

**GENERATION AND ANALYSIS OF AN INDUCIBLE  
THYROXINE-DEFICIENT MOUSE MODEL.**

**HELEN A. C. WALLACE**

Thesis presented for the degree of Doctor of Philosophy

University of Edinburgh

1992



I declare that this work is my own, except where  
otherwise stated,

## ACKNOWLEDGEMENTS.

I would like to thank my supervisor, Professor John Bishop, for the opportunity to pursue the work presented in this thesis, and for his patience, advice and encouragement over the years.

Special thanks are due to Colin Nicolson who patiently taught me histology and to Kathryn McLaren for helpful discussions on the morphology of the thyroid gland and the E.M. study. Thanks also to Melville Richardson for the TG-TK construct, to SIR Frank Johnston and Graham Brown for their excellent photography and to the staff of the Genetics animal unit and workshop.

I would like to thank Raya Al-Shawi, Stephen Harrison and Debbie Johnson for useful discussions and for reading the manuscript in its various stages of completion.

I would also like to express my gratitude to my friends and colleagues both on this side of the Atlantic and the other for their advice, help, encouragement and support over the last four years, in particular this last year. Special mention to Cindy-Lou, Bob, Gaz, Lisa, Alison, Liz, Mary and Wendy.

Lastly I would like to thank Smudge.

## ABSTRACT.

The aim of this project was to create an inducible thyroxine-deficient mouse model which could be used to investigate the role of thyroxine in gene expression. This was achieved by using the HSV1-tk ablation technique. Transgenic mice were produced containing 3.1kb of the bovine thyroglobulin promoter linked to 1.8kb of the HSV1-tk gene. Expression of the transgene was detected in the thyroid and testes. Transcription of the transgene in transgenic thyroid tissue was correctly initiated at the cap site of the thyroglobulin promoter. Treatment of female transgenic mice with 4.5mg/day of an anti-herpetic agent, such as ganciclovir, resulted in a reduction in the number of thyroid follicle cells within 3 days and their complete absence after 7 days. After 14 days of treatment the mice lacked circulating thyroid hormones, the HSV1-TK activity of the thyroid rudiments was comparable to non-transgenic controls and the total soluble protein remaining was approximately 20% of controls.

The thyroid gland comprises two cell types, thyroxine producing thyrocytes and calcitonin producing C-cells. The gland is also in intimate contact with the parathyroid gland which secretes parathyroid hormone. The expression of the transgene was restricted to the thyroid follicle epithelial cells. Transgenic ablation resulted in the precise removal of HSV1-TK expressing cells with no secondary effect on either the C-cells or the parathyroid gland. My results also demonstrate that the normal function of the C-cells and parathyroid gland are not dependent on thyroid hormones.

When treatment with ganciclovir was terminated no recovery of thyroid hormones in the circulation or HSV1-TK activity in the thyroid rudiment were observed (for up to 113 days). This suggests that young adult mice do not contain a non-differentiated stem cell that is capable of repopulating the follicle cells of the thyroid.

The mouse major urinary proteins (MUP) genes in the liver are regulated by thyroid hormones and growth hormone. I investigated the role of thyroid hormones on the expression of resident MUP genes and also a MUP transgene and demonstrated that hepatic MUP gene expression is absolutely dependent on the presence of thyroid hormones.

## CONTENTS.

	<u>PAGE.</u>
<u>CHAPTER 1: INTRODUCTION.</u>	
<u>HORMONES AND GENE EXPRESSION.</u>	1
I. <i>CIS</i> -ACTING DNA SEQUENCE ELEMENTS	3
A. ELEMENTS THAT REACT WITH INDUCERS	3
Promoter elements	3
Enhancer elements	4
B. ELEMENTS THAT REACT WITH REPRESSORS	7
II. <i>TRANS</i> -ACTING FACTORS	8
A. TRANSCRIPTIONAL ACTIVATORS	8
Helix-turn-helix proteins,	8
Helix-loop-helix proteins, octamer binding	
proteins and leucine zippers	
The zinc finger	10
Inducible <i>trans-acting</i> factors	12
B. TRANSCRIPTIONAL REPRESSORS	13
III. TISSUE-SPECIFIC GENE EXPRESSION	16
A. <i>CIS</i> -ACTING ELEMENTS	16
B. <i>TRANS</i> -ACTING ELEMENTS	19

<b><u>ANIMAL MODELS FOR ENDOCRINE STUDIES.</u></b>	21
I. HORMONE CELL TYPES OF THE THYROID AND PITUITARY AND REGULATION OF SECRETION	22
II. ALTERING THE HORMONAL STATUS OF AN ANIMAL	23
III. MUTANT HORMONE-DEFICIENT ANIMAL MODELS	24
IV. TRANSGENICS IN THE STUDY OF ENDOCRINE SYSTEMS	27
A. HOMOLOGOUS RECOMBINATION	29
B. TRANSGENIC ABLATION	29
Direct ablation	30
Conditional ablation	33
<b><u>THE THYROID GLAND.</u></b>	
I. HISTOLOGY OF THE THYROID GLAND	35
II. EXAMPLES OF THYROID HORMONE ACTION	36
III. CIRCULATING THYROID HORMONES	38
IV. THYROID HORMONE SYNTHESIS AND SECRETION	43
A. THE IODIDE PUMP	43
B. THE THYROGLOBULIN GENE	44
C. IODINATION OF THYROGLOBULIN PROTEIN	45
D. SECRETION OF THE THYROID HORMONES	46
V. REGULATION OF THE THYROID GLAND ACTIVITY	49
A. THYROTROPHIN RELEASING HORMONE	49
B. THYROID STIMULATING HORMONE	50
VI. CELLULAR ACTION AT THE TARGET TISSUE	51
THE AIM OF THE PROJECT	53

**CHAPTER 2: CHARACTERISATION OF THE THYROGLOBULIN- 54**

**THYMIDINE KINASE TRANSGENE.**

Transcription and translation of the transgene 58

**CHAPTER 3: CREATING THE INDUCIBLE THYROXINE-DEFICIENT MOUSE**

**MODEL.**

Transgenic ablation 68

Hormone levels 71

Specificity of ablation 75

Ablation studies in the male mouse 86

**CHAPTER 4: FURTHER ASPECTS OF THYROID ABLATION AND THE**

**EFFECT OF PROLONGED THYROXINE DEFICIENCY ON THE MOUSE.**

Effects of ganciclovir treatment on the thyroid 90  
gland

Circulating levels of thyroid hormones 96

Cell division 99

Regeneration 104

Effects of prolonged thyroxine deficiency 108

**CHAPTER 5: USING THE THYROXINE-DEFICIENT 112**

**MOUSE MODEL.**

Results 116

Effect of thyroxine on expression levels of 121  
a MUP transgene

<b><u>CHAPTER 6: DISCUSSION.</u></b>	130
Further applications of the thyroxine-deficient mouse model	132
Growth hormone receptor	132
Renin gene expression	134
Lactation	135
Brain development	136
<b><u>MATERIAL AND METHODS.</u></b>	
Mice	138
Enzyme and radio-labelled nucleotides	139
Urine samples	139
Serum samples	139
Drug and hormone treatment	140
Hormone assays:           ELISAs	140
RIAs	141
Tissue preparation and thymidine kinase assays	142
Protein analysis:       Western blot analysis	143
Isoelectric focusing gel	144
Histology:	144
Adipose tissue	145
Immunocytochemistry: Calcitonin	145
Thymidine kinase	146
Cell division	147
RNA preparation and Northern blotting	147
Labelling of probes	149
Hybridisation and washing of filters	150
Sub-cloning	151



<i>In vitro</i> transcription of RNA	152
Primer extension	153
<b><u>ABBREVIATIONS USED IN TEXT.</u></b>	155
<b><u>REFERENCES.</u></b>	159
<b><u>PUBLISHED PAPERS</u></b>	

TABLES.

1) Sequence comparison of hormone response elements	6
2) Zinc finger consensus sequence	11
3) Thymidine kinase activity in tissues of transgenic mice containing the TG-TK transgene	60
4) Thyroid hormone levels and HSV1-TK activity in ablated mice	70
5) Analysis of variance in the thyroid hormone levels of non-transgenic mice	74
6) Calcitonin levels in ablated mice	80
7) Parathyroid hormone levels in ablated mice	85
8) Male ablation: hormone levels and HSV1-TK activity in the testes and thyroid gland	88
9) Changes in transgenic thyroid glands during the first week of ganciclovir treatment	95
10) Thyroid hormone levels over 113 days post ablation	107
11) Thyroid hormone levels in ablated mice after hormone replacement	120
12) Levels of thyroid hormone in TG/RL transgenic mice following ablation:	
(A) male TG/RL mice	126
(B) female TG/RL mice	127

**FIGURES :**

1) Structure of mono and diiodotyrosine	40
2) Monodeiodination of thyroid hormones	42
3) Iodination of the thyroglobulin protein	48
4) The TG-TK transgene	56
5A) Primer extension of pooled thyroid total mRNA	63
B) Western blot analysis of testes and thyroid extracts	
6A) Site of HSV1-TK expression in the thyroid gland	66
B) The effect of ganciclovir treatment on the thyroid gland	
7A) Distribution of C-cells in the mouse thyroid gland	77
B) Histology of the parathyroid gland	
8A) Electron microscopy of the thyroid gland	83
B) Adipose tissue in the thyroid gland	
9) Morphology of the thyroid gland during ganciclovir treatment	92
10) Thyroid hormone levels and HSV1-TK activity during ablation	98
11) Cell division is not observed in the thyroid gland	103
12) Weight gain of thyroxine-deficient mice over 83 days	110

- 13) A) Isoelectric focusing gel of urinary proteins following ganciclovir treatment and hormone replacement 118
- B) Northern blot analysis on female TG66.19 mice
- C) Expression of the BS6- $\alpha_{2u}$  transgene in TG/RL male and female mice
- 14) The BS6- $\alpha_{2u}$  hybrid gene 123

## CHAPTER 1.

### INTRODUCTION.

#### HORMONES AND GENE EXPRESSION.

The endocrine system operates through extracellular messengers - hormones, which act on target cells to modify their activity. This control is important not only for development and growth but also in allowing the organism to adapt to changes in its internal and external environment. Hormones are synthesised in specialised cells which are either collected into distinct endocrine glands for example, the pituitary, the thyroid, and the pancreas, or are found as single cells within other organs for example, the gastrointestinal tract. Endocrine cells secrete hormones into the blood system where they are carried to target cells, which may be at some remote site in the organism. In contrast paracrine hormones for example, Insulin-like growth factor I (IGF-I), can act locally without being released into the blood system.

Hormones can be divided into three groups according to their chemical composition. Firstly the hormones which are derivatives of the amino acid tyrosine, secondly peptide/protein hormones and thirdly the steroid hormones. They act on target tissues through specific receptors found on or within the cell. The steroid hormones are soluble in lipid membranes and can therefore enter the target cell, where they bind to nuclear

receptors, by diffusion. The receptor-hormone complex binds directly to specific sequences in the gene. Due to similarities in their structures and DNA response elements the thyroid hormone receptor is included in the steroid hormone receptor superfamily (Evans, 1988). In contrast, peptide and protein hormones for example, thyroid stimulating hormone (TSH), are hydrophilic molecules that act by binding to their specific cell surface receptors inducing intracellular secondary messengers for example, cAMP (cyclic adenosine -3',5'-monophosphate) or a calcium ion. This leads to the activation of specific *trans*-acting factors (Darnell *et al.*, 1986). Many *trans*-acting factors are phosphoproteins and their actions can be modulated by phosphorylation/dephosphorylation (Rozenfurt, 1986, reviewed in Berk and Schmidt, 1990). For example, the *trans*-acting factor CREB (which binds to cAMP response elements within the promoter of cAMP responsive genes) exists as an equilibrium mixture of transcriptionally active dimers and inactive monomers. Phosphorylation shifts the equilibrium in favour of dimer formation (Yamamoto *et al.*, 1988, Gonzalez *et al.*, 1989).

The major effect of hormones on target cells is at the level of gene expression. For example in *Drosophila* development the steroid hormone ecdysone directly induces changes in chromosome structure, resulting in the appearance of chromosome puffing. This puffing has been shown to be the result of an increase in transcription at the relevant loci, suggesting a link between steroid

hormones and activation of gene expression (reviewed in Ashburner, 1980). Although hormonal control of gene expression can occur at several levels, the primary level is transcriptional via protein-DNA and protein-protein interactions around the controlled gene. This is described in more detail in the following sections.

## I. CIS-ACTING DNA SEQUENCE ELEMENTS.

### A. ELEMENTS THAT REACT WITH TRANSCRIPTIONAL ACTIVATORS.

#### Promoter elements.

Promoter elements are usually found within several hundred base pairs upstream from the transcription initiation site (the cap site) of a gene and are responsible for accurate and efficient transcription. Most of the early work on promoters concerned the  $\beta$ -globin promoter and viral promoters (for example, those of the herpes simplex virus thymidine kinase, adenoviruses and SV40). From these studies it was shown that the promoter consists of multiple *cis*-acting regulatory elements that are required for appropriate function. For example, the SV40 early promoter has three 21bp repeats that each contain two GC boxes. Each GC box has been shown to bind a *trans*-acting factor, Sp1, in a sequence-specific manner (Gidioni *et al.*, 1984).

Transcription initiation requires the assembly of a transcriptional preinitiation complex bound upstream of the cap site. This occurs at a sequence termed the TATA box, which is found 20-30bp 5' of the cap site. The TATA box has been shown to bind the *trans*-acting factor TFIID

(Savadogo and Roeder, 1985) with the sequential binding of TFIIA, TFIIB, RNA polymerase II and lastly TFIIE. In the absence of TFIID no complex can form (Buratowski *et al.*, 1989).

Studies of cellular and viral genes has revealed that many do not contain a TATA box. The promoters of these genes can be divided into two groups. Firstly the GC rich promoters mainly found in "house-keeping" genes such as hypoxanthine phosphoribosyl transferase and dihydrofolate reductase (reviewed in Dynan, 1986). These usually have several transcriptional initiation sites which act as potential binding sites for Sp1. The second class of promoters appear not to have a TATA box nor to be GC rich. The transcription of one such gene, the lymphocyte-specific terminal deoxynucleotidyl transferase gene, is dependent on the presence of a 17bp motif (the initiator) 5' to the structural gene (Smale and Baltimore, 1989). The initiator is required for the assembly of the transcription preinitiation complex, thus allowing transcription in the absence of a TATA box.

#### Enhancer elements.

These are *cis*-acting elements which determine the frequency of transcription from the promoter. They are either located 5' or 3' to the coding region of a gene or within an exon and/or intron. They act in either orientation over large distances and often determine tissue specific expression of a gene (see below). Enhancers have been shown to be composed of multiple *cis*-



acting elements. For example the SV40 enhancer comprises a tandem duplication of 72bp. The generation of point mutations within this region revealed that the enhancer comprises three functional elements. These elements are able to enhance gene transcription through the association of two different elements and tandem repetition of one of the elements can compensate for the absence of another (Herr and Clarke, 1986).

#### Inducible Enhancers.

Some genes, for example the heat shock protein (hsp) genes of *Drosophila* (Pelham & Bienz, 1982) and hormone responsive genes (Yamamoto, 1985), are activated in response to extracellular stimuli. The human metallothionein IIA gene contains both glucocorticoid and mineralocorticoid response elements (Lee *et al.*, 1987). Maximal transcription of this gene is dependent, in part, on hormonal regulation. This is discussed in more detail below.

DNA sequences responsible for regulation by hormones (hormone response elements, HRE), have been identified in many genes. For example progesterone and glucocorticoid response elements (PRE & GRE) have been found in the chicken lysozyme gene (Renkawitz *et al.*, 1984, Steiner *et al.*, 1987), in the long terminal repeats of the murine mammary tumor virus (Miksicek *et al.*, 1987, Cato *et al.*, 1988) and in the human metallothionein IIA gene (Lee *et al.*, 1987). GRE complexes have been shown to operate as enhancers that regulate the rate of transcription from

nearby promoters (Yamamoto, 1985). They act in an orientation-and-position independent manner and can confer glucocorticoid responsiveness to a heterologous promoter when linked to it (Ross and Solter, 1985, Hollenberg and Evans, 1988).

The steroid and thyroid receptors, which have related sequences, bind to the HRE as dimers. The consensus sequence of the response element is palindromic with a 6 base pair half site<sup>(Table 1)</sup>. The GRE and oestrogen response element (ERE) consensus sequences differ by two base pairs in each half site, while the ERE and thyroid response element (TRE) half-site consensus sequence are identical but are spaced differently (Beato *et al.*, 1989).

TABLE 1.

SEQUENCE COMPARISON OF HORMONE RESPONSE ELEMENTS.

OESTROGEN	5'	A	G	G	T	C	A	N	N	N	T	G	A	C	C	T	3'
GLUCOCORTICOID	5'	A	G	A	A	C	A	N	N	N	T	G	T	T	C	T	3'
THYROID	5'		A	G	G	T	C	A	T	G	A	C	C	T		3'	

Schüle *et al.*, (1988) showed that several transcription factors, for example, Sp1, octomer binding proteins and nuclear factor I, when correctly located could act synergistically with the steroid response elements, indicating that maximum gene transcription

involves interactions between *trans*-activating proteins.

#### B. ELEMENTS THAT REACT WITH REPRESSORS.

These elements are responsible for restricting the expression of a gene to a specific cell type and/or specific developmental stage. They are often in close proximity to promoter or enhancer elements. Specific types include the following: a) Silencers restrict expression in a position- and orientation-independent manner. For example a region of the GH gene located at -200 to -300 is required for the repression of transcription in non-pituitary cells (Larsen *et al.*, 1986), and a similar region has also been described in the human albumin gene (Frain *et al.*, 1990). B) Some position-dependent negative regulatory elements are located between the enhancer and the promoter elements. For example Muglia *et al.*, (1986), using chimeric flanking regions and deletion analysis, described a region between the promoter and enhancer of the  $\alpha$ -fetoprotein gene which down regulates transcription in a cell type specific manner. C) Intron repressor sequences have also been reported. For example, an intron in the 5' untranslated region of the chicken feather keratin genes represses transcription in xenopus oocytes (Koltunow *et al.*, 1986). Similarly, sequences in the immunoglobulin kappa gene major intron silence expression in non-B cells (Pierce *et al.*, 1991).

## II. TRANS-ACTING FACTORS.

*Trans*-acting factors bind *cis*-acting elements within a gene. They either act positively (activators) or negatively (repressors) to affect the rate of gene transcription by specific interaction with other proteins.

### A. TRANSCRIPTIONAL ACTIVATORS.

Activators are composed of several domains which are important for efficient transcription. An activation domain is required to interact with target protein(s). The regulatory domains include regions that determine nuclear localisation, phosphorylation and glycosylation and a DNA binding region or a region which interacts with a DNA-binding protein. The binding region is responsible for positioning the protein on or at specific *cis*-acting elements. Activators can be divided into several different classes according to their binding motifs, namely the helix-turn-helix proteins, helix-loop-helix proteins, octamer-binding proteins, leucine zipper proteins and proteins having zinc fingers.

Helix-turn-helix proteins, helix-loop-helix proteins, octamer-binding proteins and leucine zipper proteins.

Helix-turn-helix proteins were first identified in the bacteriophage lambda repressor protein, Cro and the *E. Coli* catabolic activator protein (reviewed by Pabo and Sauer, 1984). The proteins contain two  $\alpha$  helical regions separated by a  $\beta$  turn. They bind as dimers to the DNA,

using both subunits to recognise the target sequences and to stabilise the DNA-protein interaction. The developmental regulatory genes of segmented organisms contain a highly conserved region of 60 amino acids termed the homeobox. This region was shown to have sequence homology with the bacterial helix-turn-helix proteins (Laughon and Scott, 1984, Shepherd *et al.*, 1984). Specific DNA binding is specified by sequences within the homeobox (Desplan *et al.*, 1988).

The helix-loop-helix domain is a DNA-binding and dimerisation motif common to many proteins involved in cell determination. For example, the muscle determination gene MyoD (Davis *et al.*, 1987) and genes in *Drosophila* involved in cell type determination for example, *daughterless* which is involved in both neural and sex determination (Cronmiller *et al.*, 1988) and *twist* which has a role in germ line determination (Thisse *et al.*, 1988). The helix-loop-helix region consists of 2 segments which can form  $\alpha$ -helices connected by a non-conserved "loop".

The octamer transcription factor DNA binding domain contains a homeobox and another conserved region designated the POU-domain. Most of these transcription factors are cell type specific or are developmental regulatory factors.

The leucine zipper motif was first proposed for the CCAAT enhancer binding protein, C/EBP (Landschulz *et al.*, 1988). It consists of four leucine residues that are spaced exactly seven residues apart. This segment was

named the "Leucine zipper" and is required for the dimerisation and subsequent activation of the protein. In addition to the "zipper", a 30 amino acid segment located immediately N-terminal to the leucine repeat must remain intact in order for the protein to bind to the DNA (Landschulz *et al.*, 1988). Many *trans*-acting factors belong to families of proteins. Within the family the proteins can form either homodimers or heterodimers through interactions between their leucine zipper domains. Different combinations have been shown to bind DNA with altered specificity which in turn affects transcriptional activation. For example, in the *Jun/fos* family the heterodimer of *Jun* and *fos* binds to the AP-1 binding site with a higher specificity than the homodimer of *Jun*. The formation of dimers will therefore depend on the relative abundance and distribution of different members of the *Jun/fos* family and may be cell type dependent (Hirai *et al.*, 1989).

#### The zinc finger.

This motif was originally identified as a DNA binding structure in the RNA polymerase III transcription factor TFIIIA, which binds to the internal control region of the 5s RNA gene (Miller *et al.*, 1985). The motif is orientated around a zinc ion, co-ordinated to invariant cysteine and/or histidine residues, and has been termed the "zinc finger". Subsequent work has identified at least two classes of zinc fingers (Table 2).

TABLE 2.

ZINC FINGER CONSENSUS SEQUENCES

TFIIIA type proteins (C<sub>2</sub>H<sub>2</sub> fingers).

