts of effects. One class of possible effects relates to the foreign DNA itself; these include point mutation, damage due to breakage and rejoining, and effects due to the proximity and juxtaposition of more than one copy of the foreign DNA fragment. A second class of possible effects relates to the chromosomal environment into which the DNA has been inserted; regions of the same chromosome, which may be close to or far from the insertion site, may modulate the expression of the gene, perhaps in the way that enhancers modulate promoter activity or possibly in other ways. This second class has been called chromosomal position effect.

To distinguish between the two classes of effects, one a property of the foreign DNA itself and the other a property of the chromosome in the neighborhood of the foreign DNA, we recovered the foreign genes from line 64 DNA by cleavage and transgenic mice. If, on the other hand, the unusual expression is due to a chromosomal position effect, then the expression pattern of the secondary transgenic mice should be the same, in general, as that of primary transgenic mice, i.e., with expression occurring in the preputial glands in most cases. The results show that the lack of expression in

<table>
<thead>
<tr>
<th>Gland</th>
<th>Liver</th>
</tr>
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<tbody>
<tr>
<td>0.10</td>
<td>0.02 ± 0.004</td>
</tr>
<tr>
<td>0.39</td>
<td>0.05 ± 0.04</td>
</tr>
<tr>
<td>0.92</td>
<td>1.9 ± 0.32</td>
</tr>
<tr>
<td>0.6</td>
<td>5.8 ± 1.15</td>
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<tr>
<td>0.3</td>
<td>12.7 ± 1.85</td>
</tr>
<tr>
<td>0.17</td>
<td>1.3 ± 0.23</td>
</tr>
</tbody>
</table>

* Corresponding author.
† Present address: Department of Biological Sciences, UMBC, Baltimore, MD 21228.
TABLE 2. Expression of the BS6 TK-SupF hybrid gene in tissues of second-generation secondary transgenic male mouse lines

<table>
<thead>
<tr>
<th>G1 no.</th>
<th>TK activity (pmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testis</td>
</tr>
<tr>
<td>A49.1</td>
<td>121</td>
</tr>
<tr>
<td>B65.2</td>
<td>387</td>
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<tr>
<td>B56.5</td>
<td>0.74</td>
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<td>B56.11</td>
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</tr>
<tr>
<td>B56.9</td>
<td>3.74</td>
</tr>
</tbody>
</table>

secondary transgenic mice, the levels of expression in the three tissues varied independently of each other. TK activity levels were determined in five other tissues, lachrymal glands, submaxillary gland, brain, skeletal muscle, and kidneys. As in the case of the primary transgenic mice (1), the TK activities in these tissues were very low and indistinguishable from those observed with control nontransgenic mice (data not shown).

Structures of the foreign DNA in the secondary transgenic mice. As an alternative, the loss and subsequent recovery of preputial gland expression might be explained by DNA rearrangement. If the particular arrangement of the genes in line 64 caused the loss of expression, for example by interactions between the two genes, then expression might be recovered in the secondary transgenic mice if the genes became rearranged in such a way as to reverse the original effect.

It is inherently unlikely that an appropriate rearrangement would have occurred in all of the nine secondary transgenic mice examined (Tables 1 and 2). However, it is necessary to show that this DNA fragment does not undergo a consistent rearrangement and also to demonstrate that the secondary transgenic mice carry the intended DNA fragment. The organization of the DNA in several secondary transgenic mice was therefore examined by Southern blot analysis, using as a probe a fragment of the HSV TK gene. Digestion with SsrI released from line 64 DNA four fragments that hybridized with the probe. Two of these were 2.2 kb in size, and the others were 2.6 and 2.8 kb. Digestion with BglII released probe-positive fragments of 4.9 and 5.5 kb (Fig. 3A). The presence of SsrI or BglII fragments of other sizes would indicate that rearrangements had occurred, the most likely of which is a junction between the foreign DNA and the chromosomal DNA of the secondary transgenic animal.

The DNA of four G0 secondary transgenic mice was examined after separate digestion with SsrI and BglII. In each case the predominant hybridizing bands were the expected bands (Fig. 3A), showing that most of the foreign DNA fragments were not rearranged in a way that disturbed these patterns. The BglII digests of DNA samples A17 and B27 each showed an additional faint hybridizing band (Fig. 3B). These bands probably represent junction fragments between chromosomal DNA and the foreign DNA insert.

The lambda 2.1 recombinant contains a single HindIII site. If the DNA in the secondary transgenic lines takes the usual form of a direct tandem array, digestion with HindIII will produce mainly unit-size HSV TK-positive fragments. In addition, each insertional array will have a flanking junctional fragment of a different length at each end. In most cases, such fragments may be expected to hybridize with the probe. The same is true of lambda 1.2, which has two HindIII sites close together, except that the predominant fragment will be shorter than unit length by an amount equal to the 0.8-kb length of DNA between the two HindIII sites. The DNA of three secondary transgenic mice was examined after HindIII digestion. In each case, the expected band was present (Fig. 3B and data not shown).

Male descendants of secondary transgenic females. The five primary transgenic lines that carry the BS6 TK-SupF hybrid gene are all male sterile, and the founder in each case was a transgenic female (1). All of the male transgenic mice in each of the primary lines inherit their foreign genes from the mother, and it is therefore possible that the foreign gene insert in line 64 is genetically imprinted (15, 25, 29, 37) during female germ line maturation. If so, the property of being imprinted must be peculiar to the insertion of foreign DNA in line 64, since the foreign gene is expressed in the preputial glands of the other primary transgenic lines. Imprinting, if it occurs, might be due either to a chromosomal position effect or to the arrangement of the foreign DNA at the insertion site. In either case, the secondary transgenic G0 males would have expected TK activity levels that were much the same as the male mice of the founder line (1). The expression of HSV TK in these three lines each of which contains 10 or more copies of the foreign gene chromosomal insertions in lines. The map of the line 64 insertion is based on (Fig. 2); the others are based on DNA blotting patterns. Symbols: continuous line, MUP BS6 gene promoter; open box, MUP BS6 gene promoter below line, HSV TK structural gene; filled box sequence; open-ended box, uncertainty about the position of the probe; vertical wavy line, shortening of the map. Data not shown.

Expression of HSV TK in these three lines each of which contains 10 or more copies of the foreign DNA by molecular cloning. Studies have used the bacterial SupF gene to introduce foreign DNA from transfected (33) BglII digests. Lanes: 1, lambda 2.1 × SsrI (five-copy marker); 2, A6 × SsrI; 3, A6 × BglII; 4, A17 × SsrI; 5, A17 × BglII; 6, B27 × SsrI; 7, B27 × BglII; 8, B50 × SsrI; 9, B50 × BglII; 10, lambda 2.1 × BglII (five-copy marker); 11, lambda 2.1 × Smal (five-copy marker); 12, kilobase size ladder (Bethesda Research Laboratories). (B) Lanes: 1, A17 × SsrI; 2, A17 × BglII; 3, A17 × HindIII; 4, B27 × SsrI; 5, B27 × BglII.
Expression of a Foreign Gene in a Line of Transgenic Mice Is Modulated by a Chromosomal Position Effect

RAYA AL-SHAWI, JANE KINNAIRD, JOANNE BURKE, AND JOHN O. BISHOP†**

Department of Genetics and AFRC Centre for Animal Genome Research, University of Edinburgh, Edinburgh EH9 3JN, Scotland

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To distinguish between the two classes of effects, one a property of the foreign DNA itself and the other a property of the chromosome in the neighborhood of the foreign DNA, we recovered the foreign genes from line 64 DNA by molecular cloning and reintroduced them by embryonic pronuclear microinjection into the genome of mice. We refer to first cycle transgenic animals such as line 64 mice as primary transgenic mice and to transgenic mice produced in the second cycle as secondary transgenic mice. If the arrangement of the DNA at the insertion site of the primary transgenic animal is the cause of its unusual expression, we would expect to observe the same expression pattern, i.e., a lack of expression in the preputial glands, in the secondary transgenic mice. If, on the other hand, the unusual expression is due to a chromosomal position effect, then the expression pattern of the secondary transgenic mice should be the same, in general, as that of primary transgenic mice, i.e., with expression occurring in the preputial glands in most cases. The results show that the lack of expression in
the preputial glands of line 64 mice is due to a chromosomal position effect.