(tyr, phe)-X-cys-X<sub>2,4</sub>-cys-X<sub>3</sub>-phe-X<sub>5</sub>-leu-X<sub>2</sub>-  
his-X<sub>3,4</sub>-his-X<sub>n</sub>

Steroid receptor superfamily (C<sub>2</sub>C<sub>2</sub> fingers).

cys-X<sub>2</sub>-cys-X<sub>13</sub>-cys-X<sub>2</sub>-cys-X<sub>15-17</sub>-cys-X<sub>5</sub>-cys-X<sub>9</sub>-cys-  
X<sub>2</sub>-cys-X<sub>4</sub>-cys

Where X represents a variable amino acid (Taken  
from Berg, 1989).

The first class includes the TFIIIA type proteins (C<sub>2</sub>H<sub>2</sub> finger), while the second class (C<sub>2</sub>C<sub>2</sub> finger) includes the steroid receptor superfamily (Evans, 1988, Beato, 1989). It has been shown, in the case of the latter, that DNA binding and transcriptional activation require two zinc fingers (Hollenberg and Evans, 1988) which are encoded by separate exons of the receptor gene (Arriza *et al.*, 1987, Huckaby *et al.*, 1987). Although similar, these two classes of zinc fingers have been shown to differ in their secondary and tertiary structures (reviewed in Schwabe & Rhodes, 1991)

### Inducible *trans*-acting factors.

The steroid hormone receptor superfamily includes the oestrogen, progesterone, glucocorticoid, androgen, retinoic acid, vitamin D<sub>3</sub> and thyroid hormone receptors. The physiological responses to these hormones are mediated through the binding of the ligand to the receptor. Binding induces an allosteric change that allows the receptor-hormone complex to bind its DNA response element in the gene. Dalman *et al.*, (1990), using a cell free translation system, showed that the newly synthesised glucocorticoid receptor is bound to a 90 kDa heat shock protein (hsp90). Binding of the hormone to its receptor dissociates it from hsp90 and transforms the receptor to its binding form. Therefore, the hsp90 can be regarded as a repressor. In contrast, the thyroid receptor can bind to DNA in the absence of hormone. However, the hormone may be required to allow the receptor to initiate transcription by interacting with other *trans*-acting proteins or by altering the DNA structure.

Site directed mutagenesis revealed that the functional domains of the human oestrogen receptor include a hormone binding site, a dimerisation region, a domain which directs sequence-specific DNA binding and a region required for nuclear localisation which is important for hormone activation of gene expression (Kumar *et al.*, 1987, Picard & Yamamoto, 1987).



DNA binding specificity.

The DNA binding domain of the receptor contains an approximately 70 amino acid sequence which is highly conserved and folds to form two zinc fingers that mediate DNA binding. The formation of a chimaeric receptor by replacing the zinc finger region of the oestrogen receptor with that of the glucocorticoid receptor, the so called "finger swapping" experiment, changed the DNA binding specificity of the oestrogen receptor to that of the glucocorticoid receptor, demonstrating that the zinc fingers confer DNA binding specificity. In addition the replacement of either pair of cysteines with histidines within the zinc finger prevented transcriptional activation (Green and Chambon, 1987). Green *et al.*, (1988) created a series of mutations in the oestrogen receptor and showed that the first finger was responsible for binding specificity. More recently it was shown to contain 3 non-conserved amino acids at its base which are required to discriminate between the GRE and ERE (Table 1, Mader *et al.*, 1989). The second finger contains 5 non-conserved amino acids which are located in a loop on the surface of the molecule. This region is required to recognise the spacing difference between the ERE and TRE (Table 1, Umesone *et al.*, 1989).

#### B. TRANSCRIPTIONAL REPRESSORS.

As outlined above correct transcriptional initiation involves both protein-DNA interactions and protein-protein interactions. Selective repression of gene

expression relies on the same mechanisms and is also an important factor in transcriptional control.

Most of the early studies on repression of transcription were investigated in prokaryotes where the binding of a repressor protein to a region near the initiation site disrupts the binding of general *trans*-acting factors such as RNA polymerase (Jacob & Monod, 1961, Ptashne, 1986). Other models for repression have been proposed. The "Quenching" mechanism requires a repressor and an activating protein to bind adjacent DNA sequences. The repressor protein prevents correct protein-protein interaction of the activator with the transcription complex. "Squelching" repression results from the overexpression of an activating protein. This mechanism was first proposed by Gill and Ptashne, (1988) who showed that the over production of the yeast GAL4 regulatory protein resulted in down regulation of expression. This form of repression requires neither specific DNA binding sites nor an intact DNA binding domain.

A number of *Drosophila* homeotic genes have been studied in *Drosophila* cell culture. The studies reveal that these proteins can either activate or repress other homeotic genes. This mechanism is important for the temporal and spatial pattern of expression shown by these genes. For example the *Ultrabithorax* gene product can induce expression from its own promoter but inhibits expression by "quenching" from the *Antennapedia* p1 promoter (Krasnow *et al.*, 1989). In contrast the

*Engrailed* protein blocks the transcription of the *Fushi-tarazu* gene by competing for homeodomain binding sites (Jaynes and O'Farrell, 1988). Certain combinations of activating proteins have been shown to either enhance or repress transcription (Han *et al.*, 1989) suggesting that protein-protein interactions are a crucial step in regulation (Biggins and Tjian, 1989).

The steroid hormones are known to act sometimes as inducers and in other cases as repressors of transcription. For example the rat prolactin gene is induced by oestrogen (Ryan *et al.*, 1979) and repressed by glucocorticoids (Camper *et al.*, 1985). Repression was shown to be due to a region of 63 amino acids adjacent to the DNA binding domain of the glucocorticoid receptor (Alder *et al.*, 1988). Sakai *et al.*, (1988) identified a region in the bovine prolactin gene that conferred repression by glucocorticoids (negative GRE - nGRE). However unlike the GRE the nGRE enhances promoter activity in the absence of the hormone or of the receptor, presumably through interaction with a non-receptor protein. The authors speculate that the hormone-receptor complex either competes with or inactivates this second factor.

As outlined above, the thyroid hormone receptor binds to a variation of the palindromic motif 5' AGGTCATGACCT 3', which is identical to the oestrogen response element, except that the latter contains three non-palindromic nucleotides at the centre of the dyad symmetry. Glass *et al.*, (1988) showed that the thyroid

receptor did not activate transcription from an ERE *in vivo*, but that it did bind to the response element and inhibited induction by oestrogen. Similarly, Graupner *et al.*, (1989) showed that in the absence of thyroid hormone receptor the retinoic acid receptor/retinoic acid complex could induce transcription from two distinct TREs. However when the thyroid hormone receptor was present, but thyroid hormone was not, the retinoic acid receptor/retinoic acid complex failed to induce transcription. The authors therefore suggested that the thyroid receptor may have a dual regulatory role. In the presence of hormone it acts as a transcriptional activator while in the absence of hormone it functions as a TRE dependent repressor.

### III. TISSUE SPECIFIC GENE EXPRESSION.

#### A. CIS-ACTING ELEMENTS.

The first tissue specific enhancer to be identified was in an intron of the immunoglobulin heavy chain (Gillies *et al.*, 1983). Using deletion analysis and transient expression assays many other tissue specific elements have been identified. To quote a few examples of many, within the prolactin and growth hormone genes (Nelson *et al.*, 1986) and in the insulin gene (Walker *et al.*, 1983, Edlund, 1985). Analyses of the human  $\alpha$ 1-antitrypsin gene, in tissue culture, revealed a macrophage specific promoter which is located approximately 2kb upstream from a hepatocyte specific promoter, causing transcription initiation from a more 5' site and inclusion of extra

non-coding exons in the mature message (Perlino *et al.*, 1987).

The ability to introduce foreign DNA into the germ line of animals allows the evaluation in many tissues of the effects of elements responsible for developmental and tissue specific expression (Ornitz *et al.*, 1985, Grosveld *et al.*, 1987, Hammer *et al.*, 1987, Pinkert *et al.*, 1987, Behringer *et al.*, 1988). Transgenic technology also led to the discovery of sequences acting over large distances. Pinkert *et al.*, (1987) found an enhancer located 10kb upstream of the albumin gene. They proposed that this enhancer interacts with the promoter by "looping out" of the intervening sequences. The same element may also be responsible for selective repression in other tissues. These results, however, do not exclude the possibility that there are elements working at even greater distances which are used to "finely tune" the levels of expression.

For genes to be regulated their response elements have to be available to bind *trans*-acting proteins. Most chromosomal DNA is associated with histones, making it relatively insensitive to attack by DNase I. The binding of *trans*-acting factors to *cis*-acting sequences causes localised disruption of the DNA-histone complex resulting in the DNA becoming sensitive to DNase I. Thus the presence of tissue-specific elements is often associated with DNase I hypersensitivity. For example, Hammer *et al.*, (1987) identified a regulatory element in the 5' flanking region of the elastase gene, which could direct

expression of the human growth hormone gene specifically to the exocrine cells of the pancreas in transgenic mice. This element generated a coincident DNase I hypersensitive site in pancreatic DNA but not in other tissues. The sequence also generated a DNase I hypersensitive site when it was relocated 3kb upstream, or within the first intron.

The state of DNA methylation, specifically the hypo-methylation of C's in CpG dinucleotides, could be involved in allowing *trans*-acting factors to bind to the DNA (reviewed in Dynan, 1989). Studies in which methylated and non-methylated genes were introduced into cultured cells suggested that methylation is often incompatible with transcription. Similarly, *in vivo* the control regions of tissue-specific genes tend to be undermethylated in expressing relative to the same sequence in non-expressing cell types. These observations suggest that methylation may play a role in tissue-specific gene expression. Methylation could disrupt transcription in at least two ways. Firstly, it might prevent transcription factors from binding to the DNA. For example, *in vitro* methylation at a cytosine residue within a tissue-specific element in the rat tyrosine aminotransferase gene prevented a *trans*-acting protein from binding (Becker *et al.*, 1987). This mechanism is not universal, however. The GC box transcription factor, Sp1, has been shown to bind to methylated and undermethylated DNA with equal affinity (Harrington *et al.*, 1988, Höller *et al.*, 1988). Secondly, methylation might allow the

binding of repressor proteins. Saluz *et al.*, (1988) showed that in the avian vitellogenin II promoter, a CpG at position +10 was fully methylated in tissues which did not express the gene but undermethylated in expressing cells. Footprinting analysis identified a protein which bound only to methylated sites in the promoter suggesting that it may act as a repressor in non-expressing tissues. More recently Meehan *et al.*, (1989) described a protein, methyl-CpG binding protein, which binds to methylated CpG's in a variety of unrelated DNA sequences.

#### B. TRANS-ACTING FACTORS.

Growth hormone (GH) and prolactin (Prl) receptors have been shown to belong to the same family of membrane proteins (Boutin *et al.*, 1988). These receptors show heterogeneous forms. For example two major hepatic GH receptors have been identified in man (Hocquette *et al.*, 1990) and three in pregnant mice (Smith and Talamantes, 1987). To quote another example, in the rabbit different Prl receptors were found in the liver and the mammary glands (Edery *et al.*, 1989).

The thyroid hormone receptor has been shown to be encoded by two cellular genes, c-erbA $\alpha$  and c-erbA $\beta$ . In the rat the  $\alpha$  gene is alternatively spliced to give erbA $\alpha$ 1 and erbA $\alpha$ 2. The latter binds DNA but not thyroid hormones and can therefore act as an inhibitor of thyroid hormone action (Koenig *et al.*, 1989). The  $\beta$  gene also codes for more than one protein. Hodin *et al.*, (1989) showed that the erb $\beta$ 2 receptor variant was only found in

the pituitary. The biological significance of these different receptor forms is not yet clear but they may be involved in mediating the tissue-specific actions of the hormones.

Footprinting analysis has demonstrated that the expression of some *trans*-acting proteins is restricted to a limited number of cell types. For example, in the pituitary, the GH and Prl genes have been shown to bind a *trans*-acting protein pit-1/GHF-1 which is only expressed in the pituitary lactotrophs and sommatotrophs (Ingraham *et al.*, 1988, Nelson *et al.*, 1988, Castrillo *et al.*, 1989).

Similar results have been obtained for other genes expressed in a tissue specific manner for example, in the liver ( $\alpha$ 1- antitrypsin - De Simone *et al.*, 1987, Courtois *et al.*, 1988, Grayson *et al.*, 1988, Monaci *et al.*, 1988, Costa *et al.*, 1989, Albumin, Lichtsteiner *et al.*, 1987) and the pancreas (Insulin - Edlund *et al.*, 1985, Hanahan, 1985). It would appear that the binding of a tissue-specific *trans*-acting factor on its own is not sufficient for maximum transcriptional activity. For example, Costa *et al.*, (1988) identified three sites in the albumin enhancer two of which bound liver specific factors while the third bound a common factor. All three interactions were required for maximal expression. Similar results were obtained for  $\alpha$ 1-antitrypsin (Costa *et al.*, 1989). Thus transcription involves multiple interactions with tissue-specific and non-tissue-specific transcription factors.



In summary, a given gene contains binding sites for several *trans*-acting factors. These binding sites in conjunction with qualitative and quantitative differences in the *trans*-acting factors in different cell types determine the tissue-specific transcriptional activity of the gene. Transcriptional activity can be modulated by the formation of protein dimers, where homodimer and heterodimer combinations have different affinities for the target binding site. Transcriptional factor activity may also be modulated by ligand binding, (de)phosphorylation, or binding of a repressor to the factor or its target site.

#### ANIMAL MODELS FOR ENDOCRINE STUDIES.

The role of hormones in regulating the expression of hormone-responsive genes can often be examined in cultured cells. Deletion analyses and transient expression assays have led to the identification of hormone response elements. Although such studies are invaluable in providing information regarding the hormonal control of gene expression, they suffer from the disadvantage that gene regulation is examined in an isolated cell type. This situation differs from the case *in vivo* where the availability of a particular hormone may depend on the presence or absence of another hormone. Hormonal feedback loops, the developmental onset of hormone action and the effects of the hormone during

development can only be addressed *in vivo*. Our present understanding of the role a particular hormone plays *in vivo* has come from the study of naturally occurring hormone-deficient animals and from surgically or pharmacologically altered animals. In this thesis I shall discuss only examples of either GH or thyroxine deficiency.

## I. HORMONE-SECRETING CELLS OF THE THYROID AND PITUITARY AND THE REGULATION OF SECRETION.

Hormone-secreting cells are often close to or interspersed with other cell types. The thyroid gland contains thyroxine-producing thyrocytes and calcitonin-producing C-cells and is in intimate contact with the parathyroid gland which secretes parathyroid hormone. The secretion of thyroid hormones ( $T_4$  and  $T_3$ ) is under the control of the pituitary thyroid stimulating hormone (TSH). Similarly, the anterior pituitary contains phenotypically distinct cell types which express different hormones, namely somatotrophs (GH); lactotrophs (Prl); thyrotrophs (TSH); adrenocorticotrophs (adrenocorticotrophin) and gonadotrophs (luteinizing hormone and follicle stimulating hormone). Secretion of these hormones is largely under the control of the hypothalamus which releases hormones that act to stimulate hormone-secretion by specific cell types in the pituitary. For example growth hormone releasing factor (GRF) acts on the somatotrophs and TRH acts on the thyrotrophs. Some factors act in an inhibitory manner for

example, somatostatin inhibits GH secretion. Hormone synthesis and release is also modulated by negative feedback loops, initiated by the induced target cell, or by the hormone itself.

## II. ALTERING THE HORMONAL STATUS OF AN ANIMAL.

Classical methods for generating animal models with either GH or thyroxine deficiencies involved the removal of the anterior pituitary, the thyroid or the hypothalamus. This was achieved either by surgery or laser ablation. Since these tissues contain several hormone-secreting cell types, removal results in several hormone systems being affected, either directly by the removal of hormone-producing cells or indirectly by the disruption of feedback loops. This makes the attribution of an effect to a particular hormone difficult in many cases. However, if hormone replacement by a physiological administration regime reverses the induced defect completely, the problem of the potentially pleiotropic effects of surgery is averted.

Several other approaches have also been used. These can be divided into three groups according to their actions: (1) inhibition of hormone synthesis, (2) inhibition of hormone secretion and (3) inhibition of hormone action.

Synthesis of the thyroid hormones can be inhibited by the administration of anti-thyroid drugs such as methimazole. These drugs prevent the iodination of the tyrosyl residues in the thyroglobulin protein (Narayan *et*

*al.*, 1985). The drug propylthiouracil acts in a similar manner and in addition decreases the peripheral deiodination of thyroxine to its active form triiodothyronine. However these drugs are associated with side effects and show varying degrees of penetrance (Horvath *et al.*, 1989, Wilson *et al.*, 1990). Removal of the drug results in the restoration of normal thyroid hormone formation.

Secretion of hormones can be inhibited by preventing the production of the regulatory hormones. For example, treating neonatal rats with high levels of monosodium glutamate destroys the neurons in the hypothalamus required for the production of GRF. This results in the complete inhibition of GH release from the pituitary (Bakke *et al.*, 1978, Waxman *et al.*, 1990). Somatostatin can also be used to inhibit GH release but secretion of gastrin, glucagon, vasoactive intestine peptide and insulin is also inhibited (reviewed in Wass, 1988).

Antibodies or antagonists have been used to prevent the actions of a hormone. The biological activity of GRF has been neutralised by antibodies (Cella *et al.*, 1990) and its induction of GH secretion have been blocked by the use of peptide antagonists (Lumpkin *et al.*, 1989). Similarly the action of GH in rats was blocked by treatment with anti-GH antiserum (Gause *et al.*, 1983, Madon *et al.*, 1986, Flint and Gardner, 1989).

### III. MUTANT HORMONE-DEFICIENT ANIMAL MODELS.

Naturally occurring mutations have been extensively

used in endocrine studies. There are several mouse mutants which exhibit dwarfism: Ames dwarf (*df*), Snell dwarf (*dw*) and little (*lit*). All three have autosomal recessive modes of inheritance but exhibit differences in their pituitary hormone deficiencies (Phillips *et al.*, 1982). The little mouse has decreased levels of GH and Prl but can be corrected by GH treatment. However the pituitary is unresponsive to GRF suggesting that the primary defect in these mice involves the GRF receptor (Jansson *et al.*, 1986). The Ames and Snell dwarfs are phenotypically similar (they resemble the panhypopituitary dwarfs in humans) but the mutations are non-allelic. Recently the Snell dwarf has been shown to be the result of a mutation in the Pit-I gene (Camper *et al.*, 1990). Pit-I is a pituitary specific *trans*-acting factor that is required for transcription of the GH and Prl genes. The Ames and Snell dwarfs show decreased serum levels of GH, Prl and TSH. The reduced TSH level results in the mice also being hypothyroid. The pituitaries lack detectable somatotrophs and GH mRNA (Cheng *et al.*, 1983). Another dwarf mutation, Pygmy, appears to result in a lack of response to GH in the target tissue. Serum levels of GH and Prl are normal although pituitary mRNA levels are low. These mice do not respond to treatment with GH (Rimoin *et al.*, 1972) or Prl treatment (Sinha *et al.*, 1979).

There are two mouse models for hypothyroidism. The *hyt/hyt* mice show congenital hypothyroidism in the absence of goitre (enlargement of the thyroid gland is

often associated with a reduction of thyroid hormones). The mutation is first detected 15 days after conception when the thyroid becomes active (Kawaori and Tsuneda, 1985). Mature mice show decreased levels of circulating thyroid hormones and a 100 fold increase in TSH levels due to the disruption of the feedback loop whereby thyroid hormones regulate TSH production. Thyroglobulin synthesis is normal and the protein is iodinated (Stein *et al.*, 1989). The histology of the gland shows follicle structures which are smaller and fewer than normal (Beamer *et al.*, 1981). *Hyt/hyt* mice show secondary somatic and behavioral defects, for example retarded eye opening, ear raising and a decrease in body length and weight (Adams *et al.*, 1989), probably due to the deficiency of thyroid hormones *in utero* and during early neonatal life. The lesion in *hyt/hyt* mice is thought to be a lack of response of the thyroid gland to TSH. The second hypothyroid model, *cog/cog*, presents goitre. These *cog/cog* mice are a model for human hypothyroid goitre, which is caused by a defect in thyroglobulin synthesis and/or processing (Mayerhofer *et al.*, 1988).

The use of naturally occurring mutations in endocrine studies is not always ideal as other hormone systems may be affected (for example, the dwarf mice referred to above) and secondary defects can occur during development (for example, the hypothyroid mice referred to above). Furthermore all mice carrying the above mentioned mutations are characteristically sub-fertile.

#### IV. TRANSGENICS IN THE STUDY OF ENDOCRINE SYSTEMS.

Foreign DNA introduced either by proviral infection (Jaenisch, 1976, 1977) or by pronuclear injection (Gordon *et al.*, 1980) can integrate into the chromatin of the germ line and somatic cells. Microinjection usually results in multiple copies of the transgene being integrated into the chromosome at random, often forming concatemers lying in a head-to-tail configuration. Expression levels of the transgene between individual transgenic mice are highly variable due to the random integration of the transgene into the chromosome. For example, the foreign DNA may integrate into a "silent" region of genomic DNA. The level of expression of most transgenes introduced into transgenic animals does not correlate with the number of transgenes present in the genome. This is thought to be due to the influence of the DNA sequences flanking the site of transgene integration (reviewed in Bishop & Al-Shawi, 1990, Al-Shawi *et al.*, 1990). However, exceptions to this have been observed. For example, in the study of human  $\beta$ -globin genes the expression was shown to be dependent on the site of integration and to be copy-number-independent. The discovery of a locus control region which, when included, made the expression of the transgene more position-independent and more copy-number-dependent (Grosveld *et al.*, 1987, Ryan *et al.*, 1989). A similar locus control region has also been identified in the CD2 genes (Lang *et al.*, 1988) and the  $\beta$ -lactoglobulin genes (Whitelaw *et al.*, 1992). The effect of chromosomal position on the

expression of transgenes may be the result of the organisation of chromosomes into topologically constrained loops which serve as functional domains. These loops are thought to be established by scaffold attachment regions. By flanking transgenes with a 5' scaffold attachment site the expression of a transgene is protected from the positional effect of integration (Stief *et al.*, 1989). Recently the expression of a whey acidic protein transgene was shown to be accurately expressed in a position-independent, hormonal and developmental manner in transgenic mice only when the transgene was flanked with scaffold attachment sites (McKnight *et al.*, 1992).

The use of transgenic mice in studying hormonal action *in vivo* has the advantage that the effects of over expressing a particular hormone can be examined in an otherwise normal physiological context. Most of the reported transgenics, which have involved the over production of a hormone, have mainly been concerned with the regulation of somatic growth. This was investigated through the over production of foreign GH (Palmiter *et al.*, 1982, 1983, reviewed in Brem *et al.*, 1989), GRF (Hammer *et al.*, 1985) or IGF-I (Behringer *et al.*, 1990). These studies confirmed that GH plays a central role in regulating IGF-I expression and that GH and IGF-I exert separate growth promoting actions (Behringer *et al.*, 1990).

Transgenics have also been used to create animal models lacking a particular hormone. This can be achieved



by two methods namely by homologous recombination and by the selective destruction (ablation) of a particular cell type.

#### A. HOMOLOGOUS RECOMBINATION.

Mutations can be generated through gene targeting which allows mice to be generated which carry a null mutation and may theoretically allow modification of a gene to affect transcription, translation or post translational events. Gene targeting involves the use of recombinant DNA technology to introduce a mutation into the cloned DNA sequence of interest. The DNA sequence carrying the mutation is then transferred by homologous recombination to the genome of embryo-derived stem (ES) cells. Cell clones containing the mutated sequence can be selected for or identified by using the polymerase chain reaction. Cells from an appropriate clone are then microinjected into a mouse blastocyst. Germline transmission of the mutated gene from the resulting chimaeras results in offspring with the acquired characteristic (reviewed by Capecchi, 1989). To quote one example of many, using this method Dechiara *et al.*, (1990) showed IGF-II has an important physiological role in embryonic growth.

#### B. TRANSGENIC ABLATION.

Ablation of specific cell types in transgenic animals relies on the fact that specialised cells often produce a single major protein product for export. Cell

culture and deletion studies have identified *cis*-acting regulatory regions within the promoter of the genes which encode these proteins. These regulatory regions are involved in the tissue-specific expression of the protein and can be linked to the coding region of a foreign gene to drive its expression in a tissue-specific manner in transgenic animals. By linking these regulatory regions to genes coding for a toxic product the target cell is effectively knocked out.

Two methods of transgenic ablation have been successfully employed: direct and conditional. In the direct method the toxic gene product is expressed under the control of the transgene promoter and thus takes effect as soon as the promoter is developmentally activated. In the conditional method the product of the foreign gene, although expressed, shows no toxicity until a toxigenic substrate is administered.

#### Direct Ablation.

Diphtheria toxin (DT - Uchida *et al.*, 1973) and ricin are potent toxins, each of which consists of two subunits A and B. The B subunit is necessary for the entry of the toxic A subunit into cells. Thus if only the A subunit is produced, neighbouring cells cannot take it up from dying cells. In the first demonstration of direct ablation the expression of the DT-A gene was directed to the pancreatic exocrine cells under the control of the elastase I promoter (Palmiter *et al.*, 1987). This promoter becomes active at day 14 of gestation.

Transgenic mice were born with no pancreas and death followed several days later.

Other systems have been studied using the direct ablation method. These include expression of the DT-A gene in the pituitary under the control of the GH promoter (Behringer *et al.*, 1988) or the pituitary hormone glycoprotein  $\alpha$ -subunit (Kendall *et al.*, 1990) and in the lens under the control of the gamma-2-crystallin promoter (Breitman *et al.*, 1987), and expression of the ricin A subunit in the lens under the control of the  $\alpha$ -A-crystallin promoter (Landel *et al.*, 1988).

The direct method of ablation has several drawbacks. Firstly since the toxin is expressed directly under the control of the transgene promoter, it is produced and takes affect as soon as the tissue-specific promoter becomes developmentally active. This allows no experimental control over the extent or timing of ablation. This drawback has been recently addressed in *Drosophila*. By expressing a temperature-sensitive mutant ricin A subunit under the control of an eye specific enhancer, Moffat *et al.*, (1992) demonstrated controlled specific ablation of *Drosophila* eye cells at the permissive temperature. Clearly, however, this method is not applicable to mammals. A second drawback is that, since the DT-A gene cannot be removed from the transgenic animal, the regeneration potential of cell lineages can not be assessed. Thirdly, direct ablation may result in embryonic or early neonatal lethality for example, the transgenic mice in which the DT-A gene was directed to

the pancreas died a few days after birth (Palmiter *et al.*, 1987), thus allowing no pedigree lines to be established. Fourthly, the toxicity of the DT-A gene itself may pose a potential problem. It has been estimated that 1 molecule of DT-A is sufficient to kill a cell (Yamaizumi *et al.*, 1978). Due to the extreme toxicity of the DT-A gene successful ablation therefore requires that the expression of the transgene is confined to one cell type. If the promoter used for the transgene permits even low levels of expression outside the intended lineage, ablation could be lethal to the developing embryo. To overcome this problem an attenuated form of the DT-A subunit has been used in ablation studies (Maxwell *et al.*, 1987). This subunit is approximately 50-fold less toxic than the wild type A subunit (Breitman *et al.*, 1990).

Homologous recombination has the advantage over transgenic ablation that only the product of the gene of interest is disabled, as opposed to ablation of the cell-type in which it is produced. As a consequence, many questions that cannot be addressed by ablation are open to investigation by homologous recombination. However, as in the case of direct ablation this method does not allow external control over the timing of functional disruption. Furthermore in some instances the function of a cell type may be of more physiological importance than that of an individual gene. Thus transgenic ablation can be an important tool in specific cases

### Conditional Ablation.

The technique of conditional ablation presently in use is based on the expression of herpes simplex virus type 1 thymidine kinase (HSV1-TK) in specific cell types. HSV1-TK is itself not deleterious to most cells. However HSV1-TK is able to convert certain nucleoside analogues such as acyclovir, ganciclovir and FIAU, by phosphorylation, into nucleoside monophosphates and diphosphates (Elion *et al.*, 1977, Fyfe *et al.*, 1978). These are in turn phosphorylated by cellular kinases to nucleotide triphosphates, which are incorporated into the DNA resulting in cell death (St. Clair *et al.*, 1987). These analogues are phosphorylated to a lesser degree or not at all by cellular kinases. The degree of ablation is proportional to the levels of HSV1-TK expression and to the analogue concentration (Borrelli *et al.*, 1988). By altering the concentration of the analogue used and/or manipulating the duration of the time of application the degree of ablation can be controlled. The analogue can be administered at different times in the life of the animal, thus allowing some control of the time of ablation. An additional advantage is that the animal develops normally up to the time of ablation. Removal of the analogue potentially allows cell lineages to regenerate thus giving both information about and access to stem cells in a particular tissue. It appears however that one important prerequisite for the success of this method may be ongoing division of the target cells.

Heyman *et al.*, (1989) placed the HSV1-tk gene under

the control of an immunoglobulin promoter. The transgenic mice generated expressed the protein in a tissue-specific manner. The effect of ganciclovir treatment on thymic cells expressing the T-cell surface antigens L3T4 and  $\text{Lyt}^{2+}$  was investigated. Cells expressing both markers were depleted by a factor of 4, while single positives and double negatives were enriched 3- to 5-fold and 6-fold respectively. Such enriched populations contain progenitor cells capable of repopulating thymic tissue in irradiated animals (Fowifes *et al.*, 1985). Following removal of the ablating agent the thymic cells were repopulated indicating that the stem cell population that survived ablation allowed regeneration of the tissue.

Similarly, Borrelli *et al.*, (1989) directed the expression of HSV1-TK to the mouse somatotrophs and lactotrophs using the rat GH and Prl promoters respectively. In the mice carrying the GH-TK construct HSV1-TK was expressed in the sommatotrophs. Mice were treated with FIAU from embryonic day 5 to birth and from 2 days to 8-10 weeks. Weight gain of both transgenics and non-transgenic littermates were similar up to three weeks of age. Subsequently the transgenics developed as dwarfs. The pituitaries of these mice showed a massive depletion of both somatotrophs and lactotrophs. A similar effect was observed when somatotrophs were directly ablated using a GH-DT-A transgene (Palmiter *et al.*, 1987), and can be explained by supposing that the GH promoter is active in a cellular precursor of lactotrophs and somatotrophs. When the administration of FIAU to GH-TK

mice was delayed until 5 and 10 days after birth, the number of somatotrophs that developed increased progressively, although there was no increase in the number of lactotrophs. This result argues against the lineal pathway of lactotrophs differentiation from somatotrophs proposed by Behringer *et al.*, (1988).

In contrast the mice containing the Prl-TK transgene showed no morphological change to their pituitaries following FIAU treatment. Based on the assumption that ablation requires cell division in the target tissue the authors concluded that the lactotrophs escaped ablation due to differentiation being a post mitotic event. However although HSV1-TK activity was present in the pituitary of Prl-TK mice, localisation to the lactotrophs was not demonstrated. The conclusion is therefore questionable.

## THE THYROID GLAND.

### I. HISTOLOGY OF THE THYROID GLAND.

The thyroid gland consists of two lobes which lie between the thyroid cartilage and the fifth and sixth tracheal rings. The lobes are connected by a thin isthmus.

The hormone secreting unit of the thyroid is the follicle. This comprises a single layer of epithelial cells (thyrocytes) surrounding a cavity (lumen). The latter is filled with colloid containing a store of

thyroglobulin. The follicles vary in size depending on the activity of the gland. In hypothyroidism they are flat and narrow, whereas in hyperthyroidism they develop a columnar appearance. The follicles are close to a network of capillaries, which allows a large flow of blood to pass through the gland. Sympathetic nerve fibres, comprising adrenergic and cholinergic fibres, are also abundant. These nerves influence the blood flow through the gland as well as follicle activity. In the rat, immunoreactive thyroglobulin is first detected in the cytoplasm of the immature thyroid epithelium on the 15<sup>th</sup> day of gestation with formation of the thyroid hormones (thyroxine: T<sub>4</sub> and triiodothyronine: T<sub>3</sub>) two days latter (Kawaori and Tsuneda, 1985).

The thyroid gland contains another hormone producing cell type, the C-cells, which secrete calcitonin. These cells lie between the follicle structures or on the follicle wall. The thyroid is also in contact with the parathyroid gland. In the mouse there are two parathyroid glands which are bilaterally attached to the thyroid lobes.

## II. EXAMPLES OF THYROID HORMONE ACTION.

Thyroxine deficiency during development has long been associated with mental retardation. This is due to the involvement of thyroid hormones in brain maturation. For example hypothyroidism results in retardation in nerve fibre myelination, a decrease in the density of the axon network and reduced interactions between the nerves



(Eayrs, 1960, Clos *et al.*, 1973).

Thyroid hormones act on the myosin multi-chain family in a developmentally specific manner. There are at least six different myosin heavy chain (MHC) genes, which all respond to thyroid hormones (Izumo *et al.*, 1986). The  $\beta$  MHC gene is predominantly expressed during late fetal stages and declines after birth, with the subsequent activation of the  $\alpha$  MHC gene. In hypothyroid rats, the  $\beta$  MHC gene predominates but declines with the subsequent activation of the  $\alpha$  MHC gene, following  $T_4$  treatment, suggesting that the switch between  $\alpha$  and  $\beta$  MHC genes is regulated by thyroid hormones (Mahdavi *et al.*, 1984). A similar effect is observed in the expression of the ubiquitous transmembrane protein Na-K-ATPase which consists of  $\alpha$  and  $\beta$  subunits. The  $\alpha$  subunit has three distinct isoforms that are expressed in a developmental sequence which is directly regulated by both thyroid hormones and glucocorticoids (Orlowski and Lingrel, 1990).

Species-specific effects of thyroid hormones are also observed. For example, there is a functional TRE in the promoter region of the rat GH gene but not in the promoter of the human gene (Glass *et al.*, 1987, Koeing *et al.*, 1987). There is a second TRE in the third intron of the rat GH gene which can confer thyroid hormone responsiveness to a heterologous promoter *in vivo* (Sap *et al.*, 1990).

Thyroid hormones are the main product of the thyroid gland. The structure of the gland, its role in thyroid

hormone synthesis and secretion, and the regulatory influences controlling these processes are discussed in detail below.

### III. CIRCULATING THYROID HORMONES.

The structure of the iodotyrosine hormones are depicted in Figure 1. In addition to the biologically active  $T_4$  and  $T_3$ , an inactive triiodothyronine (reverse  $T_3$ ) is also found in significant concentrations in the serum.

In the thyroid gland the thyroid hormones form an integral part of the thyroid-specific protein, thyroglobulin, as part of which they are synthesised and stored. In the circulation the thyroid hormones are reversibly bound to serum liver proteins: thyroxine-binding globulin, thyroxine-binding prealbumin and albumin. Studies on the kinetics of release and metabolism of iodothyronines in the body show that  $T_4$  has a longer half-life (approximately 7 days) than has  $T_3$  (approximately 1 day: Sterling & Lazarus, 1977).

$T_4$  is the principal secretory product of the thyroid. However the active form of the hormone is  $T_3$  which is produced by a 5'-deiodination of  $T_4$ . Deiodination is also important in the inactivation of the thyroid hormones (Figure 2). Deiodination is brought about by deiodinating enzymes (see Nunez, 1988). Type I is the major enzyme found in the liver, kidney and skeletal muscle; it carries out either 5'- or 5-deiodination of  $T_4$  to yield  $T_3$  or  $rT_3$  respectively. Type

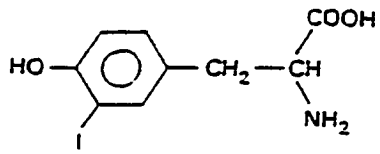
**FIGURE 1.**

**STRUCTURE OF MONO AND DIIODOTYROSINE.**

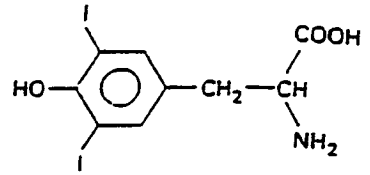
Structures of mono and diiodotyrosine, the precursors to the thyroid hormones: thyroxine ( $T_4$ ) and 3, 5, 3'-triiodothyronine ( $T_3$ ).

Also shown is the structure of the biologically inactive 3, 3',5'-triiodothyronine (reverse  $T_3$ ).

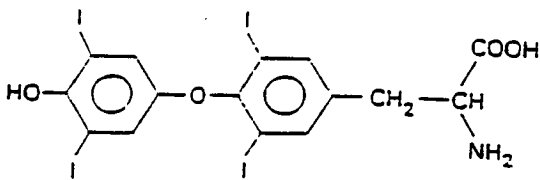
(Taken from O'Riordan *et al.*, 1988).



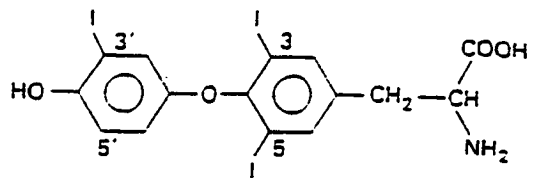
Monoiodotyrosine



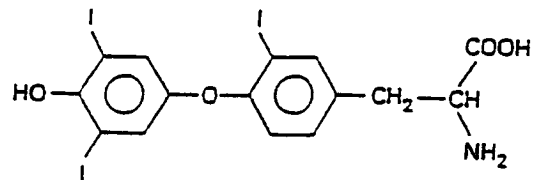
Diiodotyrosine



Thyroxine (T4)



3,5,3'-Triiodothyronine (T3)



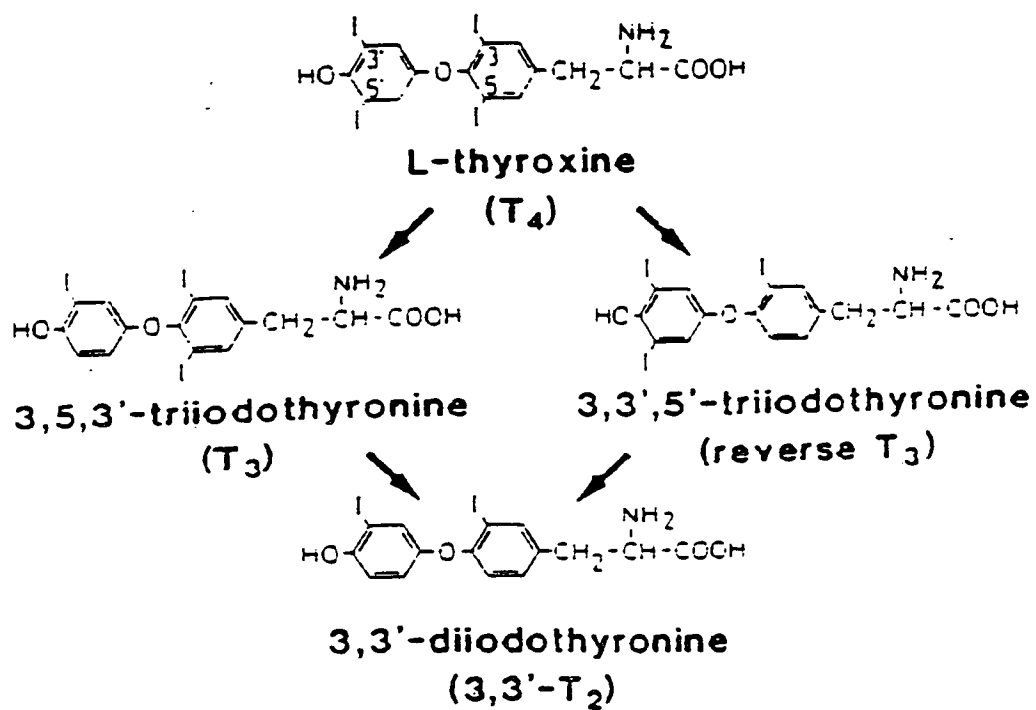
3,3',5'-Triiodothyronine  
(reverse-T3)

FIGURE 2.

MONOIODINATION OF THYROID HORMONES.

Monodeiodination of iodothyronines, giving rise to the conversion of  $T_4$  to  $T_3$  and eventually the inactivation of the hormone by conversion to 3,3'-diiodothyronine.

(Taken from Dumont *et al.*, 1989).



II is found in the brain, pituitary and brown adipose tissue. It is responsible for 5'- deiodination of  $T_4$  which is important for providing the brain with  $T_3$  and also in controlling TSH secretion and synthesis. Lastly type III is found in the heart, liver and central nervous system and is responsible for metabolising  $T_4$  to  $rT_3$ .

Selenium deficiency results in abnormal thyroid hormone metabolism. The abnormality arises because selenium is required for the synthesis of active type I deiodinase (Beckett *et al.*, 1989, 1990). The mRNA that specifies this deiodinase contains a selenocysteine<sup>TGA</sup> codon (Berry *et al.*, 1991), and when selenocysteine is unavailable an inactive enzyme is produced.

#### IV. THYROID HORMONE SYNTHESIS AND SECRETION.

Thyroid hormone synthesis is a multi-step process which includes the uptake of iodide ions, the synthesis of thyroglobulin, the iodination of thyroglobulin and the release of  $T_4$  and  $T_3$ . The mechanisms of hormone synthesis and release are discussed below.

##### A. THE IODIDE PUMP.

The kidney and the thyroid gland are the main organs in the body which can clear significant amounts of iodide ions from the circulation. In addition, the thyroid gland takes up iodide ions and concentrates it in the follicle lumen. The iodide ions pass through both the basal and the apical membrane (Andros and Wollman, 1967). Transport across the basal membrane is an active process involving

Na<sup>+</sup>/K<sup>+</sup> ATPase to pump the iodide against a concentration gradient (up to 5-300 times higher in the thyroid than the plasma, see Dumont *et al.*, 1989). Iodide ion uptake is TSH-dependant and is regulated by  $\alpha$ -adrenergic nerves (Santisteban *et al.*, 1987). Within the follicle lumen the iodide ion undergoes oxidation by a membrane-bound thyroid peroxidase, at a rate linked to the iodination of thyroglobulin.

#### B. THE THYROGLOBULIN GENE.

Thyroglobulin (Tg) is the major product of the thyroid gland. Civitareale *et al.*, (1989) identified a thyroid specific *trans*-acting factor, TTF-I, which acts positively in mediating the thyroid-specific expression of the Tg gene. Transcription of the gene constitutes approximately 10% of the total polymerase II activity of the gland (van Heuverswyn *et al.*, 1984). The bovine Tg gene spans 200 kb, and contains at least 42 exons (de Martynoff *et al.*, 1987). Transcription is regulated by the anterior pituitary hormone TSH (also known as thyrotropin, Van Heuverswyn *et al.*, 1984), which mediates its effects through the production of cAMP (Van Heuverswyn *et al.*, 1985). A cAMP response element (CRE) has been identified in the first 250bp of the Tg promoter (Christophe *et al.*, 1989). The CRE differs from those of other higher eukaryotes both in its position and sequence, having more homology with those of lower eukaryotes, where cAMP alone is not sufficient to activate transcription (Christophe *et al.*, 1989 and



references therein). This is consistent with the observation that maximal expression of the Tg gene also requires stimulation of the thyrocytes by IGF-I and insulin (Santisteban *et al.*, 1987). Thus expression of the Tg gene involves multi-hormone stimulation.

The thyroglobulin protein is synthesised on the rough endoplasmic reticulum and is primarily glycosylated as it enters the cisternae. Transport vesicles move the protein to the Golgi apparatus where it is further glycosylated (to approximately 10% of total weight). The resultant protein,  $M_r = 660,000$ , comprises two chains of equal length. The glycoprotein is transported to the apical membrane, where the protein is emptied into the lumen of the follicle by exocytosis (see below).

### C. IODINATION OF THE THYROGLOBULIN PROTEIN.

Thyroglobulin iodination occurs in the lumen of the follicle structure, primarily at the interface between the apical membrane of the thyrocyte and the lumen. The iodide ion undergoes oxidation by a membrane-bound peroxidase associated with the endoplasmic reticulum and the apical membrane. It is activated by hydrogen peroxide ( $H_2O_2$ ) and iodide ions. However little is known about how the  $H_2O_2$  is generated (see Nunez and Pommier, 1982). Oxidation of the iodide ion yields an active form which diffuses into the lumen where it is transferred to an acceptor tyrosyl residue of the thyroglobulin protein at position 3 or at position 3 and 5 to yield monoiodotyrosine (MIT) and diiodotyrosine (DIT)

respectively (Figure 1).

Approximately one third of the tyrosyl residues of thyroglobulin are iodinated and about a quarter of those iodinated are joined to form the thyroid hormones. The positions of the iodinated tyrosyls have been shown to be evolutionary invariant (Palumbo, 1987).