MATERIALS AND METHODS

Transgenic mice and TK assays. Transgenic mice were prepared by pronuclear microinjection as described previously (1). The same report contains a description of the TK assay procedure. Transgenic mice were routinely identified by the polymerase chain reaction, using tail DNA as the template and primers specific for the SupF gene.

Recovery of the foreign gene insertion from line 64 DNA. High-molecular-weight DNA was prepared from a crude preparation of liver nuclei by standard sodium dodecyl sulfate-protease K extraction and deproteinization. A 200-μg sample of DNA was partially digested with MboI and fractionated on a 10 to 40% sucrose density gradient. Fractions containing 15- to 25-kb fragments were identified by agarose gel electrophoresis, pooled, and used for library construction. DNA from the bacteriophage lambda vector EMBL3A (11) was digested with BamHI, the DNA was recovered and annealed, and the vector arms were isolated by gradient centrifugation. Each ligation was performed with 1 μg of vector arms and 0.2 μg of fractionated line 64 DNA. In vitro packaging with Gigapack Plus extract (Stratagene) gave a yield of 3.8 × 10^6 plaques per μg of mouse DNA when assayed on a SupF+ Escherichia coli host (ED8654 [3]). When plated on a Sup0 host (MC1061 [5]), the number of viable plaques obtained was lower by a factor of approximately 2.5 × 10^2. Of 12 plaques, 8 reacted with an HSV TK gene probe. In vitro packaging with Gigapack Plus extract (Stratagene) gave a yield of 3.8 × 10^6 plaques per μg of mouse DNA when assayed on a SupF+ Escherichia coli host (ED8654 [3]). When plated on a Sup0 host (MC1061 [5]), the number of viable plaques obtained was lower by a factor of approximately 2.5 × 10^2. Of 12 plaques, 8 reacted with an HSV TK gene probe.

DNA blotting procedures. To map restriction enzyme sites to genomic DNA, 10-μg samples were digested appropriately and run on 0.8 or 0.5% agarose gels. The DNA was depurinated and transferred to Hybond-N (Amersham Corp.) and fixed by UV cross-linking. HSV TK-specific bands were detected by using the PstI fragment of the HSV TK gene, labeled with 32P to a specific activity of 10 to 20, and the hybridization method of Church and Gilbert (6).

Recombinant clones were mapped by using probes specific for the TK HSV gene, the MUP BS6 gene promoter fragment, and the SupF gene. Transfers were by the alkaline blotting method of Reed and Mann (24), and hybridization was in 1× SSC (0.15 M NaCl, 0.015 M sodium citrate) containing 0.3% low-fat dried milk and 50 μg of denatured salmon sperm DNA per ml.

RESULTS

Structure of the foreign DNA and patterns of tissue-specific expression in lines 58, 64, and 78. The structures of the chromosomal insertions of foreign DNA in the primary transgenic lines 58, 64, and 78 are summarized in Fig 1. The structure of the line 64 insertion was determined by mapping bacteriophage lambda clones (see below). Those of the line 58 and line 78 insertions were deduced from Southern blots of genomic DNA (data not shown). Line 64 contains two copies of the foreign DNA in direct tandem arrangement. The two copies are separated by a short (about 500 base pairs) intervening DNA sequence of unknown origin. The two copies and the flanking regions of chromosomal DNA are contained within a 17.5-kb HindIII fragment. Line 58 contains three copies of the foreign DNA in direct tandem arrangement, with short terminal deletions at the junction points between fragments. All three copies are contained within a 20-kb HindIII fragment. Line 78 contains about 1.3 copies of the foreign DNA, again in direct tandem arrangement and with a short deletion at the junction between the nearly complete 5' copy and the truncated 3' copy. Two Smal fragments with a combined length of 17.5 kb contain the line 78 insert and extensive flanking regions. From these results it is clear that each line carries a small number of foreign genes in a unique arrangement.

The pattern of expression of HSV TK in these three lines and two others, each of which contains 10 or more copies of the gene, has been described in some detail (1). The expression observed in the liver in each case (Table 1) was expected in that the resident MUP gene promoters are active in liver. Expression was also observed in two tissues in which the resident MUP gene promoters are not active, namely, the testes and preputial glands. Expression in the preputial gland appears to be a function of the MUP gene promoter, since it has been observed in other transgenic mice carrying different MUP gene constructs (19). Of the five genes originally described, four express the hybrid gene in the preputial glands and all five express it in the testis (1). In fact, HSV TK activity in preputial glands of line 64 mice was above the background assay level observed with nontransgenic preputial glands (Table 1). However, the activity was respectively 20 and 60 times less than the activities present in line 58 and line 78 preputial glands. The liver activity level in line 64 mice was also lower than activities in lines 58 and 78. The levels of testis activity were much the same in the three lines.

Recovery of the line 64 foreign DNA by molecular cloning. Several previous studies have used the bacterial SupF gene to facilitate recovery of foreign DNA from transfected (33) and retrovirus-infected (26, 32) cells, and the principle of the method is well known (30). We have routinely included a 3'-terminal SupF gene in foreign DNA introduced into the mouse germ line and have presented evidence that in at least one case it does not influence expression of the gene with which it is associated (1).

Clones carrying the SupF gene and positive to a HSV TK gene probe were isolated from a genomic library of line 64 DNA constructed in the lambda vector EMBL3A (see Materials and Methods). The restriction sites of the cloned fragments were congruent with each other and with a restriction site map of the relevant region of line 64 genomic DNA, showing that the DNA had not become rearranged during

FIG. 1. Structures of the foreign gene chromosomal insertions in primary transgenic lines. The map of the line 64 insertion is based on cloned sequences (Fig. 2); the others are based on DNA blotting data. The lengths shown are in kilobases. Symbols: continuous line, mouse chromosomal DNA; open box, MUP BS6 gene promoter region; filled box below line, HSV TK structural gene; filled box above line, SupF sequence; open-ended box, uncertainty about the precise junction point; vertical wavy line, shortening of the map. Restriction enzyme sites: H, HindIII; M, Smal.
One clone (not shown) had the structure expected to result from a reciprocal recombination event between nonidentical copies of the foreign DNA during bacteriophage multiplication; that is, it contained a single copy of the foreign DNA sequence flanked by the same regions that flanked the tandem gene pair in the other clones. Two clones were used rather than one because a single clone might have suffered a mutation during recloning and growth in the E. coli host. If the two clones behave in the same way in the secondary transgenic mice, this may be assumed not to be the case. Both clones contained the entire (9 kb) tandem insertion and similar amounts of flanking chromosomal DNA (Fig. 2). In each case, the entire cloned fragment, consisting of the tandem insert and the flanking DNA, could be separated from the vector arms by digestion with SalI, which cleaves the small polylinker sequences of EMBL3A but does not cleave either cloned fragment. The fragments were freed from vector arms by agarose gel electrophoresis and introduced into mouse embryos by direct pronuclear microinjection.

An analysis of HSV TK activity in tissues of six G0 males is summarized in Table 1. Three of these mice were fertile but did not father transgenic offspring, and the others were sterile. Both types of reproductive behavior are characteristic of primary G0 transgenic males containing the BS6 TK-SupF hybrid gene; of five primary G0 males, two were fertile but nontransmitting and three were sterile (1). Fertile nontransmitting G0 males are mosaics, and males of the five established lines are sterile (1). HSV TK activity was present in the preputial glands of all six males (Table 1). The activity levels in different G0 males were highly variable, but the highest value (male A45) was greater than the highest value observed among the primary transgenic lines (Table 1 and reference 1). Furthermore, the distribution of values did not overlap that of seven line 64 males. The significance of this is the greater since the secondary transgenic male with the lowest preputial gland expression is a mosaic. Thus, the negligible enzyme activity in the preputial glands of line 64 mice was not preserved when the tandem gene pair was recovered and introduced into secondary transgenic animals. It may therefore be concluded that the very low preputial gland activity is due to the chromosomal environment around the insertion site in line 64, in other words, to chromosomal position effect. Since the cloned DNA contained the regions immediately flanking the line 64 foreign DNA, the position effect presumably originates from more distant regions of the chromosome.

The activity in the liver and testis of the secondary transgenic G0 males was also variable (Table 1). In this respect, the secondary transgenic lines resembled the five primary transgenic lines. Interestingly, in both primary and

### Table 1. Expression of the BS6 TK-SupF hybrid gene in tissues of primary and secondary transgenic male mice

<table>
<thead>
<tr>
<th>Group</th>
<th>Testis</th>
<th>Preputial gland</th>
<th>Liver</th>
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<tr>
<td>Control</td>
<td>0.25 ± 0.08 (6)</td>
<td>0.23 ± 0.10 (7)</td>
<td>0.02 ± 0.004 (7)</td>
</tr>
<tr>
<td>Primary transgenic line</td>
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</tr>
<tr>
<td>40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>140 ± 6.7 (3)</td>
<td>12.0 ± 0.39 (2)</td>
<td>0.05 ± 0.04 (4)</td>
</tr>
<tr>
<td>46&lt;sup&gt;b&lt;/sup&gt;</td>
<td>139 ± 17.6 (3)</td>
<td>83.1 ± 19.9 (2)</td>
<td>1.9 ± 0.32 (8)</td>
</tr>
<tr>
<td>58&lt;sup&gt;b&lt;/sup&gt;</td>
<td>31.1 ± 10.5 (3)</td>
<td>9.9 ± 2.6 (3)</td>
<td>5.8 ± 1.15 (3)</td>
</tr>
<tr>
<td>78&lt;sup&gt;b&lt;/sup&gt;</td>
<td>62.4 ± 8.4 (3)</td>
<td>32.1 ± 5.3 (3)</td>
<td>12.7 ± 1.85 (3)</td>
</tr>
<tr>
<td>64</td>
<td>40.8 ± 2.9 (7)</td>
<td>0.65 ± 0.17 (7)</td>
<td>1.3 ± 0.23 (7)</td>
</tr>
<tr>
<td>Secondary transgenic mouse nos.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(cloned line 64 insert reintroduced)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A6&lt;sup&gt;c&lt;/sup&gt; (64.1.2)</td>
<td>36.7</td>
<td>17.6</td>
<td>0.01</td>
</tr>
<tr>
<td>A17&lt;sup&gt;c&lt;/sup&gt; (64.1.2)</td>
<td>24.9</td>
<td>47.9</td>
<td>3.14</td>
</tr>
<tr>
<td>A45 (64.1.2)</td>
<td>330</td>
<td>196</td>
<td>19.6</td>
</tr>
<tr>
<td>A60 (64.1.2)</td>
<td>38.8</td>
<td>80.3</td>
<td>2.26</td>
</tr>
<tr>
<td>B27 (64.2.1)</td>
<td>50.6</td>
<td>7.8</td>
<td>0.42</td>
</tr>
<tr>
<td>B50&lt;sup&gt;c&lt;/sup&gt; (64.2.1)</td>
<td>5.6</td>
<td>1.4</td>
<td>1.20</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± standard error (number of observations).

<sup>b</sup> From Al-Shawi et al. (1).

<sup>c</sup> Fertile nontransmitting male; other males were sterile.

---

**FIG. 2.** (A) Cloned segments of line 64 DNA. Symbols: continuous line, mouse chromosomal DNA; open box, MUP BS6 gene promoter region; filled box below line, HSV TK structural gene; filled box above line, SupF sequence. Restriction enzyme sites: B, BamHI; H, HindIII; T, SstI; M, Smal; L, BglII. The precise junction points of the two foreign DNA fragments with the chromosome and with the extra fragment of DNA between them have not been precisely mapped. (B) Length of sequence present in six lambda EMBL3A recombinants.
TABLE 2. Expression of the BS6 TK-SupF hybrid gene in tissues of second-generation secondary transgenic male mouse lines

<table>
<thead>
<tr>
<th>(G_0) no.</th>
<th>TK activity (pmol/min per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Testis</td>
</tr>
<tr>
<td>A49.1</td>
<td>121</td>
</tr>
<tr>
<td>B65.2</td>
<td>387</td>
</tr>
<tr>
<td>B56.5</td>
<td>0.74</td>
</tr>
<tr>
<td>B56.11</td>
<td>0.82</td>
</tr>
<tr>
<td>B56.9</td>
<td>3.74</td>
</tr>
</tbody>
</table>

secondary transgenic mice, the levels of expression in the three tissues varied independently of each other. TK activity levels were determined in five other tissues, lachrymal gland, submaxillary gland, brain, skeletal muscle, and kidneys. As in the case of the primary transgenic mice (1), the TK activities in these tissues were very low and indistinguishable from those observed with control nontransgenic mice (data not shown).

Structures of the foreign DNA in the secondary transgenic mice. As an alternative, the loss and subsequent recovery of peptual gland expression might be explained by DNA rearrangement. If the particular arrangement of the genes in line 64 caused the loss of expression, for example by interactions between the two genes, then expression might be recovered in the secondary transgenic mice if the genes became rearranged in such a way as to reverse the original effect. It is inherently unlikely that an appropriate rearrangement would have occurred in all of the nine secondary transgenic mice examined (Tables 1 and 2). However, it is necessary to show that this DNA fragment does not undergo a consistent rearrangement and also to demonstrate that the secondary transgenic mice carry the intended DNA fragment. The organization of the DNA in several secondary transgenic mice was therefore examined by Southern blot analysis, using as a probe a fragment of the HSV TK gene. Digestion with \(SstI\) released from line 64 DNA four fragments that hybridized with the probe. Two of these were 2.2 kb in size, and the others were 2.6 and 2.8 kb. Digestion with \(BglII\) released probe-positive fragments of 4.9 and 5.5 kb (Fig. 3A). The presence of \(SstI\) or \(BglII\) fragments of other sizes would indicate that rearrangements had occurred, the most likely of which is a junction between the foreign DNA and the chromosomal DNA of the secondary transgenic animal. The DNA of four \(G_0\) secondary transgenic mice was examined after separate digestion with \(SstI\) and \(BglII\). In each case the predominant hybridizing bands were the expected bands (Fig. 3A), showing that most of the foreign DNA fragments were not rearranged in a way that disturbed these patterns. The \(BglII\) digests of DNA samples A17 and B27 each showed an additional faint hybridizing band (Fig. 3B). These bands probably represent junction fragments between chromosomal DNA and the foreign DNA insert.

The lambda 2.1 recombinant contains a single \(HindIII\) site. If the DNA in the secondary transgenic lines takes the usual form of a direct tandem array, digestion with \(HindIII\) will produce mainly unit-size HSV TK-positive fragments. In addition, each insertion array will have a flanking junctional fragment of a different length at each end. In most cases, such fragments may be expected to hybridize with the probe. The same is true of lambda 1.2, which has two \(HindIII\) sites close together, except that the predominant fragment will be shorter than unit length by an amount equal to the 0.8-kb length of DNA between the two \(HindIII\) sites. The DNA of three secondary transgenic mice was examined after \(HindIII\) digestion. In each case, the expected band was present (Fig. 3B and data not shown).

Male descendants of secondary transgenic females. The five primary transgenic lines that carry the BS6 TK-SupF hybrid gene are all male sterile, and the founder in each case was a transgenic female (1). All of the male transgenic mice in each of the primary lines inherit their foreign genes from the mother, and it is therefore possible that the foreign gene insert in line 64 is genetically imprinted (15, 25, 29, 37) during female germ line maturation. If so, the property of being imprinted must be peculiar to the insertion of foreign DNA in line 64, since the foreign gene is expressed in the preputial glands of the other primary transgenic lines. Imprinting, if it occurs, might be due either to a chromosomal position effect or to the arrangement of the foreign DNA at the insertion site. In either case, the secondary transgenic \(G_0\) males would

FIG. 3. Southern blot analysis of \(G_0\) secondary transgenic mice. (A) \(SstI\) and \(BglII\) digests. Lanes: 1, lambda 2.1 \(\times\) \(SstI\) (five-copy marker); 2, A6 \(\times\) \(SstI\); 3, A6 \(\times\) \(BglII\); 4, A17 \(\times\) \(SstI\); 5, A17 \(\times\) \(BglII\); 6, B27 \(\times\) \(SstI\); 7, B27 \(\times\) \(BglII\); 8, B50 \(\times\) \(SstI\); 9, B50 \(\times\) \(BglII\); 10, lambda 2.1 \(\times\) \(BglII\) (five-copy marker); 11, lambda 2.1 \(\times\) \(SmaI\) (five-copy marker); 12, kilobase size ladder (Bethesda Research Laboratories). (B) Lanes: 1, A17 \(\times\) \(SstI\); 2, A17 \(\times\) \(BglII\); 3, A17 \(\times\) \(HindIII\); 4, B27 \(\times\) \(SstI\); 5, B27 \(\times\) \(BglII\).
not show the imprinted phenotype (i.e., the same phenotype as line 64) because the foreign genes that they carry have not passed through the female (or any) germ line since they were recovered by molecular cloning. Thus, up to this point we have not excluded the possibility that negligible expression in the preputial gland is due to a gene arrangement or to some other alteration that invites imprinting.