Formation of  $T_4$  and  $T_3$  involves a molecular rearrangement between two iodotyrosyl residues (two molecules of DIT or one of each DIT and MIT). The bond between the aromatic ring of a mono or diiodotyrosyl residue and its alanyl side chain is broken and the ring is transferred to the phenolic hydroxyl group of another peptide-linked diiodotyrosyl residue. The coupling reaction occurs within the thyroglobulin protein (Figure 3) to yield either  $T_4$  or  $T_3$ . The iodinated protein is stored in the lumen of the follicle where some further iodination may occur. The ratio of MIT to DIT produced is dependent on dietary iodide ion intake, with greater amounts of MIT being produced during dietary deficiency.

#### D. SECRETION OF THE THYROID HORMONES.

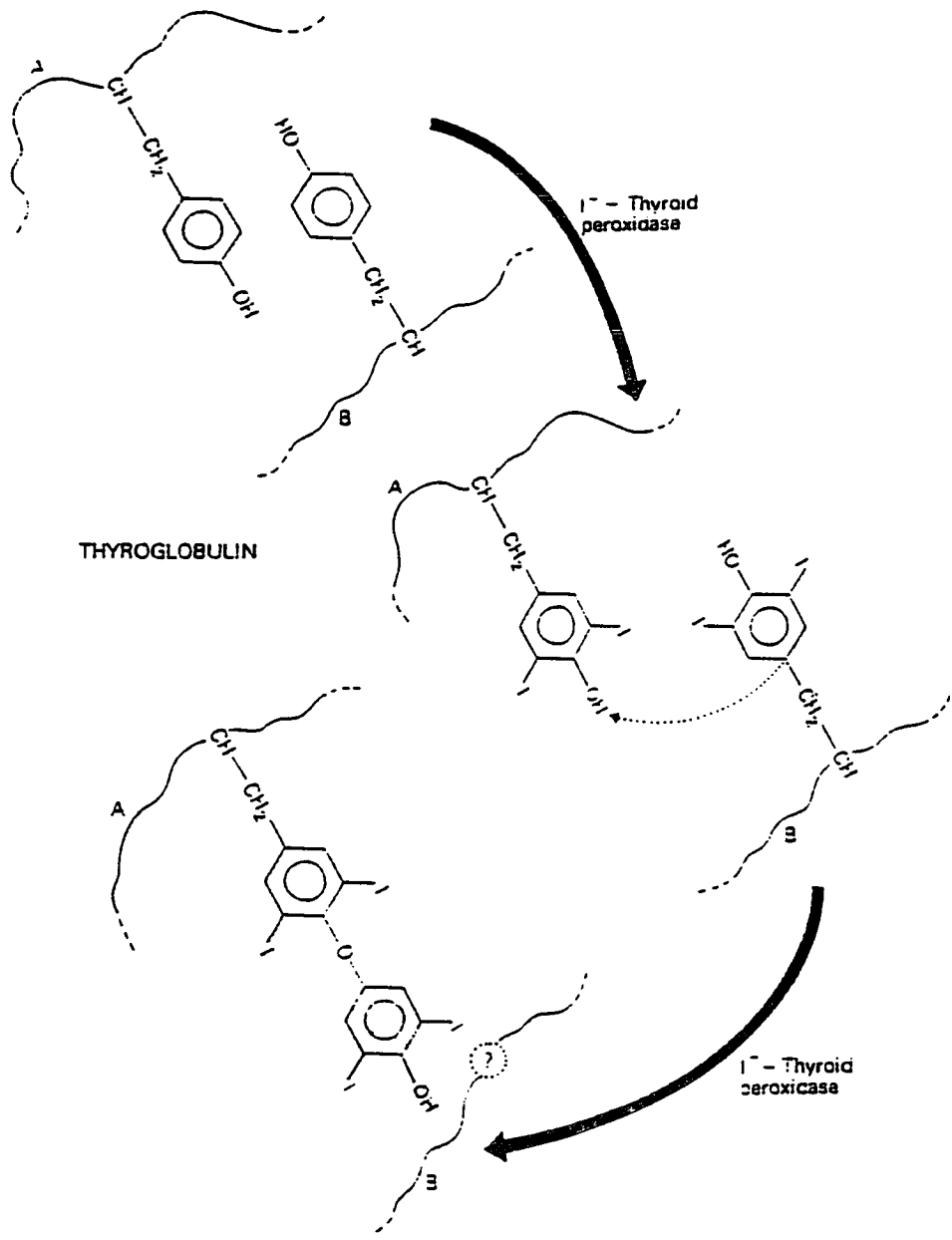
For the thyroid hormones to be released the iodinated thyroglobulin protein has to undergo proteolytic cleavage. Before this can occur the iodinated protein has to re-enter the thyroid follicle cell, and this occurs by endocytosis. Two types of endocytosis have been described: macropinocytosis and micropinocytosis. The former involves the formation of pseudopodia which trap colloid as they retreat, forming membrane-enclosed

**FIGURE 3.**

**IODINATION OF THE THYROGLOBULIN PROTEIN.**

Synthesis of the thyroid hormones. Two chains of thyroglobulin (labelled A and B) each carrying a tyrosyl residue are shown. Iodination by the thyroid peroxidase introduces two atoms of iodide into each tyrosyl residue. The dotted line indicates the molecular rearrangement which follows to yield the structure of thyroxine ( $T_4$ ). If the tyrosyl residue on the B-chain is a monoiodotyrosine then  $T_3$  is formed.

(Taken from O'Riordan *et al.*, 1988).



colloid droplets. These droplets fuse with lysosomal vesicles in the follicle cell, which leads to the breakdown of the thyroglobulin protein to yield  $T_4$ ,  $T_3$ ,  $rT_3$  and iodinated amino acids. The latter are recycled within the gland. This process involves a large turnover of the apical membrane, and is the first response of the thyroid to TSH. The thyroid hormones are released into the plasma where they are transported bound to specific serum binding proteins. In contrast, micropinocytosis is thought to be responsible for the constitutive production of the thyroid hormones which is not dependent on TSH stimulation.

## V. REGULATION OF THYROID GLAND ACTIVITY.

The activity of the follicle cell is controlled by the anterior pituitary hormone TSH. Serum levels of TSH are the result of positive hypothalamic control exerted by the thyrotrophin releasing hormone (TRH) and negatively by circulating  $T_3$  feeding back onto the pituitary. This control is described below.

### A. THYROTROPHIN RELEASING HORMONE.

TRH is a weakly basic tripeptide which is secreted by the hypothalamus into the specialised portal system that drains to the anterior pituitary where TRH binds to specific cell surface receptors on the thyrotrophs (TSH producing cells of the anterior pituitary). The action of TRH on the pituitary is rapid, resulting in the release of TSH and synthesis of new hormone. Deléan *et al.*,

(1977), using pituitary cells, showed that thyroid hormones and oestrogen respectively decrease and increase the binding capacity of the thyrotrophs for TRH. The decrease in capacity induced by thyroid hormones was subsequently shown to be due to modulation of the level of the TRH receptor (Gershengam, 1978, Hinkle *et al.*, 1981, 1982). Thyroid hormones may also act directly on the adrenergic neurons in the hypothalamus to reduce the rate of TRH secretion (Belchetz *et al.*, 1978).

#### B. THYROID STIMULATING HORMONE.

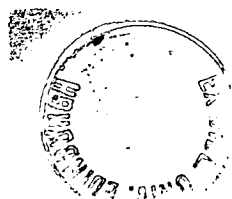
TSH comprises two subunits which are encoded by separate genes. The  $\alpha$ -subunit is shared with other pituitary hormones, follicle stimulating hormone and luteinizing hormone, and biological specificity is due to the  $\beta$ -subunit (Pierce and Parsons, 1981). The transcription of the  $\alpha$ - and, and a greater extent, the  $\beta$ -subunit genes has been shown to be induced by  $T_3$  (Shupnik *et al.*, 1985). The subunits join to form a glycoprotein with  $M_r = 28,000$ . TSH stimulation of thyrocytes leads to increased transcription of the Tg gene and to increased iodide ion uptake by the thyroid gland. TSH also controls the growth of the gland, with prolonged chronic administration inducing proliferation of thyrocytes, capillaries and fibroblasts (Dumont, 1971). TSH binds to a specific seven-pass transmembrane receptor on the plasma membrane of the thyrocyte leading to the activation of adenylate cyclase (Moore and Wolff, 1974). Transcription activation by cAMP is regulated by cAMP-

dependent protein kinase (PKA). PKA phosphorylation leads to the activation of CREB which binds to the CRE in the Tg gene leading to transcriptional activation. Although most of the actions of TSH on the thyroid gland are mediated by cAMP it has recently been shown that the binding of TSH to the membrane receptor also activates phospholipase C (Van Sande *et al.*, 1990), which leads to the hydrolysis of PIP<sub>2</sub> to IP<sub>3</sub> with the subsequent release of Ca<sup>2+</sup>. The activation of diacylglycerol activates PKC which in turn activates the serum response factor, and members of the *Jun/fos* and AP-1 groups of transcriptional factors.

The thyroid gland itself can regulate the rate of production and secretion of thyroid hormones (autoregulation). Little is known about this control mechanism but hormone secretion in the absence of TSH stimulation occurs in response to small changes in the plasma iodide ion levels or total thyroid iodide ion content (Sarne and Degroot, 1989). In addition, circulating thyroid hormones and thyroglobulin have been reported to act directly on the thyroid gland to decrease hormone production and secretion (Friedman *et al.*, 1977, Bech *et al.*, 1981).

## VI. CELLULAR ACTIONS AT THE TARGET TISSUE.

Biological responses to the thyroid hormones are initiated by the binding of the hormone to nuclear receptors in the target cell. This requires the thyroid hormones to cross the membrane of the target cell. The



uptake of the hormones involves both a carrier-mediated process, which is dependent on Na<sup>+</sup>/K<sup>+</sup> ATPase, and passive entry (Krenning *et al.*, 1978, 1980, Maxfield *et al.*, 1981). Inhibition analyses indicates that T<sub>4</sub> and T<sub>3</sub> cross the membrane via separate transport systems but also, at least in the case of the liver, that each can inhibit the transport of the other in a competitive manner (Krenning *et al.*, 1981).

In summary, the synthesis and secretion of the thyroid hormones is principally controlled by TSH which in turn is positively controlled by TRH and negatively by circulating thyroid hormones. T<sub>4</sub> functions primarily as a prohormone for the more biologically potent form T<sub>3</sub> with the majority of T<sub>4</sub> being deiodinated to T<sub>3</sub> in the peripheral tissues. T<sub>3</sub> acts directly on gene transcription in the target cell by binding to receptors which are located in the nucleus, and associated with DNA response elements.



### THE AIM OF THE PROJECT.

The aim of the work described here was to generate thyroxine-deficient mice by using the method of transgenic ablation. By linking the bovine thyroglobulin promoter to the HSV1-thymidine kinase gene the expression of the HSV1-tk gene was directed to the thyroid gland of transgenic mice. Thyroxine deficiency was achieved by treating transgenic mice with the anti-herpetic drug, ganciclovir. The thyroxine deficiency in the mice is inducible and the animal may therefore be allowed to develop normally prior to ablation.

## CHAPTER TWO.

### CHARACTERISATION OF THE THYROGLOBULIN-THYMIDINE KINASE TRANSGENE.

The thyroglobulin gene is expressed exclusively in differentiated thyroid cells under the positive control of TSH. In order to create an inducible thyroxine-deficient mouse by using the conditional ablation technique (Borrelli *et al.*, 1988, 1989, Heyman *et al.*, 1989) the expression of the HSV1-tk gene was directed to the thyroid by linking it to a bovine thyroglobulin promoter. The TG-TK transgene was constructed by M. Richardson (Figure 4).

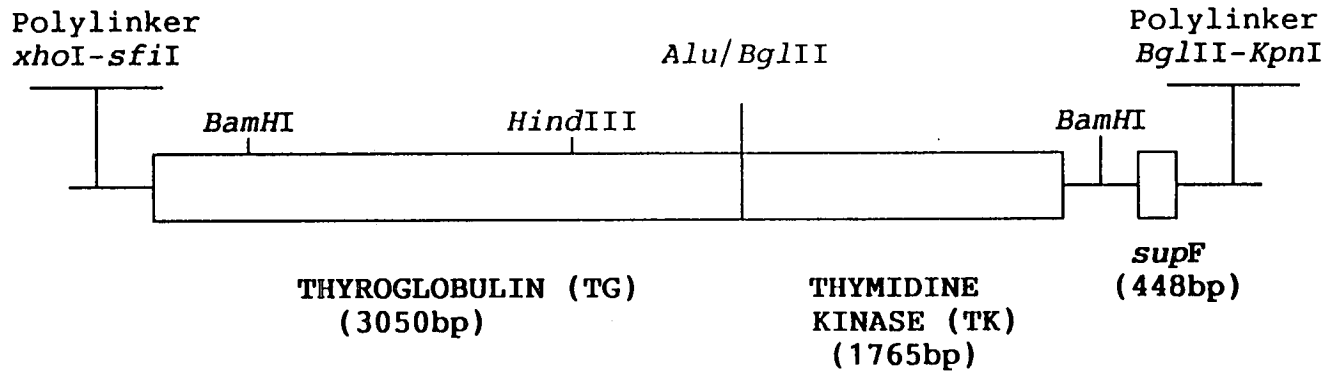
A 3050bp fragment of the bovine thyroglobulin gene contains the cap site, 13 nucleotides of the 5'-non-coding region and about 3kb 5' to the cap site. This region contains two tissue specific hypersensitive sites (Hansen *et al.*, 1988) and a cAMP response element (CRE) which is contained within the first 250bp upstream of the cap site (Christophe *et al.*, 1989). Since the TG-TK construct was made Ledent *et al.*, (1990) have shown that 2000bp of the promoter is sufficient to direct the expression of a chloramphenicol acetyltransferase gene specifically to the thyroid in transgenic mice.

The TG-TK transgene was excised by means of unique restriction sites in the polylinkers flanking the transgene and purified by gel electrophoresis prior to

## FIGURE 4.

### THE TG-TK TRANSGENE.

The fragment used for microinjection was excised by digestion with *Xho*I and *Kpn*I. 3050bp of the bovine thyroglobulin promoter (-3041bp to 13bp relative to the cap site, a gift of G. Vassart), was linked to 1765bp of the HSV1-tk gene through a junction in the non-coding regions (*Alu*I/*Bgl*III). The bacterial *supF* gene was present to allow recovery from transgenic DNA (Al-Shawi *et al.*, 1990).



microinjection into the pronuclei of C57BL/6 x CBA/ca F<sub>2</sub> eggs. 255 embryos were transferred to pseudopregnant recipients and 90 pups were born (microinjection and embryo transfers were carried out by Dr. R. Al-Shawi). Transgenic mice were identified by polymerase chain reaction on tail biopsies as described in Material and Methods. The 15 transgenic pups identified comprised 4 sterile males, 7 fertile males and 4 fertile females. The female mice were used to establish the pedigree lines TG14, TG26, TG62 and TG66.19. The sterile males plugged female mice in the normal way but failed to sire pups. Their sterility has been shown to be a direct consequence of high levels of HSV1-TK expression in the testes resulting in a low sperm count. The sperm are also morphologically defective (Al-shawi *et al.*, 1988, 1991, Braun *et al.*, 1990). The fertile males sired offspring but did not transmit the transgene and are therefore probably germ-line mosaics produced when the transgene integrates into the DNA after the first round of DNA replication. The transgenic germ cells of these animals are assumed to generate defective spermatozoa, while the non-transgenic germ cells generate functional spermatozoa (Al-Shawi *et al.*, 1991).

Southern blot analysis of the four established lines revealed that in lines TG14, TG62 and TG66.19 multiple copies of the transgene had integrated into the chromosome at a single site in a head-to-tail tandem configuration containing approximately 5, 10 and 5 copies of the transgene respectively. Line TG26 in contrast

contained 1 copy of the transgene.

Transgenic ablation requires that the expression of the transgene is limited to one tissue type. To investigate whether the TG-TK transgene is expressed only in the thyroid gland, HSV1-TK assays were carried out on a number of tissues. Expression was detected in the thyroid and testes and very low expression was detected in the submaxillary gland (Table 3). The brain, heart, kidney, muscle, ovary, liver and preputial gland showed levels of HSV1-TK activity indistinguishable from the levels of endogenous activity present in the same tissues of a non-transgenic mouse. The level of thyroid HSV1-TK activity varied between the lines with line TG66.19 having a level more than 10 fold higher than the other lines (Table 3).

#### **TRANSCRIPTION AND TRANSLATION OF THE TRANSGENE.**

Transgenes containing the same sequences of the HSV1-tk gene as TG-TK, but linked to a variety of different promoters, have been studied in transgenic mice. They consistently show HSV1-TK activity in the testes, suggesting that testes expression is a property of the HSV1-tk gene (Al-Shawi *et al.*, 1991). Nuclease protection assays showed that the HSV1-TK transcripts in the testes map to sites downstream of the normal initiation codon of HSV1-tk (ATG<sup>1</sup>), most frequently at positions +150 and +160. Examination of the translation products in the testes by Western blot analysis revealed two major immunoreactive polypeptides of M<sub>r</sub> 39,000 and

**TABLE 3**

**THYMIDINE KINASE ACTIVITY IN THE TISSUES OF TRANSGENIC  
MICE CONTAINING THE TG-TK TRANSGENE.**

Thymidine kinase activities are expressed in pmol/min/mg. TG14, TG26, TG62 and TG66.19 refer to established pedigree lines, derived from progenitor female mice, where M is male, F is female, non-T is a F<sub>2</sub> C57BL/6 x CBA/ca, non-transgenic control, na, not applicable. -, not determined.

Submaxillary gland is abbreviated to submax.

The number of mice analysed is shown in subscript.

Tissue	Transgenic Lines									
	TG14		TG26		TG62		TG66.19		Non-T	
	M	F	M	F	M	F	M	F	M	F
Thyroid	11.90 <sup>3</sup>	10.60 <sup>5</sup>	18.05 <sup>1</sup>	11.40 <sup>2</sup>	0.65 <sup>1</sup>	1.20 <sup>2</sup>	219.10 <sup>2</sup>	178.80 <sup>5</sup>	0.64 <sup>2</sup>	0.03 <sup>5</sup>
Liver	0.04 <sup>1</sup>	0.05 <sup>2</sup>	0.03 <sup>1</sup>	0.04 <sup>1</sup>	0.04 <sup>1</sup>	0.03 <sup>1</sup>	0.02 <sup>1</sup>	0.04 <sup>2</sup>	0.03 <sup>2</sup>	0.03 <sup>2</sup>
Brain	0.30 <sup>1</sup>	0.28 <sup>2</sup>	0.07 <sup>1</sup>	0.17 <sup>1</sup>	0.09 <sup>1</sup>	0.05 <sup>1</sup>	0.09 <sup>1</sup>	0.37 <sup>2</sup>	0.22 <sup>2</sup>	0.27 <sup>1</sup>
Muscle	0.08 <sup>1</sup>	0.03 <sup>1</sup>	0.02 <sup>1</sup>	0.06 <sup>1</sup>	0.30 <sup>1</sup>	0.01 <sup>1</sup>	0.02 <sup>1</sup>	0.01 <sup>1</sup>	0.03 <sup>2</sup>	0.03 <sup>2</sup>
Submax.	0.06 <sup>1</sup>	0.17 <sup>2</sup>	0.06 <sup>1</sup>	0.11 <sup>1</sup>	0.04 <sup>1</sup>	0.11 <sup>1</sup>	0.13 <sup>1</sup>	0.32 <sup>2</sup>	0.06 <sup>1</sup>	0.01 <sup>2</sup>
Kidney	0.06 <sup>1</sup>	0.05 <sup>1</sup>	0.04 <sup>1</sup>	0.12 <sup>1</sup>	0.04 <sup>1</sup>	0.24 <sup>1</sup>	0.03 <sup>1</sup>	0.05 <sup>1</sup>	0.06 <sup>2</sup>	0.18 <sup>2</sup>
Heart	0.04 <sup>1</sup>	0.07 <sup>1</sup>	-	0.07 <sup>1</sup>	-	0.08 <sup>1</sup>	-	0.11 <sup>1</sup>	0.03 <sup>1</sup>	0.09 <sup>2</sup>
ovary	na	0.51 <sup>2</sup>	na	0.29 <sup>1</sup>	na	0.30 <sup>1</sup>	na	0.43 <sup>2</sup>	na	0.33 <sup>2</sup>
Testes	124.70 <sup>3</sup>	na	35.80 <sup>2</sup>	na	0.79 <sup>1</sup>	na	53.40 <sup>2</sup>	na	0.29 <sup>3</sup>	na
Preputial	0.06 <sup>1</sup>	na	0.06 <sup>1</sup>	na	0.20 <sup>1</sup>	na	0.08 <sup>1</sup>	na	0.15 <sup>2</sup>	na



37,000; both shorter than the expected full length peptide of  $M_r$  43,000. These truncated products probably result from translational initiation occurring from the second and third ATG codons of the HSV1-tk reading frame (Al-Shawi *et al.*, 1991). These results suggest that the testes contain one or more trans-acting factors that activate a cryptic promoter element in the HSV1-tk gene, initiating transcription downstream of the first ATG codon.

To determine whether the TG-TK transgene initiates transcription correctly in the thyroid, primer extension analysis was carried out. A 20 nucleotide oligonucleotide complementary to the region +438 to +419 of the HSV1-tk gene was used as a probe (Al-Shawi *et al.*, 1991). A positive control was generated by cloning a 312bp fragment (*MscI-EcoRV*), which spans the cap site of the TG-TK transgene, into a Bluescript SK<sup>+</sup> vector. This clone was used to generate a positive control template by *in vitro* RNA transcription. Figure 5A shows primer extension products obtained with thyroid RNA and positive control template. The expected lengths of the primer extension products are 114bp and 208bp respectively. Lanes 1-3 show a 208bp product obtained with the control template while lanes 5+6 show a 114bp product obtained with thyroid RNA. As expected lane 4 shows no extension product with thyroid RNA from non-transgenic mice. Thus transcription of the TG-TK gene in the transgenic thyroid is correctly initiated at the cap-site of the thyroglobulin gene. The limit of detection of the assay is between 1pg (lane 2)

**FIGURE 5.**

**A) PRIMER EXTENSION OF POOLED THYROID TOTAL mRNA.**

Annealing of the primer and the extension reaction are described in Material and Methods. Autoradiograph exposed for 7 days.

- lanes:- 1) 10pg *in vitro* transcribed RNA.  
2) 1pg *in vitro* transcribed RNA.  
3) 0.2pg *in vitro* transcribed RNA.  
4) 5µg non transgenic pooled male thyroid RNA.  
5) 5µg TG66.19 pooled male thyroid RNA.  
6) 1µg TG66.19 pooled male thyroid RNA.  
7-10) sequence reaction of M13mp18 with ddTTP;  
ddCTP; ddGTP and ddATP respectively.

**B) WESTERN BLOT ANALYSIS OF TESTES AND THYROID EXTRACTS.**

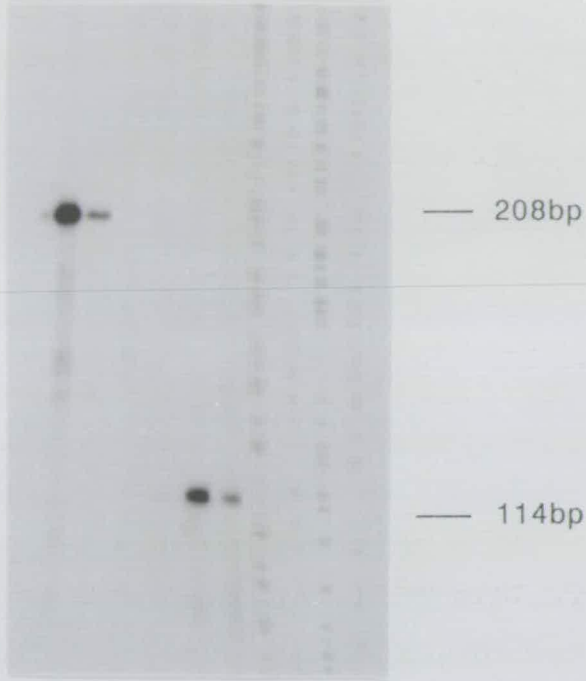
Blot was challenged with anti-HSV1-TK serum (Al-Shawi *et al.*, 1991). Detection system is biotin/streptavidin alkaline phosphatase.

- lanes:- 1) 50µg TG62 testes extract.  
2) 50µg TG14 testes extract.  
3) 50µg TG26 testes extract.  
4 & 5) 50µg TG66.19 testes extract.  
6) 50µg non-transgenic testes extract.  
7) 50µg HSV1-tk infected cell extract.  
8) 20µg TG14 female thyroid extract.  
9) 20µg TG66.19 male thyroid extract.  
10) 20µg TG66.19 female thyroid extract.  
11) 20µg non-transgenic female thyroid extract.

C = control; TG = transgenic; F = female; M = male; T<sub>3</sub> = T<sub>3</sub> RNA polymerase transcripts.

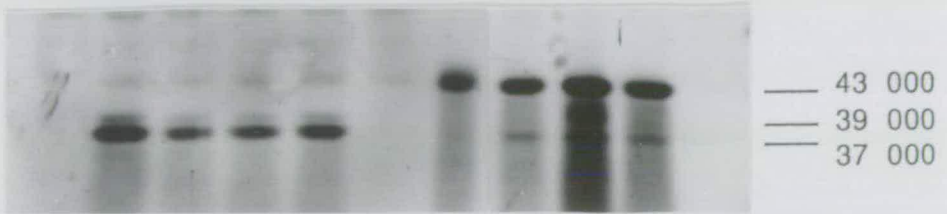
A.

<u>T3</u>		<u>C</u>	<u>TG</u>		<u>T</u>	<u>C</u>	<u>G</u>	<u>A</u>	
1	2	3	4	5	6	7	8	9	10



B.

<u>TESTES</u>						<u>H</u>	<u>THYROID</u>				
TG						S	TG		C		
						V	F		M	F	F
					C	t					
						k					
1	2	3	4	5	6	7	8	9	10	11	



and 0.2pg (lane 3). The intensities of the signals obtained with thyroid RNA indicates that the TG-TK mRNA in the thyroid constitutes approximately 0.002% of the total thyroid RNA. Translation products of the transgene in thyroid and testes extracts were detected by Western blot analysis, challenging with anti-HSV1-TK serum (a gift of Dr W. Summers). The main reactive protein present in the thyroid extracts was full length 43kDa HSV1-TK (Figure 5B, lanes 8, 9 & 10) suggesting that translation initiation occurs at the first ATG codon of the HSV1-tk reading frame. In contrast, the majority of immunoreactive protein products in the testes were shorter than the expected full length peptide (Figure 5B, lanes 1-5). The pattern of immunoreactive proteins present in the testes of TG-TK mice was similar to the patterns obtained from other transgenic lines which express the HSV1-tk gene in the testes (Al-Shawi *et al.*, 1991). Thus the mechanism by which the HSV1-tk gene is expressed in the testes of mice containing the TG-TK transgene would appear to be the same as in other transgenic mice which express HSV1-tk in their testes.

The site of HSV1-TK expression in the thyroid was determined by immunocytochemistry using anti-HSV1-TK serum. In an untreated transgenic mouse staining was confined to thyroid follicle epithelial cells (Figure 6A) showing that significant transgene expression was directed exclusively to the thyrocytes.

In conclusion it would appear that 3050bp of the bovine thyroglobulin promoter is sufficient to direct the

## FIGURE 6.

### A. SITE OF HSV1-TK EXPRESSION IN THE THYROID GLAND.

Immunohistochemical visualisation of HSV1-TK expression (positively stained cells) in 3 $\mu$ m sections of female thyroid gland. Sections were challenged with an anti-HSV1-TK serum and developed using horseradish peroxidase. The sections were counter stained with haematoxylin.

- 1) Non-transgenic control.
- 2) Untreated TG66.19 female.

Magnification: x100.

### B. THE EFFECT OF GANCICLOVIR TREATMENT ON THE THYROID GLAND.

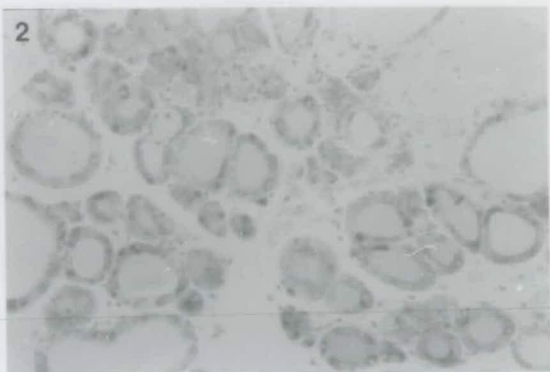
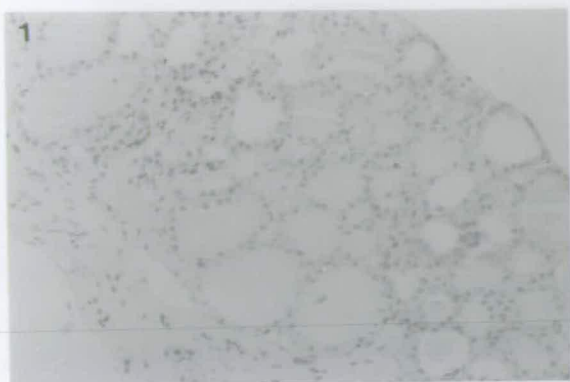
Thyroid glands of:

- 1) Non-transgenic mouse.
- 2) Untreated transgenic.
- 3) Transgenic mouse treated with 4.5mg/day ganciclovir for two weeks.

In each case the right lobe of the thyroid gland, indicated with an arrow, is exposed above the trachea.

*(Also depicted in "specific ablation of thyroid follicle cells in adult transgenic mice" - see published papers).*

A



B



majority of HSV1-tk gene expression to the thyroid. Although the submaxillary gland also has detectable levels of HSV1-TK activity the level is approximately 0.2% of that observed in the thyroid gland of transgenic mice. In the thyroid the transgene is transcribed from the correct start site and is translated correctly to yield a full length TK polypeptide of 43kDa. In contrast the majority of HSV1-TK expression in the testes results from transcription initiated at sites downstream of the first ATG codon, giving rise to truncated polypeptides of 39kDa and 37kDa.

To avoid any complications that may be caused by HSV1-TK expression in the testes, most of the experiments were carried out on female mice from line TG66.19 which showed the highest HSV1-TK activity in the thyroid gland.

### CHAPTER THREE.

#### **CREATING THE INDUCIBLE THYROXINE-DEFICIENT MOUSE MODEL.**

##### TRANSGENIC ABLATION.

Mature female transgenic mice (up to 18 weeks of age), containing the TG-TK transgene, were implanted with Alzet osmotic mini-pumps for two weeks. These pumps are designed to allow a constant rate of infusion over a set period of time. The mini-pumps were filled either with phosphate-buffered saline (PBS) or with ganciclovir. The concentration of the drug was set to give a delivery rate of 4.5mg/day.

At the end of two weeks the animals were sacrificed. Figure 6B shows the effects of ganciclovir on the thyroid gland over this period of time. In each case the right lobe was exposed above the trachea. The mouse exposed to ganciclovir shows an almost complete regression of the thyroid gland. This correlates with the total soluble protein obtained from the rudiment, which is less than 20% of the control value (Table 4). Due to the difficulty in dissecting out the rudiment without removing neck muscle, this value is likely to overestimate the remaining protein.

Any thyrocytes which escaped ablation might be expected to retain HSV1-TK activity. HSV1-TK activity was therefore measured in residual thyroid tissue to assess the extent of thyrocyte ablation. Table 4 shows the total



**TABLE 4.**

**THYROID HORMONE LEVELS AND HSV1-TK ACTIVITY IN ABLATED MICE.**

Mice were treated with ganciclovir (4.5mg/day) or PBS by minipump for two weeks. Circulating thyroid hormone levels were measured using ELISA competitive assays (Kodak Diagnostics). The total protein obtained from the thyroid is expressed in  $\mu\text{g}$ . The HSV1-TK activity is in units of TK activity/thyroid.

Treatment & Exp. Nos.		T <sub>4</sub> (nmol/L)		T <sub>3</sub> (nmol/L)		TK Activity	Total Protein (μg)
		Before	After	Before	After		
Transgenic & PBS	1	44.9	15.8	-	0.00	8.60	60
	1	44.1	24.0	0.86	0.27	-	-
	1	20.3	20.6	-	-	11.50	30
	<b>Average</b>	<b>36.4</b>	<b>20.1</b>	<b>0.86</b>	<b>0.14</b>	<b>10.05</b>	<b>45</b>
Transgenic & ganciclovir	1	52.3	0.0	0.96	0.00	0.01	7
	1	51.4	0.0	0.98	0.00	0.03	10
	1	50.7	0.0	0.83	0.00	0.01	10
	3	57.7	0.0	-	-	-	-
	5	48.8	0.0	-	-	0.04	10
	<b>Average</b>	<b>52.2</b>	<b>0.0</b>	<b>0.92</b>	<b>0.00</b>	<b>0.02</b>	<b>9</b>
Non-Transgenic & ganciclovir	3	66.4	40.4	0.73	0.48	0.01	100
	5	49.1	33.4	-	-	0.01	100
	<b>Average</b>	<b>57.8</b>	<b>36.9</b>	<b>0.73</b>	<b>0.48</b>	<b>0.01</b>	<b>100</b>

units of HSV1-TK activity and protein per thyroid rudiment in the ablated mice. In each case the ablated mice had essentially zero HSV1-TK values. Thus after two weeks of ganciclovir treatment there appears to be little or no expression of the transgene. From the results in Table 4 the ablated mice have an average HSV1-TK value of 0.02 units/thyroid compared to 10.05 units/thyroid in a PBS treated transgenic control. The background level of HSV1-TK activity in the assay is 0.01 units/thyroid (non-transgenic control). Based on the assumption that any remaining thyrocyte is expected to retain HSV1-TK activity the value of 0.02 units/thyroid in the ablated mice suggest that no more than 0.1% of the thyrocytes escape ablation during the two weeks of ganciclovir treatment.

#### HORMONE LEVELS.

To determine whether the mice were deficient in thyroxine after two weeks of drug administration, serum samples were analysed for the presence of circulating  $T_4$  and  $T_3$ . Circulating  $T_4$  and to a lesser degree  $T_3$  are mainly bound to serum proteins (see Chapter 1). The blocking agent 8-anilino-1-naphthalene sulphonic acid was included in the assay to dissociate the hormones from the binding proteins so that total circulating hormone could be measured by a competitive ELISA system (enzyme linked immunoabsorbent assay). I used the Amerlite system (Kodak Diagnostics) which depends on competition for a limited number of binding sites on a specific antibody between

antigen in the sample and a horseradish peroxidase-labelled antigen conjugate. The amount of bound labelled antigen is inversely proportional to the concentration of antigen in the sample. In each case (Table 4) mice treated with ganciclovir had levels of serum  $T_4$  and  $T_3$  below the sensitivities of the assays (8.0nmol/L and 0.15nmol/L for  $T_4$  and  $T_3$  respectively) with actual readings of 0.0 being obtained. The control mice, that is transgenics with PBS or non-transgenics with ganciclovir pumps, still had detectable thyroid hormone levels after two weeks. However the values obtained were generally lower at the end of the experiment ( $T_4$  fell by about 40% on average). This appears to be a direct affect of the experiment due to surgery or from mini-pump implantation.

The levels of  $T_4$  in untreated non-transgenic control mice, sampled at the same time of day on different days, were also quite variable (Table 5). However in these mice, which were not implanted with minipumps, the variation between samples and within mice was similar to the variation between mice, suggesting that differences in  $T_4$  levels did not follow any obvious pattern. This contrasts with the consistent difference observed before and after minipump implantation.

In conclusion, administration of 4.5mg/day of ganciclovir over a period of two weeks resulted in mice with undetectable levels of circulating  $T_4$  and  $T_3$ . The thyroid gland showed almost complete regression with residual tissue having negligible HSV1-TK activity, suggesting that few if any thyrocytes escaped ablation.

TABLE 5.

ANALYSIS OF VARIANCE IN THYROID HORMONE LEVELS IN NON-TRANSGENIC MICE.

Five age-matched male and female mice were tail bled several time over a period of eight days. T<sub>4</sub> levels were assessed using a commercial assay kit (Amerlite- Kodak Diagnostics).

The F-distribution analysis (F) was applied with degrees of freedom (DF) as indicated in Table 5. The difference between male mice, between female mice and between all mice was not significant (NS) at the 1% level of significance.

Sex of mouse	T <sub>4</sub> (nmol/L)			
	Day 1	Day 4	Day 6	Day 8
Male	22.5	80.0	60.0	55.0
Male	35.0	57.5	72.5	47.5
Male	37.5	82.5	80.0	47.5
male	20.0	62.5	80.0	50.0
Male	47.5	75.0	40.0	62.5
Female	70.0	45.0	35.0	12.5
Female	70.0	40.0	37.5	30.0
Female	60.0	50.0	40.0	5.0
Female	40.0	70.0	47.5	30.0
Female	52.5	87.5	52.5	42.5

	F	Significance	DF
Male mice/Diff	0.94	NS	4 & 12
Female mice/Diff	1.27	NS	4 & 12
All mice/Diff	0.90	NS	9 & 27

## SPECIFICITY OF ABLATION.

The thyroid contains two cell types of different embryonic origin: thyrocytes, which secrete  $T_4$  and  $T_3$  and C-cells which secrete calcitonin (CT). The C-cells are distinct endocrine cells found on the follicle wall or interspersed with the follicle cells and hence are often referred to as parafollicular cells (Figure 7A). Morphologically they differ from thyrocytes in being larger with a more granular appearance. The thyroid gland is also in intimate contact with the parathyroid gland which secretes parathyroid hormone (PTH). The two parathyroid glands are bilaterally attached to the thyroid lobes, and each consists of a densely packed mass of cells separated from the thyroid gland by a connective tissue capsule. In conjunction with CT and  $1,25,(\text{OH})_2$ -vitamin  $D_3$ , PTH is involved in calcium homeostasis and bone remodelling in the adult.

The results shown in Figure 6A demonstrate that the expression of the transgene is specifically directed to the thyrocytes in female transgenic mice with administration of ganciclovir resulting in regression of the thyroid gland (Figure 6B). I investigated the specificity of ablation in the thyroid gland to determine whether ablation was restricted to one cell type or whether it caused the destruction of the other cell types. Destruction of the other cell types could occur in at least two ways. Firstly the thyrocytes and the C-cells may be connected by gap junctions allowing the transfer of phosphorylated nucleotides from one cell type to

## FIGURE 7.

### A. DISTRIBUTION OF C-CELLS IN THE MOUSE THYROID GLAND.

Immunohistochemical visualisation of C-cells (positively stained cells) in 3µm sections of female thyroid gland. Sections were challenged with an anti-human calcitonin antibody and developed using horseradish peroxidase. The sections were counter stained with haematoxylin.

- 1) Non-transgenic mouse.
- 2) Untreated transgenic.
- 3) Transgenic mouse treated with 4.5mg/day ganciclovir for two weeks.

Sections show areas of C-cells that have been brought together by the removal of the thyrocytes and by the overall shrinkage of the gland.

Magnification: x100.

### B. HISTOLOGY OF THE PARATHYROID GLAND.

Histological examination of the thyroid gland showing the parathyroid gland (P)

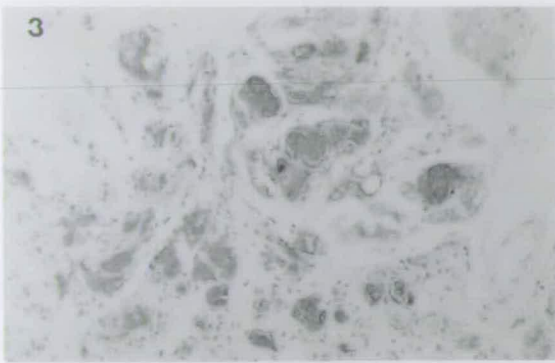
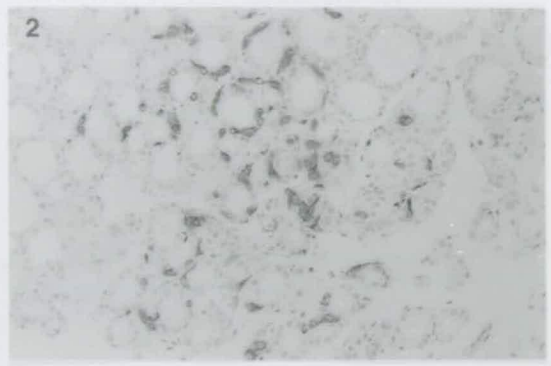
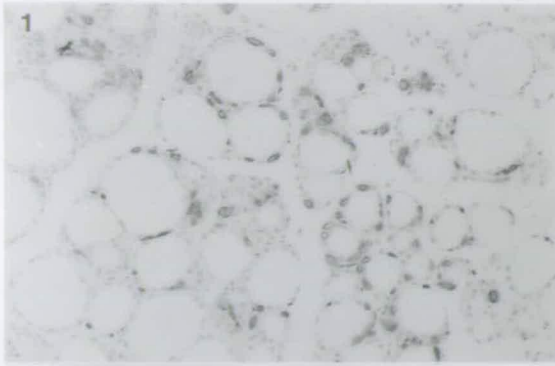
- 1) Non-transgenic control.
- 2) Untreated transgenic.
- 3) Transgenic treated with 4.5mg/day ganciclovir for two weeks.

Sections were cut at 5µm and stained with H&E. Surrounding thyroid tissue is marked T.

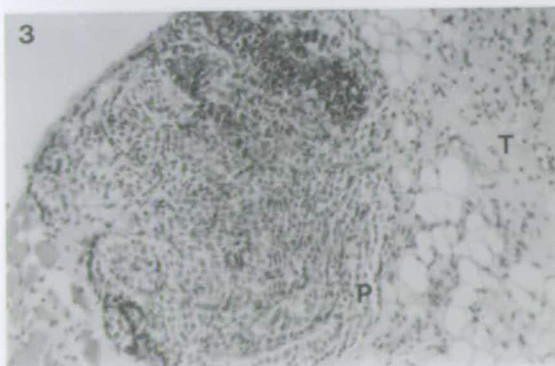
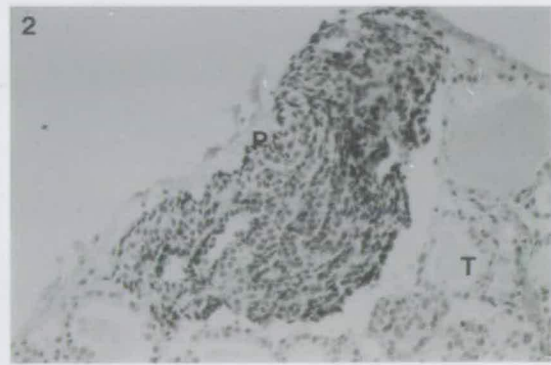
Magnification x100.



A.



B.



another. Secondly leakage of toxic compounds which could be taken up by surrounding cells might occur. Although cells do not take up nucleotides, other toxic compounds might escape from the dying thyrocytes.

#### C-CELLS.

CT levels were assessed, using a radioimmunoassay (RIA), in transgenic mice treated with ganciclovir for two weeks. The assay is based on competition between a radioactive standard antigen and non-radioactive experimental antigen for a limited number of antibody binding sites. The amount of bound radioactive antigen is inversely proportional to the concentration of antigen in the sample. The hormone levels observed before and after ablation showed little variation (Table 6), demonstrating that the C-cells were still functional in the absence of detectable levels of thyroid hormones. In contrast, a thyroidectomised mouse showed no circulating CT (Table 6). The persistence of normal levels of CT is consistent with the identification of functional C-cells in thyroid sections taken from a transgenic mouse treated with ganciclovir (Figure 7A). The distribution of the cells had altered. After ablation the C-cells were found clustered together rather than distributed around the follicles as before (Figure 7A). This configuration would be expected if, when the thyrocytes disappear, the follicles themselves collapse. Electron microscopy indicated that ablation had not caused any intracellular

TABLE 6.

CALCITONIN LEVELS IN ABLATED MICE.

Serum samples, collected at the start and completion of the experiment were pooled and assessed for circulating calcitonin using a commercial RIA kit (Kodak Diagnostics). A thyroidectomised mouse was included as a negative control.  $T_4$  was determined using an ELISA system.