This possibility was tested by analyzing the progeny of three secondary G0 transgenic females. Three transgenic male descendants of B36 and also transgenic male descendants of A49 and B65 were analyzed (Table 2). As was the case with two of the G0 males, expression in the preputial glands of all of these males was high. This experiment excludes the possibility that the low preputial gland expression in line 64 is related to a genomic imprinting effect dependent on the structure of the foreign gene or to the immediate chromosomal environment. It remains possible that the chromosomal position effect that we have identified acts through imprinting, but this does not affect the central conclusion that it is a position effect.

**DISCUSSION**

When a foreign DNA fragment is introduced by embryo pronuclear microinjection, the chromosomal integration sites usually contain many copies of the fragment. These are generally arranged in a direct tandem array (other arrangements are found infrequently), and the arrays probably arise by homologous recombination between different identical copies of the foreign DNA fragment. The process of integration is essentially stochastic and cannot be controlled. Consequently, any set of transgenic mice made by introducing the same foreign DNA fragment is inevitably made up of individuals that carry different numbers of fragments at the chromosomal integration site (or at each site if there are more than one). It is generally found that the level of expression of the foreign gene is independent of its copy number. Underlying this, each individual chromosomal integration complex is associated with a different level of expression. As we (1) and others (9, 39) have shown by comparing transgenic lines, the expression level is a heritable property of the integration complex.

Integration complexes differ in a number of ways. First, there is the copy number referred to above. Then, although most arrays of foreign DNA consist of direct tandem repeats, some contain other arrangements such as inverted repeats and different sorts of fusions between internal parts of two copies of the foreign DNA (rearrangements). The junctions between the array and the chromosome generally involve sequences internal to the foreign DNA (7, 8, 40). Arrays may contain fragments of DNA from elsewhere in the genome or from unknown sources (40). Finally, the DNA may in principle be damaged by nucleotide substitutions or small deletions. Any or all of these changes may affect expression in different cases, and changes that affect the expression of one gene may not affect another. Copy-number-dependent expression in erythroid fetal liver and in T cells has been observed in transgenic mice carrying particular chromosomal regions associated with the beta-globin and CD2 loci (13, 14, 28). In neither case is it clear whether these act by overcoming effects due to the chromosomal environment or effects due to the arrangement of the gene array, where the copy number is greater than one.

The question we have addressed here is whether the level of expression is in some way dictated by chromosomal location, as opposed to the effects listed above. We selected a transgenic line with unusual expression and with a low copy number so that the foreign DNA could be isolated easily. The expression pattern of the foreign gene in this line is unusual in that the level of expression is extremely low in just one of the three tissues in which it is expressed in other lines. Thus, the gene is not totally inactive as it would be if it had suffered a major lesion or if it were uniformly methylated. When the foreign gene pair was reisolated from line 64 and reintroduced into secondary transgenic mice, expression in the preputial gland was restored. The same general approach was previously used to study the cause of the inactivation of an HSV TK gene that had become expressationally inert while inserted into the chromosomes of an L-cell line. In that case too, the activity of the gene was restored when it was recovered and reintroduced into the genome of a cell (4). At least two of our secondary transgenics showed some rearrangement of the foreign DNA. Any one of these rearrangements could in principle have reversed the effect of a previous rearrangement. It is highly unlikely, however, that this could have happened in every case, while on the other hand it is very likely indeed that the foreign genes were integrated into entirely new chromosomal locations in the secondary transgenic lines. Thus, the conclusion that negligible preputial gland expression in line 64 is due to a chromosomal position effect is a strong one. Since the immediate chromosomal preputial regions were reisolated and reintroduced along with the insert, the position effect must be due to influences originating from more distant parts of the chromosome.

P-element-mediated transformation of the *Drosophila* genome leads in most cases to the insertion of solitary foreign DNA molecules into the chromosome. This system therefore lends itself to the detection of chromosomal position effects without the complication of tandem arrays and major rearrangements of the foreign DNA. Moderate negative effects attributed to chromosomal position have been reported (12, 36). In these cases, mutational changes and small rearrangements of the foreign DNA were not excluded as possible causes of the reduced expression.

The provirus form of a retrovirus is inserted into the chromosome by a special mechanism at any one of a very large number of potential sites (32) as a solitary sequence between direct repeats of the proviral long terminal repeat. Since it does not form integrated arrays, evidence of line-specific variation in the expression of a retrovirus suggests the existence of chromosomal position effects (34). The introduction into the mouse germ line of single copies of a retroviral provirus (e.g., Moloney murine leukemia virus) leads in different lines to different patterns of expression of the viral RNA (20, 21). This has been attributed to chromosomal position effect (20). The ectopic expression of retrovirus-borne cellular genes (35) may be a related phenomenon. However, as pointed out by Soriano et al. (35), the high mutation rate of retroviruses, including Moloney murine leukemia virus (27, 31), makes it impossible to draw a firm conclusion on these grounds. A proof that chromosomal position effect, or indeed any effect other than mutational alteration, is operating in these cases would require a demonstration that the viral genome is unchanged in at least some of the cited instances. In one case (Mov-3), the proviral genome was recovered from the infected line, together with flanking sequences, and microinjected into the cytoplasm of mouse embryos (18). A single mouse that had integrated the provirus into its genome was recovered and analyzed. Viral sequences were expressed at a high level in skeletal muscle, an ectopic tissue with respect to normal...
Moloney murine leukemia virus expression. This experiment has crucial weaknesses, however, as a demonstration of chromosomal position effect, principally because only a single mouse (integration event) was studied. The route by which the microinjected provirus became integrated into the genome of the embryo is unclear, but the flanking chromosomal sequences are known to have been removed in some way. If integration involved transcription from the proviral genome, followed by reverse transcription and integration by the normal retroviral route, then it is possible that the properties of the virus were altered by point mutation, frameshift, or deletion (27). If it was integrated by illegitimate recombination between the incoming provirus (with flanking sequences) and chromosomal DNA, the loss of flanking sequences shows that DNA rearrangement has occurred, and this could have extended to the viral long terminal repeat. We conclude that while effects of chromosomal position may modulate the expression of retroviral proviruses, this explanation has the status of a hypothesis at this time.

In the few well-studied cases of foreign genes that are expressionally inactive in some but not all transgenic animal or cell lines, the foreign DNA was hypermethylated. The HSV TK gene which spawned expressionally inactive variants while resident in L cells was hypermethylated in most cases (4). Similarly, the murine line-specific inactive Mov proviruses studied by Jähner and Jaenisch (21) become hypermethylated during early development and are later selectively demethylated in a line- and tissue-specific manner. As pointed out above, expression of the line 64 foreign hybrid genes cannot be uniformly and irreversibly inactivated by methylation, since these genes are expressed in the liver and testis. However, it is possible that there is tissue-specific hypermethylation (or failure of demethylation) in the preputial glands of this strain. This possibility is now under investigation.

Causes of chromosomal position effect have been indicated or identified in some special cases. None of these findings exclude hypermethylation as a concomitant of inactivation. The powerful selection possible in cell transfection experiments has allowed the identification of chromosomal enhancers in the region of hyperactive foreign genes (2, 16). Similarly, cell lines transfected with retroviruses have been used to study chromosomal position effects. In one study, a transcriptionally active provirus was found to have been integrated at a chromosomal site which, in uninfected cells, was within a DNase-sensitive region of the chromatin (10). In another, an enhancer sequence that activated the proviral long terminal repeat was found close to the site of integration of a provirus that was unexpectedly active in an EC cell line (38). These examples relate to gene activation, as do the tissue-specific DNase-hypersensitive sites which activate the transcription of globin and other genes (13, 14, 28). The causes of tissue-specific inactivation, like that observed in line 64, must be of a different nature.