Treatment & Exp. Nos.	Calcitonin (pg/ml)		T <sub>4</sub> (nmol/L)	
	Before	After	Before	After
Transgenic & PBS				
1	-	34	44.9	15.8
3	36	29	20.3	20.6
5	25	28	47.5	28.5
<b>Average</b>	<b>30</b>	<b>30</b>	<b>37.6</b>	<b>21.6</b>
Transgenic & ganciclovir				
1	-	22	51.4	0.0
3	24	26	48.3	0.0
	38	33		
5	30	27	-	-
<b>Average</b>	<b>31</b>	<b>27</b>	<b>49.8</b>	<b>0.0</b>
Non-Transgenic & ganciclovir				
3	23	25	-	-
5	42	39	49.1	33.6
<b>Average</b>	<b>32</b>	<b>32</b>	<b>49.1</b>	<b>33.6</b>
Thyroid-ectomized	27	0.0	56.5	8.1

alterations to the C-cells (Figure 8A - Dr.C. McLaren, Pathology Department, Edinburgh University). Thus the C-cells are unaffected by ablation of the thyroid gland. The serum assays also demonstrate that the activity of C-cells is independent of thyroid hormones.

#### PARATHYROID GLAND.

The effect of ganciclovir treatment on the parathyroid gland was assessed. Circulating levels of PTH were measured by RIA. Table 7 shows that ganciclovir treatment had little or no effect on the circulating levels of PTH over the two week ablation period. The integrity of the gland was confirmed by histology (Figure 7B). These results indicate that there is no direct or indirect damage to the parathyroid during thyrocyte ablation and, furthermore, that thyroxine has no role in regulating the function of the parathyroid gland.

In conclusion, the results indicate that the expression of the TG-TK transgene is directed to the thyrocytes. The administration of ganciclovir results in the destruction of HSV1-TK expressing cells and affects only one hormone secreting cell type in the thyroid gland, with no secondary effect on either the C-cells or the parathyroid gland. Thus ablation is restricted to the site of HSV1-TK expression. This is in agreement with the observations of Borrelli *et al.*, (1989) who demonstrated the precise ablation of somatotrophs and lactotrophs in the pituitary, while other pituitary cell types were unaffected. The results also show that the normal

**FIGURE 8.**

**A. ELECTRON MICROSCOPY OF THE THYROID GLAND.**

Transgenic mice were administered ganciclovir (4.5mg/day) by minipump for two weeks. The thyroid glands were removed and prepared for EM. Sections 1-3 show granulated C-cells (C). However after two weeks of ganciclovir treatment the C-cells form clusters.

- 1) Non-transgenic control (x 6200).
- 2) Untreated transgenic (x 8200).
- 3) Transgenic treated with ganciclovir for two weeks (x 8200).

(K. McLaren, Pathology Department, University of Edinburgh).

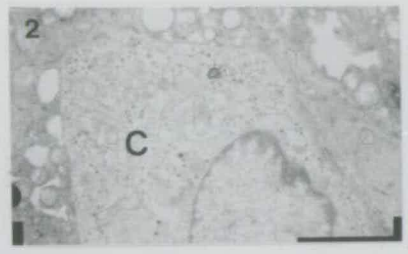
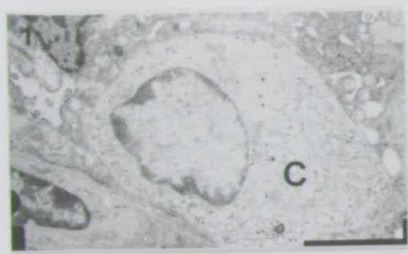
**B. ADIPOSE TISSUE IN THE THYROID GLAND.**

Cryosections (10µm) of thyroid glands stained for adipose tissue (Sudan black).

- 1) Non-transgenic control.
- 2) Untreated transgenic.
- 3) Transgenic treated with ganciclovir for two weeks.

Magnification x100.

A.



B.

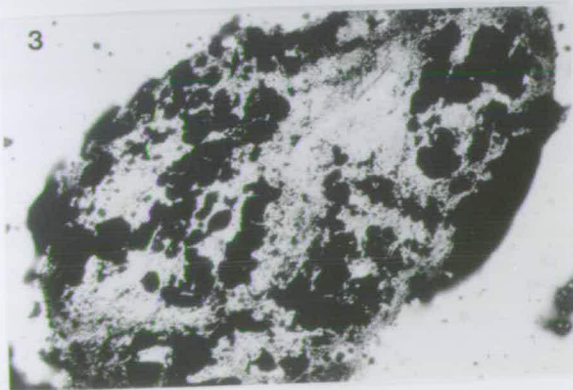
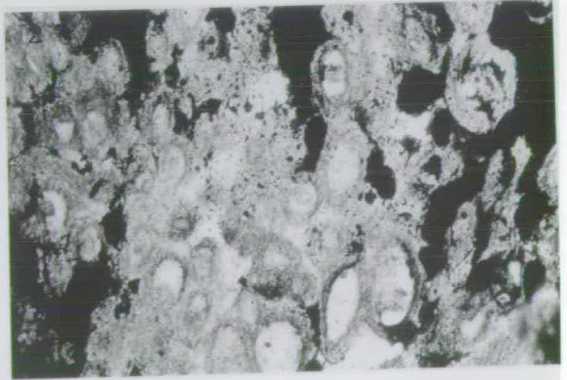
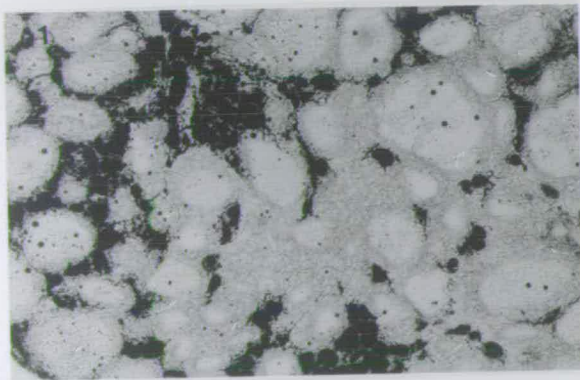


TABLE 7.

PARATHYROID HORMONE LEVELS IN ABLATED MICE.

Circulating levels of parathyroid hormone (PTH) from pooled samples, were assessed using a commercial RIA kit (Kodak Diagnostics).  $T_4$  was determined by competitive ELISAs (Kodak Diagnostics).



Treatment & Exp. Nos.	PTH (pmol/L)		T <sub>4</sub> (nmol/L)	
	Before	After	Before	After
Transgenic & PBS				
1	-	36	44.9	15.8
2	54	49	44.1	39.5
<b>Average</b>	<b>54</b>	<b>42</b>	<b>44.5</b>	<b>27.6</b>
Transgenic & ganciclovir				
1	-	48	51.4	0.0
2	71	65	29.8	0.0
<b>Average</b>	<b>71</b>	<b>56</b>	<b>40.6</b>	<b>0.0</b>
Non-Transgenic & ganciclovir				
2	63	84	21.0	10.8

functioning of the C-cells and the parathyroid gland are not dependent on thyroid hormones.

#### ABLATION STUDIES IN THE MALE MOUSE.

Male mice carrying a transgene with the HSV1-tk gene as a reporter have significant levels of HSV1-TK activity in the testes (Chapter 2). Moderate and high levels of HSV1-TK expression, but not low levels, cause male sterility. The mechanism by which HSV1-TK activity in the testes causes sterility is not understood (Al-Shawi *et al.*, 1988, 1991, Braun *et al.*, 1990)

I investigated whether ganciclovir could ablate the cells in the testes (spermatids) that express the HSV1-tk gene. A line of transgenic mice in which HSV1-TK expression is confined to the testes and preputial gland (J. Whitaker, PhD Thesis Edinburgh University 1989) was used in the first instance to avoid complications which might arise due to thyroid ablation. HSV1-TK activity in the testes was analysed after two weeks of drug treatment and found to be unchanged compared to a PBS treated control transgenic of the same line. TG66.19 male mice were then examined. The effect on the level of thyroid hormones was similar to that observed in female transgenic mice treated in the same way. At the end of two weeks of ganciclovir treatment there was no detectable  $T_4$  or  $T_3$  in the serum and the HSV1-TK activity of the thyroid had effectively decreased to zero (Table 8). In contrast, HSV1-TK expression in the testes was unchanged by ganciclovir treatment, levels of transgene

TABLE 8.

MALE ABLATION: HORMONE LEVELS AND HSV1-TK ACTIVITY IN THE TESTES AND THYROID GLAND.

Male TG66.19 mice were treated with 4.5mg/day ganciclovir or PBS by minipump for two weeks. Serum levels were measured using ELISA competitive assays (Kodak Diagnostics). The total protein obtained from the thyroid is expressed in  $\mu\text{g}$  and the HSV1-TK activity is in units/thyroid. The HSV1-TK activity in the testes is expressed in units/mg

Treatment	T <sub>4</sub> (nmol/L)		T <sub>3</sub> (nmol/L)		Testes TK Activity (units/mg)	Thyroid	
	Before	After	Before	After		TK activity per gland	Total Protein (μg)
Transgenic & PBS	43.2	46.2	0.61	0.56	84.4	31.4	90
Average					74.4	33.1	147
					79.4	32.2	118
Transgenic & ganciclovir	48.2	0.0	0.72	0.00	74.2	0.0	20
					104.9	0.0	45
	25.1	0.0	0.29	0.00	59.5	0.2	49
Average	36.6	0.0	0.50	0.00	97.9	0.0	35
					84.1	0.05	37
Non-Transgenic & ganciclovir	67.4	54.3	0.81	0.69	0.79	0.1	140

expression being similar to that of a PBS treated control transgenic animal (Table 8). Histological studies revealed no morphological alteration to the structure of the testes following ganciclovir treatment (Department of Pathology, Western General Hospital, Edinburgh). It would therefore appear that male mice can be used in ablation studies without any complications caused by the expression of HSV1-tk in the testes.

The persistence of HSV1-TK expression in the testes following ganciclovir treatment could be due to any of several causes. (1) HSV1-TK activity is mainly detected in haploid spermatids (Al-Shawi *et al.*, 1991) at which time the sperm are near maturity and thus may be insensitive to the affects of ganciclovir. (2) The cells expressing the HSV1-tk gene may be inaccessible to the ablating agent due to the possible presence of a blood membrane barrier in the testes. (3) The truncated proteins present in the testes may not be able to phosphorylate the drug. This latter question could be addressed in a tissue culture system by manipulating the HSV1-tk gene so that only truncated protein products were produced. Cell viability following drug treatment could then be assessed.

## CHAPTER FOUR.

### FURTHER ASPECTS OF THYROID ABLATION AND THE EFFECT OF PROLONGED THYROXINE DEFICIENCY ON THE MOUSE.

#### EFFECTS OF GANCICLOVIR TREATMENT ON THE THYROID GLAND.

The thyroxine-deficient mouse (Chapter 3) was generated by treating transgenic mice continuously with ganciclovir for a period of two weeks. I was therefore interested to determine the time-course of ablation. Accordingly, ganciclovir was administered to transgenic female mice for 1, 2, 3, 7 and 14 days, at which times the mice were sacrificed and the thyroid gland removed. The glands were analysed for changes in morphology and HSV1-TK activity

The effects of ganciclovir treatment on thyroid gland morphology were investigated. A non-transgenic control thyroid (Figure 9A) and an untreated transgenic thyroid (Figure 9B) show typical follicle structures, with a layer of epithelial cells (thyrocytes) surrounding a cavity (lumen). The latter is filled with stained colloid. A change in the morphology of the thyroid gland was observed after two days of drug treatment (Figure 9D) with areas of the gland becoming devoid of follicle structures. By the third day (Figure 9E) follicle structures which contain colloid were nearly eliminated. After 7 days no follicle structures were observed in any of the sections studied (Figure 9F). Figure 9G shows a

**FIGURE 9.**

**MORPHOLOGY OF THE THYROID GLAND DURING GANCICLOVIR  
TREATMENT.**

Histological sections (5 $\mu$ m) of thyroids from TG66.19 female mice treated with ganciclovir (4.5mg/day) by minipump. Sections show typical follicle structures.

A) Non-transgenic.

B) Untreated transgenic.

C) 1-day of ganciclovir treatment.

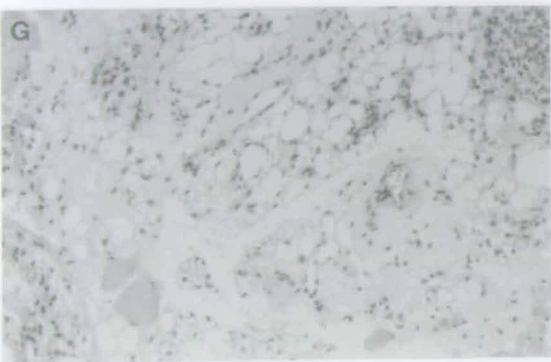
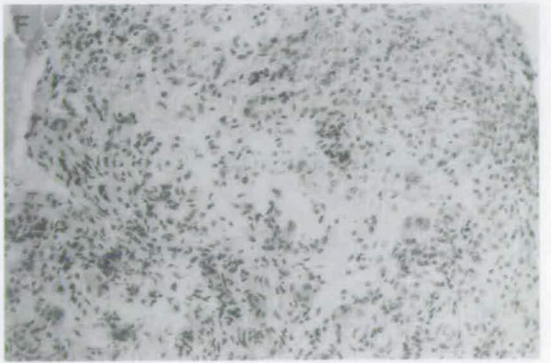
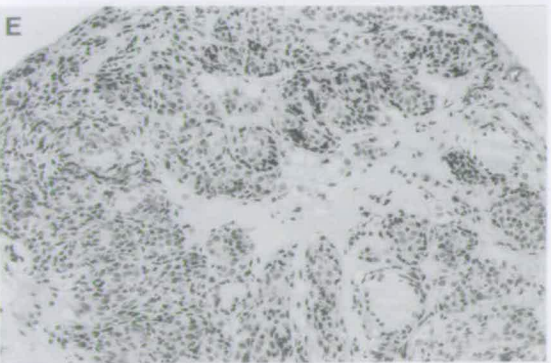
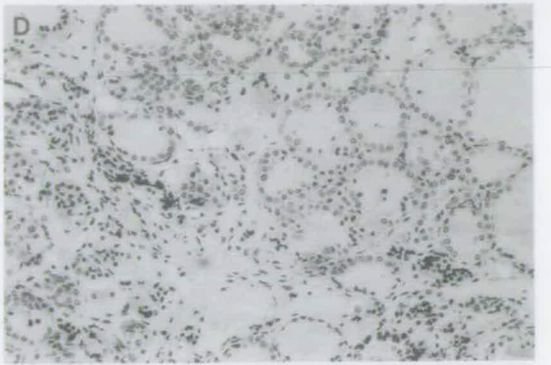
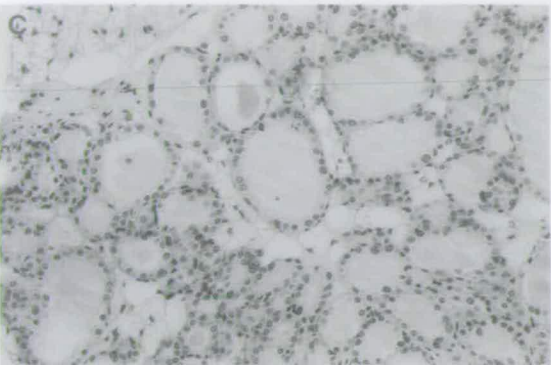
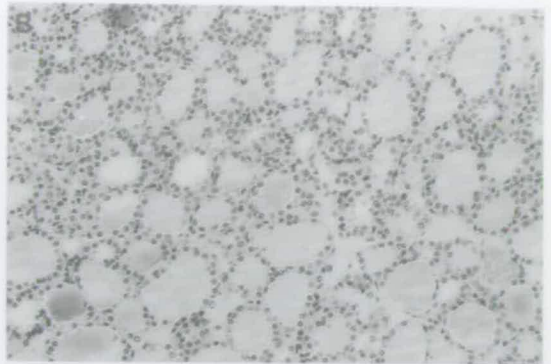
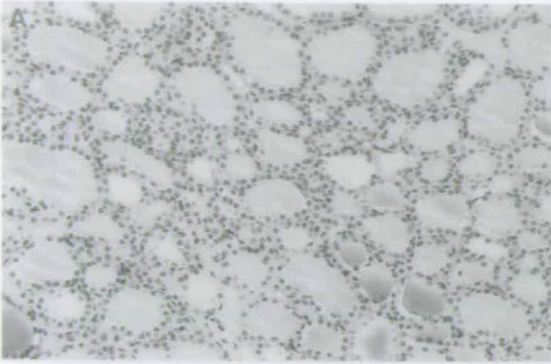
D) 2-days of ganciclovir treatment.

E) 3-days of ganciclovir treatment.

F) 7-days of ganciclovir treatment.

G) Two weeks ganciclovir treatment.

The sections were stained with H&E. Magnification x100.





typical section taken from a transgenic mouse treated with ganciclovir for fourteen days. The section contains structures which resemble empty follicles. However these structures appear *de novo* between days seven and fourteen and are therefore unlikely to be empty follicles, indeed most have been identified as adipose tissue (Figure 8B). Figure 9G also demonstrates the presence of endothelial-like cells and C-cells (Figure 7A). Others remain unidentified, but they are present in very much lower numbers than the follicle cells of the control thyroid. All the sections show nerve cells and blood vessels which were unaffected by ganciclovir treatment.

The total protein and total HSV1-TK activity recovered from excised glands were consistent with the morphological changes observed (Table 9). Total protein was significantly reduced after 3 days of treatment and after 7 days had fallen to about 20% of the control value. Total HSV1-TK activity fell more rapidly and was already significantly reduced after just 1 day of treatment. Although the accuracy of the total protein values is uncertain, due to the difficulties of excising thyroid tissue, the specific activity (units/mg) provides an indicator of HSV1-TK activity which will be reliable if recovery of thyroid tissue is incomplete, provided there is no contamination by muscle or other non-thyroid tissue. The specific HSV1-TK activity was significantly reduced after 1 day (Table 9), lending support to the reduction in total HSV1-TK activity observed at that time. After 7 days of ganciclovir treatment total HSV1-TK

TABLE 9.

CHANGES IN TRANSGENIC THYROID GLANDS DURING THE FIRST WEEK OF GANCICLOVIR TREATMENT.

Female transgenic mice were administered ganciclovir by minipump at a rate of 4.5mg/day for 1, 2, 3, and 7 days. At the end of these times the mice were sacrificed. Thyroid glands were removed and assessed for HSV1-TK activity. HSV1-TK activity is expressed in units/thyroid and also as specific activity (per mg). Total soluble protein is expressed in  $\mu\text{g}$ .

$T_4$  and  $T_3$  levels were measured by competitive ELISAs on serum samples collected at the start of the experiment and at the time of sacrifice.

Treatment	T <sub>4</sub> (nmol/L)		T <sub>3</sub> (nmol/L)		TK Activity		Total Protein (µg)
	Before	After	Before	After	Units/gland	Units/mg	
Control Transgenic	52.4	-	0.52	-	21.8	310.4	70
	58.2	-	0.68	-	15.7	112.2	140
	<b>Average</b>	55.3	-	0.60	-	18.8	211.3
Transgenic & ganciclovir 1 day	40.7	16.6	0.74	0.42	13.6	113.4	120
	50.0	18.5	0.65	0.42	11.5	117.9	196
	<b>Average</b>	45.4	17.6	0.70	0.42	12.6	115.6
Transgenic & ganciclovir 2 days	55.8	11.3	0.63	0.22	9.2	152.8	60
	34.3	29.0	0.47	0.71	8.8	79.7	110
	<b>Average</b>	45.0	20.2	0.55	0.46	9.0	116.2
Transgenic & ganciclovir 3 days	48.8	66.1	0.34	0.97	1.7	28.7	60
	26.1	10.7	0.29	0.20	3.7	104.7	35
	<b>Average</b>	37.4	38.4	0.32	0.58	2.7	66.7
Transgenic & ganciclovir 7 days	34.4	30.6	-	-	0.01	0.80	20
	32.4	0.0	-	-	0.00	0.00	20
	<b>Average</b>	33.4	15.3	-	-	0.01	0.4
Non-Transgenic & ganciclovir 7 days	55.8	34.7	0.43	0.27	0.00	0.00	40
	42.1	34.7	0.74	0.17	0.01	0.02	260
	<b>Average</b>	48.9	34.7	0.58	0.22	0.01	0.01

activity in the thyroid rudiment had fallen to zero. Extrapolation of the data suggests that the HSV1-TK activity in the thyroid gland fell to zero by day 5 of ganciclovir treatment (Figure 10C).

Histological examination of the glands showed that disintegration of follicle cells preceded the loss of follicle contents. Since the follicle contents are likely to contribute a significant proportion of the total protein recovered, this can explain the fact that the total HSV1-TK activity and the HSV1-TK specific activity fell before there was a significant change in total protein.

If, as seems likely, the fall in total HSV1-TK activity after 1 day of treatment is significant, it would appear that the expression of HSV1-TK was depressed before any morphological changes became apparent. This suggests that there may be a significant time delay between the metabolic death of the follicle cells and their disintegration.

#### CIRCULATING LEVELS OF THYROID HORMONES.

Circulating levels of  $T_4$  and  $T_3$  fell below the limits of detection after 14 days of drug treatment (Table 4). The variation in circulating  $T_4$  and  $T_3$  (Table 5) and the marked effect of surgery and the manipulation on control animals (Table 4) makes it difficult to define the time-course of hormone levels during ganciclovir treatment with accuracy. Keeping this important reservation in mind, and despite considerable variation in the response

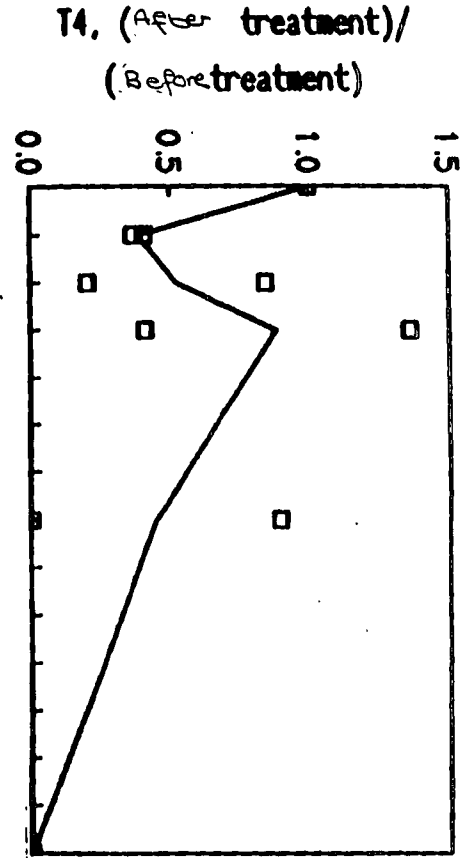
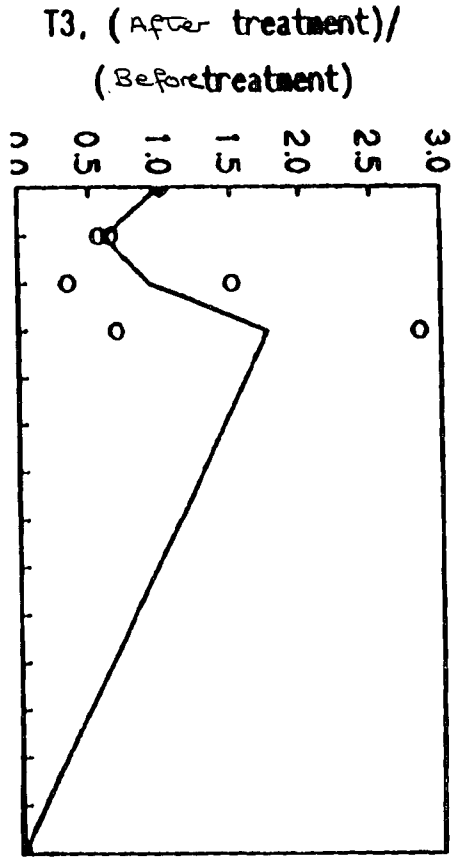
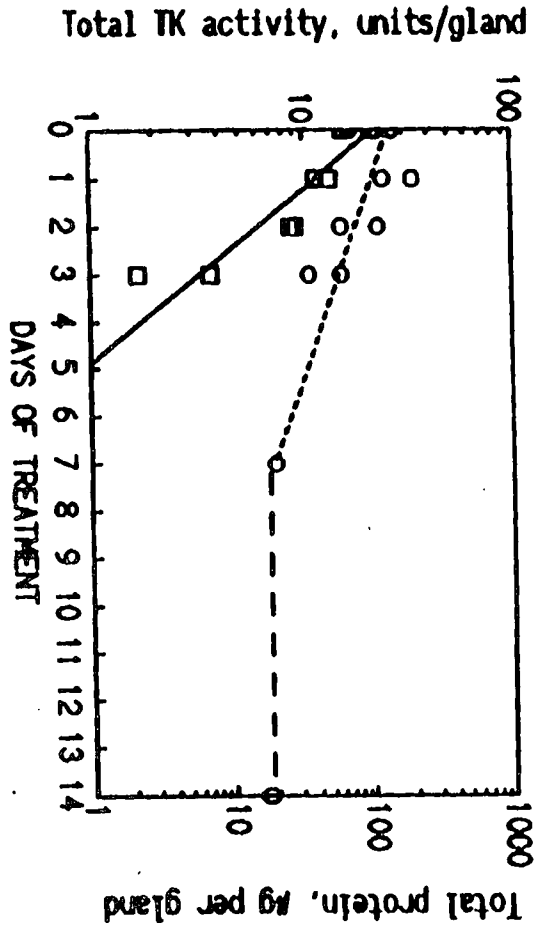
FIGURE 10.

THYROID HORMONE LEVELS AND HSV1-TK ACTIVITY DURING  
ABLATION.

A) T<sub>4</sub> levels and B) T<sub>3</sub> levels in transgenic mice treated with 4.5mg/day ganciclovir for 1, 2, 3, 7 and 14 days. Points represent the ratio of hormone levels obtained ~~after~~ and ~~before~~ the experiment. Graph shows line of best fit between data points.

C) Total protein and HSV1-tk activity obtained from the thyroid glands of transgenic mice treated with 4.5mg/day ganciclovir for 1, 2, 3, 7 and 14 days. Curve represents an exponential decay curve where  $Y = A(B^{-x})$ .

(Courtesy of J. Bishop)



of different mice, the data suggests that after an initial decline the circulating levels of the thyroid hormones may recover after 2-3 days (Table 9). If so, this recovery is followed by a second decline which is complete before the 14 day time-point (Table 4 and Figure 10A and 10B).

The recovery, if it does in fact occur, could be due to any of several possible mechanisms: (1) Circulating levels of TSH may increase in response to declining levels of thyroid hormones. (2) At short times of treatment the residual thyrocytes might take up and process iodinated thyroglobulin more rapidly and/or iodinate intrafollicular thyroglobulin more actively. (3) The death of follicle cells might release into the follicles the enzymes which process iodinated thyroglobulin, leading to a surge in hormone production. The release of processing enzymes might also explain the eventual dispersal of the follicle content. (4) Iodinated thyroglobulin released from the follicles might be processed in the body fluids to release thyroid hormones.

#### CELL DIVISION.

Toxicity of the anti-herpetic agent is due to phosphorylation of the drug by HSV1-TK. The phosphorylated products are then converted to nucleoside triphosphates by the viral and cellular kinases and are incorporated into the DNA resulting in chain termination and ultimately cell death. Experiments *in vitro* demonstrated that the phosphorylated drug products are

capable of preventing DNA replication (Borrelli *et al.*, 1988), while *in vivo* experiments, for example, ablation of thymic tissue (Heyman *et al.*, 1989), suggested that cell populations with high mitotic indices ablate more efficiently than those with a low mitotic activity. However prior investigations of the conditional transgenic ablation technique have always studied the effects of ablation in dividing cells (Borrelli *et al.*, 1988;1989, Heyman *et al.*, 1989). This leaves open the question of whether the conditional ablation technique can be applied to non-dividing cells. The thyroid gland is an ideal tissue with which to address this question. The follicle cells in the adult thyroid are essentially non-dividing with mitotic indices less than  $10^{-3}$  (Santler, 1957, Wollman *et al.*, 1968). TSH and EGF when present at elevated levels, can stimulate cell division (Dumont, 1971). I therefore investigated whether either the expression of the TG-TK transgene or the addition of ganciclovir to a transgenic mouse induced cell division in the thyroid gland or whether instead ablation was occurring in the absence of cell division. Thyroid sections from transgenic female mice treated with ganciclovir for 1-3 days were challenged with an antibody directed to proliferating cell nuclear antigen (PCNA). PCNA (also called cyclin) is an intranuclear protein found in normal proliferating cells. The level of PCNA increases during the G1 phase of the cell cycle, reaching a maximum during S phase and declining during the G2/M phase (Kurki *et al.*, 1988). PCNA is also induced during



the repair of DNA damage (Shivji *et al.*, 1992). The section in Figure 11B (compare Figure 11A) shows thyroid tissue from an untreated transgenic mouse. No PCNA-positive cells were identified, indicating that the expression of the transgene in the thyroid gland does not induce a higher rate of cell-division or cause damage to the DNA. Treating transgenic mice with ganciclovir revealed PCNA-positive cells on day 2 of drug treatment (Figure 11D). The positively-stained cells were not present in follicle structures, suggesting that they may not be thyrocytes. As shown previously, at this time of treatment the morphology of the thyroid gland has altered with fewer follicle structures being present (Figure 9) while the total HSV1-TK activity has fallen by 50% (Table 9). This data therefore suggests that ablation of thyrocytes can occur in the absence of cell division and that the appearance of PCNA-positively stained cells at day 2 of ganciclovir treatment probably occurs as a result of ablation rather than a cause of ablation. Cell death results in the release of lysosomal enzymes which attach cellular structures, including the DNA. It is possible that an attack on the DNA induces PCNA in the dying cell.

To conclude, the results above suggest that ganciclovir can kill non-dividing cells, bringing into question the general assumption that the incorporation of phosphorylated ganciclovir into the DNA of dividing cells is the only cause of cell death. However it is possible that phosphorylated ganciclovir is incorporated into the

FIGURE 11.

CELL DIVISION IS NOT OBSERVED IN THE THYROID GLAND.

Thyroids sections (3 $\mu$ m) from female transgenic mice, treated with ganciclovir (4.5mg/day), were challenged with a mouse monoclonal proliferating cell nuclear antibody (Boehringer). Sections were developed using horseradish-peroxidase and counter stained with haematoxylin. Examples of positively stained cells are indicated by an arrow.

A) Non-transgenic.

B) Untreated transgenic.

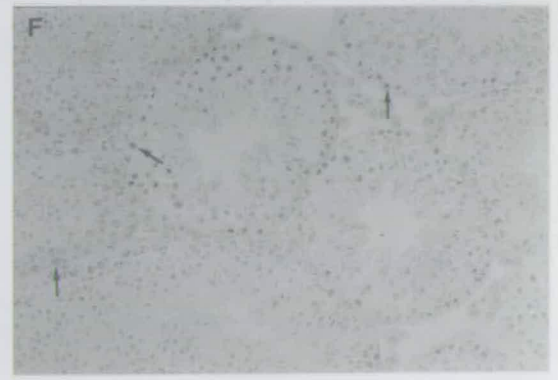
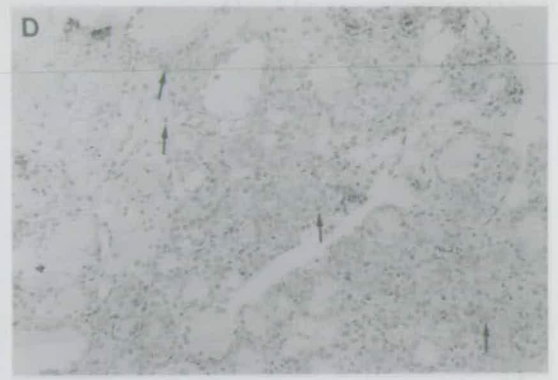
C) 1-day of ganciclovir treatment.

D) 2-days of ganciclovir treatment.

E) 3-days of ganciclovir treatment.

F) Non-transgenic testes

Magnification x100.



DNA by a DNA repair mechanism. Recently it has been reported that PCNA is required for the DNA synthesis that repairs nicked DNA intermediates (Shivji *et al.*, 1992). Alternatively, as ganciclovir is an analogue of guanosine and different G-proteins are involved in many intracellular processes, ganciclovir may be acting at a level other than incorporation into the DNA, for example, on a secondary messenger system within the thyrocyte.

It is quite possible that ablation by ganciclovir of non-dividing cells that express HSV1-TK is unique to thyrocytes. The mechanism of cell death induced by ganciclovir in the presence of HSV1-TK requires further investigation.

#### **REGENERATION.**

Treatment with ganciclovir of transgenic mice carrying an immunoglobulin promoter linked to the HSV1-tk gene resulted in the ablation of specific lineages in the thymus (double positive cells for the cell surface T-cell markers  $\text{Lyt}^{2+}$  and L3T4) while other populations showed selective enrichment (single positives and double negatives). Seven days following the removal of the ablating agent both the  $\text{Lyt}^{2+}$  and L3T4 populations in the thymus were similar to PBS treated control transgenics demonstrating that stem cells sufficient to repopulate the cell lineages had survived the treatment (Heyman *et al.*, 1989). Similarly, Borrelli *et al.*, (1989) observed regeneration of both somatotrophs and lactotrophs when the administration of FIAU was discontinued in mice

carrying a GH-TK transgene.

The potential of the thyroid to regenerate after treatment with ganciclovir was investigated. The minipumps were removed after two weeks of drug administration. In the first instance, the mice were sacrificed at 10 day intervals for up to 30 days. At the end of 30 days the mice had no detectable levels of  $T_4$  or  $T_3$  in the serum and HSV1-TK activity in the thyroid gland was equivalent to a non-transgenic control (Table 10). IEF urinary protein gels were run in conjunction with the hormone assays to assess MUP expression (Chapter 5). The results were consistent with a lack of thyroid hormones.

The experiment was therefore extended to a period of 4 months. At the end of this time there was no indication that thyroid gland regeneration was occurring:  $T_4$  and  $T_3$  levels were still zero, the HSV1-TK activity of the gland was comparable to a non-transgenic control and the total soluble protein was approximately 30% of the control. These results suggest that young adult mice do not contain a non-differentiated stem cell that is capable of repopulating the follicle cells of the thyroid. This is consistent with the view that after thyroidectomy, in man, regeneration of the thyroid gland occurs through slow division of residual thyrocytes. However we cannot exclude the possibility that a distinct stem cell population exists because if the stem cells express the TG-TK transgene they will be sensitive to ganciclovir.

TABLE 10.

THYROID HORMONE LEVELS OVER 113 DAYS POST ABLATION.

Mice were administered ganciclovir (4.5mg/day) by minipump for two weeks at the end of this time the pumps were removed. The mice were sacrificed at interval indicated in Table 10. T<sub>4</sub> and T<sub>3</sub> levels in mice were measured using a competitive ELISA, values are given in nmol/L. The units of HSV1-TK activity in the thyroid rudiment was also assessed, as was the total protein content of the rudiment (µg).

Days following mini-pump removal.	T <sub>4</sub> (nmol/L)		T <sub>3</sub> (nmol/L)		TK Activity	Total Protein (µg)
	Before	After	Before	After		
10 Post Ablation	37.7	0.0	0.92	0.00	0.03	10
20 Post Ablation	61.5	0.0	1.01	0.00	0.00	20
30 Post Ablation	73.5	0.0	0.97	0.00	0.00	7
	61.8	0.0	0.87	0.00	0.00	16
<b>Average</b>	<b>67.6</b>	<b>0.0</b>	<b>0.92</b>	<b>0.00</b>	<b>0.00</b>	<b>12</b>
Non-Transgenic & PBS	53.7	27.9	0.72	0.60	0.01	66
57 Post Ablation	57.1	0.0	0.85	0.00	0.01	25
Non-Transgenic & PBS	54.0	124.0	0.54	0.23	0.01	25
85 Post Ablation	33.3	0.0	0.47	0.00	-	-
	60.1	0.0	0.34	0.00	0.03	40
<b>Average</b>	<b>46.7</b>	<b>0.0</b>	<b>0.40</b>	<b>0.00</b>	<b>0.03</b>	<b>40</b>
Non-Transgenic & PBS	37.0	31.8	0.18	0.43	0.10	100
	35.7	54.2	0.60	0.51	0.02	180
<b>Average</b>	<b>36.4</b>	<b>43.0</b>	<b>0.39</b>	<b>0.47</b>	<b>0.06</b>	<b>140</b>
113 Post Ablation	52.5	0.0	-	-	-	-
	44.7	0.0	0.36	0.00	0.02	40
<b>Average</b>	<b>48.6</b>	<b>0.0</b>	<b>0.36</b>	<b>0.00</b>	<b>0.02</b>	<b>40</b>
Non-Transgenic & PBS	59.5	43.3	-	-	-	-
	42.3	43.1	0.66	0.49	0.02	130
<b>Average</b>	<b>50.9</b>	<b>43.2</b>	<b>0.66</b>	<b>0.49</b>	<b>0.02</b>	<b>130</b>

## EFFECTS OF PROLONGED THYROXINE DEFICIENCY.

Thyroid hormone deficiency in man results in deteriorated functioning of several bodily systems, namely the cardiovascular, gastrointestinal and neuromuscular systems. Symptoms of hypothyroidism include weight gain, cold intolerance, weakness, slow movements and reflexes and coarse skin and hair.

Weight gain was monitored during and after ganciclovir and PBS administration for a period of 83 days (Figure 12). In contrast to the weight-gain observed in man traditional methods of creating hypothyroidism in animals, for example, by the anti-thyroid drug, methimazole (Narayan *et al.*, 1985), or surgery, results in a cessation of weight-gain. Rather than a cessation of weight-gain the mean weight of the transgenic thyroxine-deficient mice at any particular time point showed slowed weight-gain compared with the control mice. The reduced weight-gain is similar to that seen in immature GH-deficient animals (Behringer *et al.*, 1988, 1990), suggesting that the slowing down of growth observed in the transgenic ablated mice may be due to a lack or deficiency of both GH and IGF-I. As mentioned earlier  $T_3$ , in the rat, is required for full expression of the GH gene (Glass *et al.*, 1987, Koeing *et al.*, 1987, Sap *et al.*, 1990). The actions of GH are often direct. However its effect on growth are thought to be mediated partly directly and partly by induction of IGF-1. The production of IGF-1 is modulated by GH (Mathews *et al.*, 1986, Roberts *et al.*, 1986) with the main site of synthesis

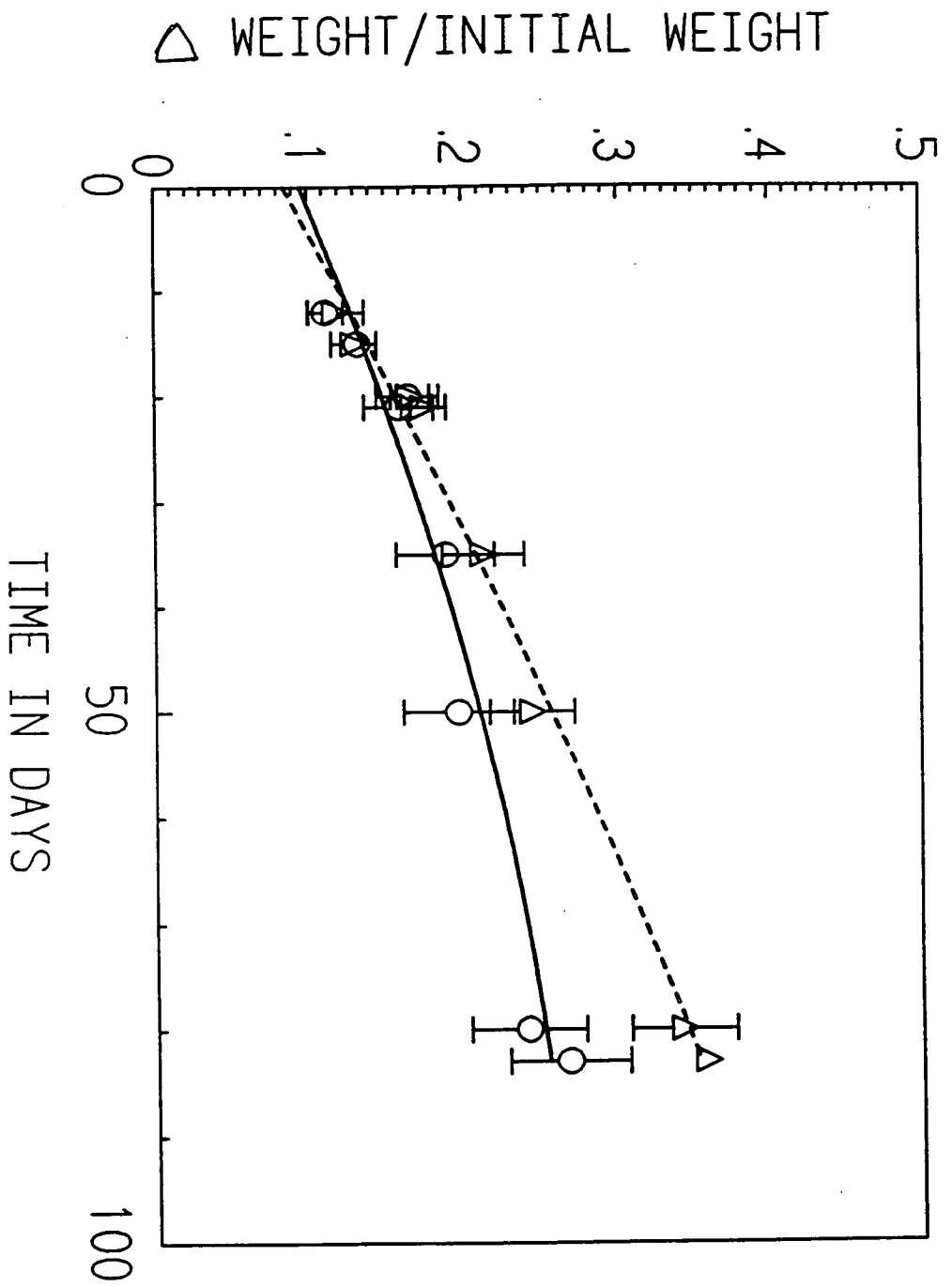


FIGURE 12.

WEIGHT GAIN OF THYROXINE-DEFICIENT MICE OVER 83 DAYS.

Effects of prolonged thyroxine deficiency on weight over 83 days. Data analysed by second degree polynomial regression analysis where  $Y = A + BX + CX^2 + DX^3 + EX^4$ , and D and E are constants.

Weight gain (g) is measured as the change of weight/initial weight, where circles represent the mean of weights in ablated mice at a particular time point and triangles represent control mice. Bars represent the deviation from the mean. (courtesy of J. Bishop)



being the liver. Transgenic animals which over-express GH and IGF-I (Chapter 1) have been created in an attempt to elucidate their individual effects on growth (Palmiter *et al.*, 1982, 1983, Hammer *et al.*, 1985, Behringer *et al.*, 1988, 1990, Mathews *et al.*, 1988a, 1988b, Brem *et al.*, 1989). The studies confirmed that both hormones exert growth promoting actions.

The muscle weakness and shivering which are associated with methimazole treatment were not observed in the transgenic thyroxine-deficient mice. The movement of the mice appeared sluggish compared to their controls and their fur was dishevelled, in appearance which correlates with the signs of hypothyroidism mentioned earlier.

Female mice which had been thyroxine-deficient for 1 month successfully mated and maintained pregnancy to term. The resulting litters were developmentally normal.

In conclusion, transgenic ablated mice exhibited a lower rate of weight gain which may be explained by a deficiency of GH and hence IGF-I. The thyroxine-deficient mice were fertile and able to rear successful litters.

## CHAPTER FIVE.

### USING THE THYROXINE-DEFICIENT MOUSE MODEL.

In this chapter the effect of administering thyroid hormones to thyroxine-deficient mice is examined and the use of the model to examine the role of thyroid hormones in the regulation of gene expression is explored.

If ablation has induced a single hormonal deficiency, replacement of the hormone should restore the animal to normal. In order to address this issue I selected a single phenotypic characteristic known to be dependent on the thyroid hormone status of the animal. I chose to examine the role of thyroid hormones in the expression of hepatic mouse major urinary protein (MUP) genes and also of a MUP transgene. The results shown below demonstrate that thyroid hormone treatment of mice exposed to ganciclovir restores the animal to normal. In addition the results clarify the role of thyroxine in MUP hepatic gene expression.