ACKNOWLEDGMENTS

We are grateful to Melville Richardson, Helen McIlroy, and Garry Brown for technical assistance, to J. Paul Simons for discussions, and to Noreen Murray for providing hosts for EMBL3A and advice on their use.

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LITERATURE CITED

introduction at abnormal chromosomal positions and expression in inappropriate tissues. Cell 34:343-358.


The Herpes Simplex Virus Type 1 Thymidine Kinase Is Expressed in the Testes of Transgenic Mice under the Control of a Cryptic Promoter

RAYA AL-SHAWI, 1, 2 JOANNE BURKE, 3 HELEN WALLACE, 1 CHERYL JONES, 1 STEPHEN HARRISON, 2 DAVID BUXTON, 3 STEPHEN MALEY, 3 ANN CHANDLEY, 4 AND JOHN O. BISHOP 1, 2, 5

Institute of Cell and Population Biology, Division of Biology, 1 and AFRC Centre for Genome Research, 2 University of Edinburgh, Moredun Research Institute, 3 and Medical Research Council Human Genetics Unit, Western General Hospital, 3 Edinburgh, United Kingdom, and Department of Biological Sciences, University of Maryland, Baltimore, Maryland 21228

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We reported previously that the herpes simplex virus type 1 (HSV-1) thymidine kinase reporter gene (tk) was expressed in the testes of transgenic mice when coupled to the promoter of a liver-specific mouse major urinary protein (MUP) gene. Here we show that HSV-1 tk is also expressed in the testis when coupled to a MUP pseudogene promoter, to a truncated MUP promoter that is not active in the liver, and to the promoter of the bovine thyroglobulin gene. Furthermore, HSV-1 tk itself was expressed in the testis, although its normal expression had been disabled by removing an upstream regulator of transcription. In every case, the same multiple transcripts were observed, with their 5' ends located downstream of the normal HSV-1 tk translation initiation codon. We conclude that the transcription of HSV-1 tk in the testis is directed by a cryptic TATA box-independent promoter located in the coding region of the gene. The longest HSV-1 thymidine kinase (TK) polypeptides synthesized in the testis were shorter than full-length TK and probably result from translational initiation at Met46 and Met48, the second and third ATG codons of the tk reading frame. Male mice of most transgenic lines were sterile, and the severity of the lesion in spermatogenesis was directly related to the level of TK expression. In the most highly expressing lines, sperm counts were low and morphologically defective sperm were common. In other sterile lines, TK was expressed at a lower level and sperm counts were normal but sperm motility was greatly reduced. Lines with the lowest levels of HSV-1 TK expression were fertile. HSV-1 TK was expressed in germ line cells, mainly in the haploid spermatids. However, low-level HSV-1 TK activity was found in the testis before the first germ cells entered meiosis, showing that if expression is confined to the germ cells, it also occurs in spermatagonia.

The coding region of the herpes simplex type 1 (HSV-1) virus thymidine kinase gene (tk) has been used extensively as a reporter sequence in cell transfection (30, 45) and was one of the first reporters used in work with transgenic animals (8, 43, 44). More recently it was adopted as a component of a system for destroying (ablatting) specific cell types in transgenic animals (4, 5, 22). The basis of the procedure is to place HSV-1 tk under the control of a tissue-specific promoter and introduce the compound gene into the mouse genome. HSV-1 thymidine kinase (TK) is expressed in target tissues when the promoter becomes active. At this time, target cells may be killed by administering an antitherpetic agent to the transgenic mouse. Several potentially useful antitherpetic agents are available, the most effective of which seems to be Ganciclovir (9-1,3-dihydroxy-2-propoxy(methyl)-guanine [DHPG]). The agent is phosphorylated quite efficiently by HSV-1 TK, and the phosphorylated DHPG derivatives are lethal to the cell. DHPG is phosphorylated very inefficiently by cellular nucleoside kinases, and this explains its lack of toxic effects on nonexpressing tissues and nontransgenic animals.

In addition to an agent which is nontoxic to nonexpressing cells, the ideal ablation system would employ an enzyme which has no deleterious effect in the absence of the agent. The apparent lack of deleterious effects of HSV-1 TK (4) is quite surprising in view of its nonphysiological mode of action: unlike the major cellular enzyme, it phosphorylates cytidine as well as thymidine (11, 26), and it also phosphorylates pyrimidine nucleotides (10, 41). We reported previously that in combination with a mouse major urinary protein (MUP) gene promoter sequence, HSV-1 tk was expressed in the testis and caused male sterility (1). We have now coupled HSV-1 tk to other promoters and find that it is invariably expressed in the testis. The severity of the defect in spermatogenesis correlates positively with the level of TK expression.

MATERIALS AND METHODS

Plasmid constructs and transgenic mice. The different hybrid genes with animal promoters and the HSV-1 tk coding region are described elsewhere (1, 2, 44a, 44b). All contain the same 1,759-bp fragment of HSV-1 tk between a BglII site in the 5' noncoding region and a BstEII site 430 bp downstream of the polyadenylation signal (Fig. 1). The BglII site was joined to each cellular promoter at a site in the 5' noncoding region of the transcription unit and in each case the bacterial supF gene was attached beyond the BstEII site as described previously (1). The 1,887-bp EcoRI-BstEII fragment of HSV-1 tk (Fig. 1) was excised from plasmid pTK1 (46). DNA fragments for microinjection were prepared as described. Transgenic mice were identified by polymerase chain reaction of tail-cut DNA.
RNA analysis. Testis RNA was prepared and Northern (RNA) blot analysis was performed as described previously (1). The probes used were (i) pTK1, which could react with up to 1,256 nucleotides of full-length transcript (BglII site to polyadenylation site), (ii) probe A (Fig. 1), an 841-bp PstI fragment of pTK1, and (iii) probe B. RNA transcribed from a 419-bp EcoRI-EcoRV fragment of HSV-1 tk was hybridized overnight at 45°C with 5 μg of total testis RNA. Digestion was with RNase T1. RNase protection assays were carried out as described from a 450-bp Asp700I-EcoRV fragment of BS6-tk-supF (−142 to +348) cloned in pBluescript II SK+ (Stratagene Ltd.). Approximately 5 × 10^6 dpm of antisense probe was hybridized overnight at 45°C with 5 μg of total testis RNA. Digestion was with 40 μg of RNase A and 650 U of RNase T1 per ml for 30 min at 37°C.

Protein and enzyme analysis. Tissue extracts were prepared and HSV TK assays were performed as described previously (1). Where indicated, the protease inhibitors 3,4-dichloroisocumarin (DCI) or tosyl phenyl chloroketone (TPC) and p-hydroxymercuriphenylazoate (PHMB) were added at the time of homogenization. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out with 12% separating and 4% stacking gels. Proteins were transferred to nitrocellulose (0.2-μm pore size; Schleicher & Schuell), and Western immunoblots were carried out as described previously (27), using a 1/250 dilution of rabbit anti-HSV-1 TK serum (provided by W. P. Summers) as the primary antibody and biotinylated goat anti-rabbit immunoglobulin G (Amersham) as the secondary antibody. Filters were treated with streptavidin-alkaline phosphatase conjugate (1/1000 dilution; Amersham) for 45 min at room temperature and developed with 7 mM 5-bromo-4-chloro-3-indolyl phosphate and 6 mM nitroblue tetrazolium in 0.1 M Tris-HCl, pH 9.5, at room temperature for about 20 min.

Immunostaining of testis sections. Deeply anesthetized mice were perfused with 2% paraformaldehyde, and tissues were postfixed with modified Bouin fixative, dehydrated in graded alcohols, and embedded in paraffin wax. Dewaxed 4-μm sections were treated with 1% H2O2 in methanol to block endogenous peroxidase, treated with 1 mM EDTA–0.05% Tween 20 in phosphate-buffered saline for 10 min, flooded with 2% egg albumen, and incubated overnight at 4°C after addition of rabbit anti-HSV-1 TK serum. Peroxidase-conjugated goat anti-rabbit immunoglobulin G was added for 90 min, and slides were developed with 3,3'-diaminobenzidine.

Results

Expression of HSV-1 tk in the testis in conjunction with active and inactive tissue-specific promoters. We previously described a construct (BS6-tk-supF) in which the expression of HSV-1 tk was directed by a MUP gene promoter (1). This gene was expressed consistently in the livers and preputial glands of transgenic mice (four of five transgenic lines in each case). It was also expressed very consistently in the testis (five of five transgenic lines), and males of all five transgenic lines were sterile. We next made a comparative study of a number of other constructs in which the same HSV-1 tk reporter sequence was coupled to different promoters (Fig. 1). Two of these contained defective MUP promoters: BS2-tk-supF contained a fragment of a MUP pseudogene homologous to the BS6 promoter, and BS6Sat2-tk-supF contained only the proximal part (to nucleotide -314) of the BS6 promoter. Another, TG-tk-supF, contained a 3.05-kb promoter fragment of the bovine thyro-
TABLE 1. Expression of HSV-1 TK in tissues of lines of transgenic mice carrying constructs with different promoters linked to the HSV-1 tk reporter

<table>
<thead>
<tr>
<th>Construct</th>
<th>Fertilitya</th>
<th>n</th>
<th>TK activity (pmol/min/mg of protein)</th>
<th>Testis</th>
<th>Liver</th>
<th>Preputial gland</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± SE</td>
<td>Range</td>
<td>Mean ± SE</td>
<td>Range</td>
</tr>
<tr>
<td>BS6-tk-supF</td>
<td>S</td>
<td>5</td>
<td>84.0 ± 20.7</td>
<td>31-140</td>
<td>4.39 ± 2.04</td>
<td>0.05-12.7</td>
</tr>
<tr>
<td>BS6-tk-supF (TR)</td>
<td>S</td>
<td>4</td>
<td>195 ± 40.8</td>
<td>68-286</td>
<td>5.86 ± 3.68</td>
<td>0.62-18.5</td>
</tr>
<tr>
<td>BS2-tk-supF</td>
<td>F</td>
<td>2</td>
<td>2.52</td>
<td>1.77-3.26</td>
<td>0.047 ± 0.005</td>
<td>0.46-0.48</td>
</tr>
<tr>
<td>BS6 Sau2-tk-supF</td>
<td>S</td>
<td>6</td>
<td>60.6 ± 10.9</td>
<td>17.4-91</td>
<td>0.09 ± 0.03</td>
<td>0.01-0.16</td>
</tr>
<tr>
<td>TG-tk-supF</td>
<td>S</td>
<td>3</td>
<td>54.5 ± 9.0</td>
<td>40.4-73.3</td>
<td>0.06</td>
<td>3.06</td>
</tr>
</tbody>
</table>

a S, sterile males; F, fertile males.
b Number of transgenic lines studied.
c Mean of line means.
d Range of line means.
e Mean of two lines. Values typically observed in extracts from nontransgenic mice are shown in Table 3.

* RNA and HSV-1 tk protein are expressed in testes of transgenic mice. We reported previously that two main size classes of HSV-1 tk-specific RNA were present in testes of transgenic mice carrying BS6-tk-supF (1). A 900-bp transcript, first observed by Roberts and Axel (3) was not labelled by an antisense probe (Fig. 2A and C, lane 1) and must therefore be a reverse transcript. Possibly related to a reverse transcript previously observed in cells infected with HSV-1 (18, 25). Line L78 carries a full-length copy and a second truncated copy of the 5' end of the BS6-tk-supF gene (2). L78 uniquely has a third short (500-bp) testis transcript (Fig. 2A, lane 1) which was labelled by a probe directed against the 5' part of the HSV-1

FIG. 2. Mapping of HSV-1 tk-specific RNA in extracts of line 78 testis. Identical RNA transfers were probed with a full-length HSV-1 tk double-stranded DNA probe (A), a 5' double-stranded DNA probe (B), and a 5' single-stranded antisense RNA probe (C). Lanes contain RNA samples as follows: 1. line 78 testis; 2. BHK cells stably transformed with a plasmid with an active BS6-tk gene; and 3, nontransgenic testis. Lane 4, runoff RNA size markers. 1.830, 1.320, 957, and 444 nucleotides long.
tk gene (Fig. 2B). We interpret the 500-bp transcript which is peculiar to L78 as being from the second truncated copy of the gene. A 1,200-bp transcript is the predominant transcript in testis from all transgenic lines examined. It is the only transcript that can act as template for the synthesis of active TK but is shorter than the 1.350-hp transcript expected if transcription was initiated at the cap site of the BS6 gene.

Primer extension (not shown) and nucleic protection experiments were carried out to determine the effective cap site of the 1,200-bp transcript. Lines L40, L46, and L78 (all carrying BS6-tk-supF) were used in different experiments. Apart from the additional short transcript present in L78 RNA, these lines showed the same band pattern in Northern blots. In liver RNA, the 5' ends of transcripts mapped in approximately equal amounts to two sites, one within 1 nucleotide of the predicted MUP gene cap site (-1) and a second site 73 nucleotides downstream (Fig. 3, lane 3, markers B and D). In testis RNA, no transcripts originated at the MUP gene cap site. At least six different 5' ends mapped to sites between about +130 and +205, the most prominent being at about +150 and +160 (Table 2 and Fig. 3, lane 6, markers E to I). All of these sites lie between the normal initiation codon of HSV-1 tk (M4; Fig. 1) and the second methionine codon (M46; Fig. 1). The same 5' ends were observed in experiments with testis RNA from transgenic mice carrying BS6-Sau2-tk-supF and TG-tk-supF (Fig. 3, lanes 10 and 12).

In L46, the BS6-tk hybrid gene was highly expressed in both the testis and the male preputial glands. We examined the pattern of anti-TK antibody-reactive polypeptides in extracts of these two tissues. Several polypeptides present in a standard preputial gland extract reacted with anti-TK antiserum (Fig. 4, lane 3). The most prominent was a proteolytic product, since it was absent when the extracts were prepared in the presence of protease inhibitors (Fig. 4, lanes 4 to 6). In the presence of protease inhibitors (19), the most prominent component was the full-length M43000 (43K) HSV-1 TK. A similar amount of a 37K polypeptide and a smaller amount of a 39K polypeptide were also present. These polypeptides are present in cells infected with HSV-1 (31). The truncated forms are due to initiation of translation at two internal ATG codons corresponding to M49 and M60. They were produced in increased amounts when M1 was deleted (20) or if a stop codon was introduced between M3 and M46 (19, 23), and at least one of them has TK activity (12, 19). The origin of an additional 35K polypeptide is not known, but it is also observed in extracts of HSV-1-infected cells and may result from initiation at the fourth ATG codon of the reading frame, M85. Overall, the pattern of polypeptide expression in the preputial gland was similar to that observed in HSV-1-infected cells (Fig. 4, lanes 1 and 6).

The testis pattern was unaffected by the addition of protease inhibitors, indicating that testis extracts do not proteolyze HSV-1 TK (Fig. 4, lanes 7 to 10). The predominant components were the 39K and 37K polypeptides, consistent with the initiation of transcription between ATG1 and ATG2. In most cases full-length (43K) HSV-1 TK was not observed, but in some experiments a very small amount of antigenically reactive protein migrated with the appropriate mobility. This may represent the translation of a small amount of the 43K protein from rare transcripts initiated upstream of ATG1. Testis RNA from lines carrying MUP gene constructs contains a small amount of transcript which may originate within the MUP promoter sequence upstream of the TATA box (Fig. 3, lanes 6, 9 and 10, marker A).

Similarly, the RNA from TG-tk-supF mice shows a protected band at the point at which homology between probe and target ends, presumably derived from transcripts originating upstream of the junction (Fig. 3, lane 12, marker C). These longer transcripts, which are present in very small amounts relative to the shorter transcripts, may account for the production of small amounts of full-length HSV TK.

Since the testes of transgenic mice carrying different HSV-1 tk constructs contained the same truncated RNA transcripts, it was expected that they would also contain the same HSV-1 TK polypeptides. This was found to be the case. Testis extracts from five different transgenic lines which carry the BS6-tk-supF hybrid gene (lines L40, L46, L78) showed the same band pattern as testis extracts from transgenic mice carrying the TG-tk-supF hybrid gene (Fig. 4, lanes 3 to 6). The three most prominent components are present in all of the testis extracts, although their relative amounts vary from one line to another.
L58, L64, and L78) and from lines carrying the different constructs listed in Table 1 exhibited the same pattern of truncated HSV-1 TK polypeptides (Fig. 5).

Expression of a promoterless HSV-1 tk gene in testes of transgenic mice. The identical patterns of aberrant transcription in the testes of transgenic mice carrying HSV-1 tk with different tissue-specific promoters suggested that the cellular promoters are not involved in the initiation of transcription in the testis. To test this possibility, an 1,887-bp EcoRI-BstEII fragment (−79 to +1808) of the HSV-1 tk gene (Fig. 1) was excised and introduced into the mouse genome by microinjection. This fragment (HSV-1 tkD52−) terminates at the same 3′ site in the HSV-1 genome as the HSV-1 tk fragment used in the constructs listed in Table 1. At the 5′ end it carries the HSV tk TATA box, but it lacks the more distal of two upstream control elements which are both required for full expression of the gene in frog oocytes and HSV-1-infected cells (14, 34). Removal of the distal element by deleting the 5′ end of the gene down to position −85 reduces expression in frog oocytes by approximately 20-fold (33).

HSV-1 TK expression was measured in four G0 transgenic males carrying the HSV-1 tkD52− fragment and in male transgenic offspring of a G0 female (founder of line TK16). Three of the G0 males and the line TK16 males expressed HSV-1 TK at moderate to high levels in the testis (Table 3). Expression in other tissues was sporadic and, other than in the brain and lachrymal glands of TK16 males, at very low levels. Two of the expressing males were fertile nontransmitting, like many G0 males carrying tk constructs (1). Such males are probably mosaics with nontransgenic precursor cells in their germ line (see below). The third expressing G0 male and line TK16 males were sterile (Table 3). In nuclease protection experiments, testis RNA from line TK16 showed the same pattern of 5′ ends within the HSV tk coding region as did testis RNA from mice carrying the promoter constructs (Fig. 3, lane 11). Furthermore, line TK16 testis contained the same shorter HSV TK polypeptides that were observed in the testis of those mice (Fig. 5, lane 5). These results argue convincingly that the expression of HSV-1 TK in the testes of transgenic mice is not due to the various tissue-specific promoters when these are present. Instead, it appears to be due to a cryptic promoter within the HSV-1 tk reporter gene, located downstream of the TATA box-containing promoter which is utilized during HSV infection.

Sites of HSV-1 TK expression in the testis. To determine whether HSV-1 TK is expressed in spermatogenic cells or in ancillary cells, sections of testis from mature male transgenic mice were immunostained with anti-TK antiserum as the primary antibody. The sections consistently showed expression in the postmeiotic haploid spermatids (Fig. 6). No evidence was obtained of expression at earlier stages of spermatocyte development or in Sertoli cells, and staining was observed only rarely and sporadically outside the tubules.

Developmental onset of HSV-1 TK expression. During postnatal development in the male mouse, some germ cells in all of the testicular tubules begin to advance toward the first meiosis at about the same time (35). The first spermatocytes in meiotic prophase are observed between 8 and 10 days after birth. Between days 10 and 12, the most advanced cells observed are in mid-pachytene; at 15 to 17 days, 30% of tubules contain cells in late pachytene; and at 22 to 24 days, spermatids are observed for the first time in about 35% of tubules. Thus, by assaying the testis at different times during the first 3 weeks of life, an indication can be obtained of the developmental stage at which a gene product is first formed. At 7 days after birth, when the testis first becomes large enough to permit the assay, the specific HSV-1 TK activity (units per milligram of testis protein) was significant (Fig. 7).
TABLE 3. TK activities in tissues of transgenic mice carrying the promoter-truncated HSV-1 tkDS2- fragment

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Nontransgenic control</th>
<th>G0 transgenic males</th>
<th>Line TK16 male, sterile</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TK10, fertile*</td>
<td>TK22, fertile</td>
<td>TK39, sterile</td>
</tr>
<tr>
<td>Testis</td>
<td>0.11</td>
<td>23.15</td>
<td>0.16</td>
</tr>
<tr>
<td>Liver</td>
<td>0.06</td>
<td>0.46</td>
<td>0.00</td>
</tr>
<tr>
<td>Preputial gland</td>
<td>0.16</td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.08</td>
<td>0.27</td>
<td>0.20</td>
</tr>
<tr>
<td>Brain</td>
<td>0.20</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.00</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Lachrymal gland</td>
<td>0.07</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>Submaxillary gland</td>
<td>0.03</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Fertile nontransmitting.  
ND, not determined.

The lack of HSV-1 TK immunostaining in ancillary cells of mature testis suggests that this TK activity is present in the spermatogonia. However, the possibility that HSV-1 TK is expressed in ancillary cells in immature testis has not been excluded. Between 10 and 20 days after birth, a higher level of HSV-1 TK was present, possibly due to a continuing increase in the proportion of the testis made up of spermatogonia and spermatocytes (35). At about 21 days, the time at which the first haploid spermatids appear, a more abrupt increase in HSV-1 TK activity occurred. This finding suggests that there may be renewed expression in the round spermatids, a stage at which de novo synthesis of spermiogenesis-specific proteins is frequently observed (17, 21, 36, 40).

Effects of HSV-1 TK expression on spermatogenesis. In lines which expressed HSV-1 TK at a low level, the male mice were fertile. One of seven BS2-tk-supF lines and two of six BS6-tk-supF(TR) lines had relatively low levels of HSV-1 TK expression and were male fertile, and males transmitted the foreign gene to their progeny (Table 1). This finding suggests that sterility ensues only above a threshold level of HSV TK (7). However, the highest level of expression among the male-fertile lines was five times less than the lowest level among male-sterile lines (3.3 versus 17.4 U/mg).

FIG. 6. Testes of BS6-tk-supF transgenic mice immunostained with anti-HSV-1 TK serum. (A) Nontransgenic testis; (B) transgenic testis from line TG66 (TG-tk-supF); (C and D) transgenic testis from line 78 (BS6-tk-supF). Magnifications: A, B, and C, ×70; D, ×280.
This large discontinuity raises the possibility that there are two modes of expression of HSV-1 tk in the testis, for example two independent mechanisms of transcriptional activation or possibly expression in two different cell lineages. If so, it is possible that only one of the two modes is active in the male-fertile lines while the other causes sterility.

Although the levels of testis expression due to different constructs were similar when averaged over several lines, different lines carrying the same construct exhibited substantial differences. Three transgenic lines carrying the BS6-tk-supF gene were therefore examined. Line L78 mice have an intermediate level of expression in the testis (62 U/mg of protein). In L78, development of the spermatozoa appeared normal (Fig. 6). The sperm counts of L78 mice were also normal, but the motility of the spermatozoa was reduced and furthermore faded very rapidly in vitro.

In lines L40 and L46, which have a high level of HSV TK expression in the testis (140 U/mg of testis protein), testis weight was lower than in nontransgenic controls and the sperm content of the epididymides was lower by a factor of 25 to 500 (Table 4). Very few sperm were found in the vas deferentia of these animals and those observed had very abnormal morphology and were nonmotile (Fig. 8). Thus the severity of the spermatogenic lesion is greater when the expression level of HSV-1 TK in the testis is higher.

Sterility of G0 transgenic males. In the lines that express moderate levels of HSV-1 TK and produce normal numbers of nonmotile sperm, the heterozygous male offspring of transgenic females were sterile although half of the sperm should not carry the foreign gene. This outcome could be expected if the lesion which results in sperm incapacitation occurs prior to meiosis. However, the cells descended by mitosis and meiosis from each committed spermatogonium remain together as a syncytium with cytoplasmic bridges between the cells (6). Transfer of HSV TK across the bridges could be expected to lead to complete sterility of heterozygous males even if the lesion due to HSV-1 TK expression occurred only in the haploid spermatids (7).

Two classes of G1 transgenic males were obtained with each of the constructs listed in Table 1. Taken together, 47% of the transgenic G1 males were sterile, while 53% were fertile but did not transmit the foreign gene to any of their offspring. The sterile transgenic males are taken to be descended from injected embryos in which at least one insertion event occurred prior to DNA replication at the one-cell stage, leading to a uniformly heterozygous individual G0 pup, comparable to the heterozygous male offspring of transgenic females. The fertile nontransmitting males are taken to be germ line mosaics, in which integration of foreign DNA occurred at some time after the first round of DNA synthesis. The transgenic cells of such individuals are het-

| Table 4. Defects of spermiogenesis in severely affected sterile mice |
|------------------|-------------|-----------------|-------------------|
| Determination    | Controls    | Line L40 Mouse 1 | Line L40 Mouse 2 |
| Sperm weight (mg)| 91, 95      | 72              | 74               |
| Epididymal sperm | 2–3 × 10⁶    | 10³             | 5 × 10³           |
| Contents of vas deferens | Numerous sperm |                 | None with normal morphology |
| HSV-1 TK activity* (line mean ± SE) | 80% normal | 140 ± 6.7 | 139 ± 17.6 |

* From Al-Shawi et al. (1).
offspring of transgenic females, are assumed to generate only homozygous and, by analogy with the heterozygous male expression to the testis. The conclusion that the HSV-1 sumably develop separate syncytia which generate compe-

ents of transcription initiation, possesses properties of this promoter sequence is presently being investigated. 

dent transcription of the metallothionein 1 gene is initiated at several quite widely dispersed sites, and these are located within a region with a high G+C content which contains no discernible TATA box but resem-

bly develop separate syncytia which generate compet-

ent spermatooza.

DISCUSSION

HSV-1 tk contains a cryptic promoter which directs its expression to the testis. The observations described lead to the conclusion that the HSV-1 tk gene contains a cryptic promoter, independent of its own TATA box, which is particularly active in postmeiotic spermiogenic cells. The putative cryptic promoter, assumed to be located upstream of the sites of transcription initiation, possesses properties associated with promoters of housekeeping genes. Thus, transcription is initiated at several quite widely dispersed sites, and these are located within a region with a high G+C content which contains no discernible TATA box but resembles an HTF (HpaII tiny fragment) island (3) in containing many potential methylation sites. However it is clearly not a true housekeeping promoter since it is consistently ex-

ressed at a detectable level only in the testis. The location of this promoter sequence is presently being investigated.

In an interesting parallel, vigorous TATA box-independent transcription of the metallothionein I gene is initiated at several sites between −134 and −28 in spermatocytes and spermatids (15). This observation and those reported here, together with at least one other example (42), suggest that the transcriptional activation of cryptic promoters which share properties with housekeeping promoters may be a property of postmeiotic male germ cells.

In the testes of mice carrying a metallothionein promoter–β-interferon–tk construct, Iwakura et al. (24) observed minority transcripts (about 20%) which hybridized with both β-interferon and tk probes, while about 80% of the transcripts reacted only with the tk probe and closely resembled the testis tk transcripts that we observed (Fig. 2). The cryptic tk promoter evidently did not abrogate (or abrogate com-
pletely) the activity of the upstream metallothionein promoter, which was itself active in the testis. In contrast, our MUP and TG constructs produce minute amounts, if any, of full-length mRNA, presumably because these tissue-specific promoters are inactive in the testis.

Synthesis of truncated HSV-1 TK polypeptides in the testis. In HSV-1-infected cells, the synthesis of HSV TK is nor-

maly initiated not only at the first ATG codon but also, at much lower frequencies, at the second and third ATG codons (19, 31), possibly because both the first and second ATG codons lie within consensus sequences which are inefficient translational initiators (19). The pattern of HSV TK synthesis in the preputial glands of MUP-tk transgenic mice closely resembled that observed in HSV-1-infected cells.

In cells infected with HSV-1 carrying a deletion that removes the first ATG codon of the tk reading frame, the second and third ATG codons are utilized more efficiently (19). The truncated HSV-1 tk RNA molecules synthesized in the testis lack the first ATG codon, and to this extent they are equivalent to the mRNA synthesized from the deletion mutant. Accordingly, the most abundant HSV-1 TK poly-

peptide in the testis comigrates with the most abundant TK polypeptide (37K) present in cells infected with the deletion mutant (19). Translation of the 37K polypeptide is presumed to be initiated at M^{60}, the third methionine of the reading frame, which lies within the strongest translational initiation consensus of the first three ATG codons. M^{60} lies within a region of the enzyme shown by site-directed mutagenesis to be the ATP-binding pocket (29). Consequently, the enzymatic activity of the 37K protein is likely to be less than that of full-length TK, and the level of TK expression in the testis may be considerably higher than indicated by enzyme as-
says. Supporting this view, the level of HSV-1 tk mRNA in testis RNA is disproportionately high relative to that in preputial gland RNA in comparison with the ratio of TK activities in the two tissues (1).

TK expression in the testis causes sterility. We previously concluded that male sterility in mice carrying an HSV-1 tk reporter gene was due to a high level of ectopic HSV-1 TK expression in the testis (1), and this was recently confirmed (7). The results presented here strengthen our previous conclusion. While the truncated HSV-1 tkΔDS2− gene caused male sterility (Table 3), transgenic mice carrying the HSV-1 tk gene with the promoter region intact were male fertile (7). Thus, the property of causing sterility seems to be neutral-

ized by the HSV-1 tk promoter. In these fertile mice, the onset of TK expression was at a later stage in sperm development and the level of enzyme activity attained was lower than in sterile mice with similar levels of HSV-1 tk mRNA (7). This finding suggests that the effect of the HSV-1 tk promoter was to postpone expression of the gene to a later stage in spermatocyte development when protein synthesis is less vigorous. Since transgenic mice which exhibit a low level of TK expression in the testis are fertile (Table 1), the fertility of the mice that carry the HSV-1 tk gene with its promoter intact may be due simply to reduced enzyme activity.

The ectopic expression of HSV-1 tk in the testis has in the past led to erroneous conclusions being drawn. In one case, male sterility in mice transgenic for a cosmids containing the major histocompatibility complex class II E_{α} gene was at
first attributed to the expression of E, (37). However the cosmid also carried an HSV-1 tk gene, and sterility was later found to be due to the expression of this gratuitous component of the construct (7). In another case, a metallothionein-β-interferon-tk foreign gene caused male sterility, which was attributed to the expression of β-interferon (24). HSV-1 TK activity at levels similar to those we have observed was detected in the testes of the transgenic mice, and it was not convincingly demonstrated that β-interferon rather than HSV-1 TK was the cause of sterility.

Impaired fertility has been reported in transgenic mice carrying HSV tk directed by the immunoglobulin gene promoter and enhancer (22). Although it is unlikely that testis expression of HSV tk influenced the ablation of the lymphocytes by DHPG or of the somatotrophs of mice carrying a growth hormone promoter-HSV tk reporter gene (5), it is clearly an undesirable potential complication in experimental animals.

Causes of male sterility. Our data indicate that sterility in mice with an intermediate level of TK expression may be due to expression in the postmeiotic spermatids. Since mammalian sperm do not undergo postmeiotic mitosis, this would exclude effects involving the synthesis of DNA, such as hypermutation. The nonmotile sperm produced by these sterile mice are present in normal numbers and are mainly normal in appearance, suggesting that their lack of motility may be due to an impoverished energy source. HSV-1 TK is an aggressively promiscuous nucleoside kinase, is only poorly susceptible to end-product inhibition, and vigorously phosphorylates mononucleotides as well as nucleosides (10, 41). Extracts of transgenic testis show elevated levels of both nucleoside kinase and nucleotide kinase activity (34a).

Normal mouse sperm remain motile for several hours in the absence of an exogenous energy source, suggesting that they possess a sizable energy store (9). Thus, the lack of sperm motility may derive from a failure of energy storage due to ATP depletion during maturation.

The gross morphological defects and almost complete failure of sperm maturation that are observed in mice with higher levels of testis HSV-1 TK activity are likely to have a more radical cause. Possibly the low-level TK activity present prior to the first meioses, and tentatively attributed to expression in premeiotic germ cells, interferes with DNA synthesis or some other vital cellular function in lines with intermediate expression levels.

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REFERENCES