The MUP genes can be divided into four groups. Group 1 contains approximately 15 genes. Many of the group 1 genes are known to be expressed in the liver and, with the exception of one of these genes which is also expressed in the mammary gland (Held *et al.*, 1987), none are expressed in other tissues. The proteins are synthesised as pre-proteins, secreted into the blood and ultimately excreted into the urine (Finlayson *et al.*,

1965). MUP expression in the liver is regulated by thyroxine and growth hormone (Knopf *et al.*, 1983, Shaw *et al.*, 1983, Norstedt and Palmiter, 1984, Spiegelberg and Bishop, 1988, Al-Shawi *et al.*, 1992). The expression pattern of group 1 MUP genes in the liver is sexually dimorphic. The difference between the sexes can be examined by studying urinary proteins or liver mRNA. Male mice excrete 20 to 30 times more MUP protein than females (Szoka & Paigen 1978), and the spectrum of MUP proteins excreted by the two sexes is different (Knopf *et al.*, 1983, Clissold *et al.*, 1984). The level of MUP mRNA in the male liver is at least 5 times greater than in the female due to differences in the transcription rate (Hastie *et al.*, 1979, Derman, 1981, Derman *et al.*, 1981). By probing liver mRNA with gene specific oligonucleotide probes it was shown that different genes have different levels of expression in the two sexes (McIntosh and Bishop, 1989).

Secretion of GH from the pituitary, in the rat, is controlled by the hypothalamic hormones growth hormone releasing factor (GRF) which stimulates secretion and somatostatin which is inhibitory. It is generally assumed that this control mechanism is universal. The different hypothalamic secretion patterns are determined mainly by the sex steroid status of the animal (Jansson *et al.*, 1984, Jansson and Frohman, 1987). The sexually dimorphic expression of hepatic genes, for example the MUP genes, is thought to be due to differences in the pattern of GH secretion in the two sexes. The pattern of GH secretion

in the mouse shows no sex-dependent difference in either the amplitude of the GH peak or of the basal secretion level (MacLeod *et al.*, 1991). In male mice, however, the level of circulating GH shows a sharp peak, on average, at intervals of 2.5 hours while in female mice the peaks occur at intervals of 1.4 hours. This contrasts to the situation in the rat where the male has regular high amplitude secretion bursts every 3-4 hours, and females show a more continuous, but highly variable secretion pattern, characterised by low amplitude secretion (Tannenbaum and Martin, 1978, Robinson and Clark, 1987). It has therefore been suggested the time interval between GH peaks is important in GH mediated masculinisation.

By manipulating the levels of circulating GH in mice the levels of MUP mRNA can be masculinised or feminised. For example, the continuous infusion of GH by osmotic minipump decreased MUP mRNA synthesis in male mice (Norstedt and Palmiter, 1984). Similarly females given periodic treatment of GH (by injection) have increased levels of MUP mRNA (Al-Shawi *et al.*, 1992). Mice homozygous for the recessive mutation little (*lit*) have low levels of circulating GH (Eicher and Beamer, 1976) and express MUP at a very low level (Knopf *et al.*, 1983, Norstedt and Palmiter, 1984). GH administered to *lit/lit* mice of either sex, by injection to mimic the male GH pattern, induced a male level of MUP expression (Knopf *et al.*, 1983, Norstedt and Palmiter, 1984, Al-Shawi *et al.*, 1992).

Species specific actions of thyroid hormones are

observed. For example, there is a functional TRE in the promoter region of the rat GH gene but not in the promoter of the human gene (Glass *et al.*, 1987, Koeing *et al.*, 1987). There is a second TRE in the third intron of the rat GH gene which can confer thyroid hormone responsiveness to a heterologous promoter *in vivo* (Sap *et al.*, 1990). The effect of thyroid hormones on GH transcription in the rat may explain the long-established observation that hypothyroidism results in reduced growth. In the mouse the role thyroid hormones play in GH transcription is unknown. However if thyroid hormones play a role analogous to their role in the rat, thyroid hormone deprivation would also affect GH levels and therefore MUP expression.

Thyroid hormones may also affect MUP gene expression by a second route. This was indicated from experiments in which GH and T<sub>4</sub> were administered separately and together to hypophysectomised female mice. Hypophysectomy eliminates GH secretion, and greatly reduces or abolishes thyroid hormone secretion because of a lack of TSH. MUP levels in hypophysectomised mice were reduced approximately 100 fold compared to normal female levels. The administration of supra-physiological levels of either GH or T<sub>4</sub> increased MUP mRNA levels 2-fold compared to normal female levels while the administration of both hormones increased MUP mRNA 14-fold (Knopf *et al.*, 1983). This data suggest that the two hormones act synergistically, and also that each one on its own has an inducing effect. However it was not clear whether after

hypophysectomy the levels of either hormone were zero. Low levels of thyroid hormones are produced in the absence of TSH (autoregulation - see Chapter 1). Thus the effect seen upon administration of GH alone may have depended upon the presence of a low residual level of thyroid hormones.

The aims of the experiments described below are two fold, firstly to determine whether thyroid hormone treatment can reverse the suppression of MUP gene expression induced by ganciclovir ablation of TG-TK mice and secondly to determine whether GH induces MUP expression in the total absence of  $T_4$ .

## RESULTS.

Mice treated with ganciclovir for two weeks had very low levels of urinary MUP (Figure 13A lanes 3 and 4). In contrast, MUP levels were unaffected in a transgenic mouse treated with PBS and a non-transgenic mouse treated with ganciclovir (Figure 13A, lanes 1, and 2, 13 and 14). Administration of  $T_4$  during the last five days of ganciclovir treatment restored the circulating level of  $T_3$  to near-normal levels (Table 11) and also restored the urinary MUP level (Figure 13A lanes 11 and 12). Ablation of the thyrocytes was complete as shown by the very low level of HSV1-TK activity in the residual thyroid tissue (Table 11).

As would be expected administration of GH to transgenic mice during the last 5 days of ganciclovir treatment did not induce a detectable level of



### FIGURE 13.

#### A) ISOELECTRIC FOCUSING GEL OF URINARY PROTEINS FOLLOWING GANCICLOVIR TREATMENT AND HORMONE REPLACEMENT.

Mouse urine samples were collected before and after treatment. Odd number lanes, before treatment, even number lanes, after treatment. Mice were TG66.19 transgenic females unless otherwise indicated. Lanes 1 to 14 were as follows:

1+2) PBS treated; 3+4) ganciclovir treated; 5+6) ganciclovir and T<sub>4</sub> diluent treated; 7+8) ganciclovir and GH treated; 9+10) non-transgenic and GH treated; 11+12) ganciclovir and T<sub>4</sub> treated; 13+14) Non-transgenic and ganciclovir treated.

Variation in the MUP pattern between individual mice is due to heterozygosity at the MUP locus in C57/BL x CBA cross.

#### B) NORTHERN BLOT ANALYSIS ON FEMALE TG66.19 MICE.

Northern blot analysis of total liver RNA (30µg/lane). Probed using Mup11 (Group 1 gene). Autoradiograph exposed for 8 hours.

Lanes 1) PBS treated female; 2) ganciclovir treated female; 3) ganciclovir and T<sub>4</sub> treated female; 4) ganciclovir and GH treated female; 5) GH treated non-transgenic female; 6) non-transgenic male; 7) ganciclovir treated non-transgenic female.

#### C) EXPRESSION OF THE BS6- $\alpha_{2u}$ TRANSGENE IN TG/RL MALE AND FEMALE MICE.

Northern blot of total liver RNA (30µg/lane), probed with a 50 mer  $\alpha_{2u}$  oligonucleotide (see Material and Methods) autoradiograph exposed for 8 hours. Mice were TG/RL males and females unless otherwise indicated.

Lanes: 1) BS6-24 male; 2) PBS treated male; 3) PBS treated female; 4) ganciclovir treated male; 5) ganciclovir treated female; 6) ganciclovir and T<sub>4</sub> treated male; 7) ganciclovir and T<sub>4</sub> treated female; 8) ganciclovir and GH treated male; 9) ganciclovir and GH treated female; 10) ganciclovir, T<sub>4</sub> and testosterone treated female; 11) ganciclovir and testosterone treated female; 12) ganciclovir treated non-transgenic male; 13) ganciclovir treated non-transgenic female.

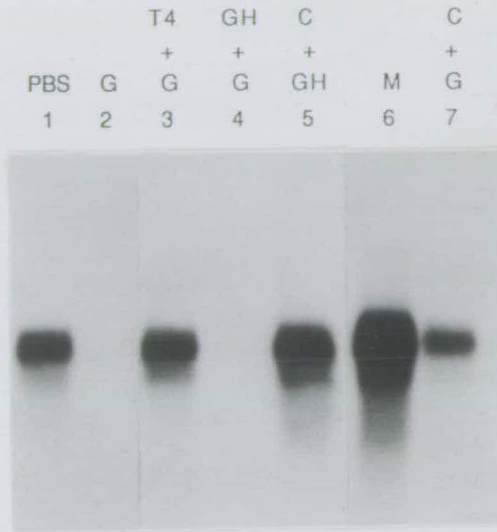
Testosterone (T) pellets (30mg) were implanted in female mice at the time of ganciclovir administration and continued for a further two weeks post ablation. Growth hormone (GH) was administered i.p. (100µg/20g body weight) every 12 hours during the last 5 days of ablation. T<sub>4</sub> (5µg/20g body weight) was injected every 24 hours over the same time period.

M = male; F = female; C = control; DIL = T<sub>4</sub> diluent G = ganciclovir.

A.



B.



C.

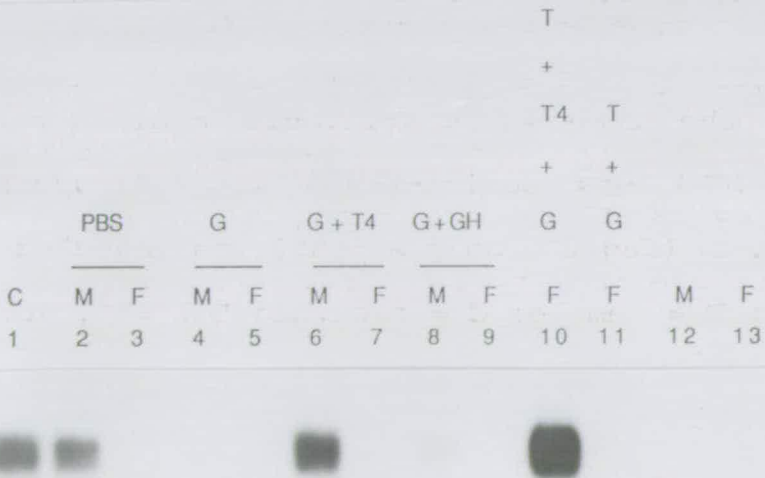


TABLE 11.

THYROID HORMONE LEVELS IN ABLATED MICE AFTER HORMONE REPLACEMENT.

Pooled serum samples were analysed for T<sub>4</sub> and T<sub>3</sub> using a competitive ELISA (Kodak Diagnostics). The average HSV1-TK activity per gland is given in units/thyroid. Total protein is the total recovered from the gland.

Growth hormone was administered i.p. (100µg/20g body weight) every 12 hours during the last 5 days of ablation. T<sub>4</sub> (5µg/20g body weight) was injected every 24 hours over the same time period.

Treatment & Exp. Nos	T <sub>4</sub> (nmol/L)		T <sub>3</sub> (nmol/L)		TK Activity	Total Protein (µg)
	Before	After	Before	After		
Transgenic & PBS 2	44.1	39.5	0.95	0.58	17.50	144
4	57.1	34.5		-	20.40	95
<b>Average</b>	<b>50.6</b>	<b>37.0</b>		<b>0.58</b>	<b>18.95</b>	<b>220</b>
Transgenic & ganciclovir 2	29.8	0.0		-	0.03	10
4	38.1	0.0		-	0.02	30
<b>Average</b>	<b>33.9</b>	<b>0.0</b>		<b>-</b>	<b>0.02</b>	<b>20</b>
Transgenic & ganciclovir/GH 2	20.1	0.0		0.00	0.03	8
4	47.3	0.0		-	0.01	3
<b>Average</b>	<b>33.7</b>	<b>0.0</b>		<b>0.00</b>	<b>0.02</b>	<b>6</b>
Transgenic & ganciclovir/T <sub>4</sub> 2	50.1	64.2		0.33	0.04	8
4	47.5	266.3		-	0.02	7
<b>Average</b>	<b>48.8</b>	<b>165.2</b>		<b>0.33</b>	<b>0.03</b>	<b>8</b>
Non-Transgenic & ganciclovir 2	21.0	10.8		3.90	0.02	170
4	55.1	41.6		-	0.01	105
<b>Average</b>	<b>38.0</b>	<b>26.2</b>	<b>3.90</b>	<b>0.02</b>	<b>138</b>	

circulating  $T_4$  or  $T_3$ . Again ablation of the thyrocytes was complete as indicated by the lack of HSV1-TK activity (Table 11). In contrast to  $T_4$  treatment, GH did not restore the urinary MUP levels (Figure 13A lanes 7 and 8). The biological potency of the GH was assessed by treating a female control mouse. This resulted in a male pattern of MUP protein (Figure 13A lanes 9 and 10). Thus, replacement of GH alone, in the absence of thyroid hormones, did not induce MUP gene expression. Availability of thyroid hormones is evidently an absolute requirement for the expression of MUP genes.

The level of MUP mRNA present in the liver of control and treated mice correlated with the levels of urinary MUP (Figure 13B) and demonstrate that the analysis of urine provides a method for assessing the role of hormone regulation on MUP gene expression, and consequently the hormonal status of the mouse.

#### EFFECTS OF THYROXINE ON EXPRESSION LEVELS OF A MUP TRANSGENE.

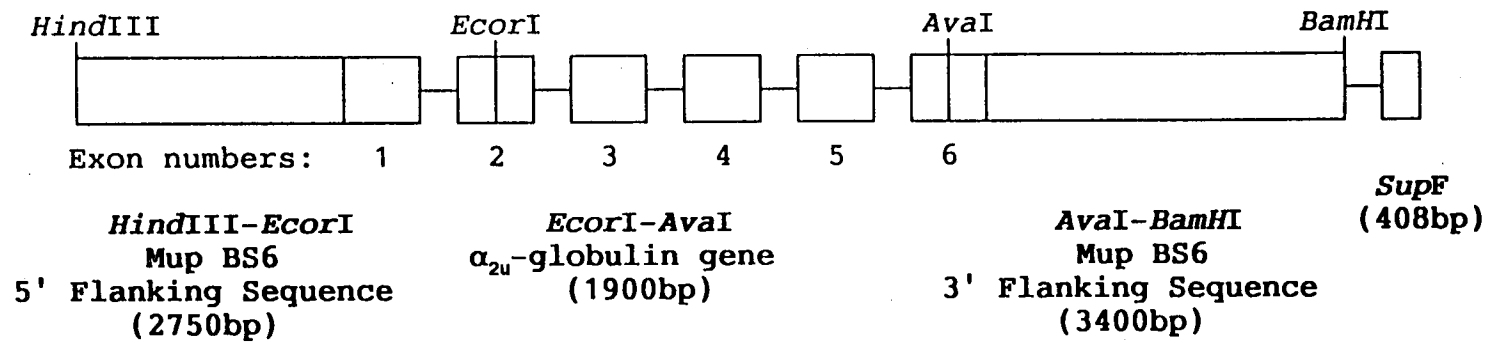
To investigate *cis*-acting elements which are involved in  $T_4$  and GH induction of MUP gene expression a hybrid gene was constructed. It comprised 3 fragments as described in the Figure legend of Figure 14. Although  $\alpha_{2u}$ -globulin genes are 81% homologous to MUP genes the degree of dissimilarity is sufficient to allow the RNA and protein products of the hybrid gene to be distinguished from endogenous MUP mRNA and protein.

The expression of the hybrid gene was examined in

**FIGURE 14.**

**BS6- $\alpha_{2U}$  HYBRID GENE.**

A hybrid gene was constructed comprising of 3 fragments: a 2.75kb 5' fragment (*HindIII/EcoRI*) of the group 1 MUP gene BS6 (Clarke *et al.*, 1984), which extends 2.2kb upstream of the cap site to a site in exon 2 (*EcoRI*). The region extending from the restriction site in exon 2 to a restriction site in exon 6 (*AvaI*) was replaced by the homologous region of the  $\alpha_{2U}$ -globulin gene  $\alpha_{207}$ . This was flanked with a 3.4kb fragment (*AvaI/BamHI*) which includes 1.8kb 3' flanking sequences of the group 1 MUP gene BS6. A 408bp sequence was included which contained the bacterial gene *supF* to allow recovery from transgenic DNA (Al-Shawi *et al.*, 1990).



transgenic mice of both sexes. High levels of expression were detected in the male liver whereas in the female liver there was no expression of the transgene, or expression at a very much lower level than in the male (Al-Shawi *et al.*, 1992). Thus the sexually dimorphic expression of the hybrid transgene is similar to that of the resident  $\alpha_{2u}$ -globulin genes in the rat (Kulkarni *et al.*, 1985).

The role of thyroid hormones in regulating the expression of the BS6- $\alpha_{2u}$  transgene was investigated by crossing mice carrying the BS6- $\alpha_{2u}$  transgene with transgenic mice carrying the TG-TK transgene. F<sub>1</sub> mice were designated (TG/RL). TG/RL mice of both sexes were treated with ganciclovir for two weeks. Some of the ganciclovir treated mice were exposed to T<sub>4</sub>, GH and testosterone as described in the legend to Table 12A and 12B. Expression of the BS6- $\alpha_{2u}$  transgene was examined by Northern blot analysis of total liver mRNA (Figure 13C). Expression of the transgene in TG/RL control mice treated with PBS was sexually dimorphic (Figure 13C lanes 2 and 3) and indistinguishable from its expression in the BS6- $\alpha_{2u}$  parental line (Al-Shawi *et al.*, 1992).

The expression of the transgene in male TG/RL mice treated with ganciclovir was abolished just as expression of the resident MUP genes was abolished in ablated female mice (Figures 13C lane 4). The absence of circulating T<sub>4</sub> and T<sub>3</sub> was confirmed by ELISA assays (Table 12A). Administration of T<sub>4</sub> treatment during the last 5 days of



TABLE 12A & B.

LEVELS OF THYROID HORMONES IN TG/RL TRANSGENIC MICE  
FOLLOWING ABLATION.

12A experiments on male TG/RL mice

12B experiments on female TG/RL mice

Ganciclovir (4.5mg/day) was administered by minipump to TG-TK mice crossed with BS6-24 mice, (TG/RL), for two weeks. Serum samples were analysed for T<sub>4</sub> and T<sub>3</sub> levels. Average HSV1-TK activity per gland is expressed in units/thyroid and total protein is given in µg. A testosterone pellet (30mg) was implanted at time of ganciclovir administration and continued for a further two weeks post ablation. Growth hormone was administered i.p. (100µg/20g body weight) every 12 hours during the last 5 days of ablation. T<sub>4</sub> (5µg/20g body weight) was injected every 24 hours over the same time period.

G = ganciclovir; M = male; F = female; C = control; T<sub>4</sub> = thyroxine; GH = growth hormone.

A.

Treatment	Hormones Administered	T <sub>4</sub> (nmol/L)		T <sub>3</sub> (nmol/L)		TK Activity	Total protein (µg)
		Before	After	Before	After		
Male TG/RL							
PBS		51.1	35.2	0.77	0.46	28.5	40
		40.1	16.3			30.6	150
<b>Average</b>		<b>45.6</b>	<b>25.8</b>			<b>29.6</b>	<b>95</b>
ganciclovir		58.5	1.3	0.55	0.00	0.00	21
		38.7	0.0			0.01	20
<b>Average</b>		<b>48.6</b>	<b>0.6</b>			<b>0.01</b>	<b>20</b>
ganciclovir	GH	47.0	0.0	0.69	0.00	0.00	10
		44.0	0.0			0.03	30
<b>Average</b>		<b>45.5</b>	<b>0.0</b>			<b>0.02</b>	<b>20</b>
ganciclovir	T <sub>4</sub>	47.8	197.3	0.78	1.53	0.00	10
		34.6	279.1			0.01	10
<b>Average</b>		<b>41.2</b>	<b>238.2</b>			<b>0.01</b>	<b>10</b>
Non-Transgenic. & ganciclovir		69.7	30.2	0.65	0.45	0.00	60
		53.0	17.9			0.07	110
<b>Average</b>		<b>61.4</b>	<b>24.0</b>			<b>0.04</b>	<b>85</b>

8.

Treatment	Hormones Administered	T <sub>4</sub> (nmol/L)		T <sub>3</sub> (nmol/L)		TK Activity	Total protein (µg)
		Before	After	Before	After		
Female TG/RL							
PBS		66.8	34.2	0.77	0.40	17.6	60
		62.9	26.6			25.5	80
<b>Average</b>		<b>64.8</b>	<b>30.4</b>			<b>21.6</b>	<b>70</b>
ganciclovir		54.6	0.0	0.80	0.00	0.00	20
		46.7	0.0			0.01	4
<b>Average</b>		<b>50.6</b>	<b>0.0</b>			<b>0.01</b>	<b>12</b>
ganciclovir	GH	55.3	2.9	0.53	0.00	0.00	2
		44.1	0.0			0.04	20
<b>Average</b>		<b>49.7</b>	<b>1.4</b>			<b>0.02</b>	<b>11</b>
ganciclovir	T <sub>4</sub>	47.5	210.0	0.82	4.81	0.00	7
		49.5	243.2			0.11	30
<b>Average</b>		<b>48.5</b>	<b>226.6</b>			<b>0.06</b>	<b>18</b>
ganciclovir	T <sub>4</sub> & Testosterone	36.3	151.6	0.59	0.58	0.00	10
		38.3	287.9			0.04	
<b>Average</b>		<b>37.3</b>	<b>219.8</b>			<b>0.02</b>	
ganciclovir	Testosterone	31.2	3.2	0.19	0.00	0.00	7
		33.4	0.0			0.03	20
<b>Average</b>		<b>32.3</b>	<b>1.6</b>			<b>0.02</b>	<b>14</b>
Non-Transgenic & ganciclovir		52.2	33.7	0.57	0.56	0.00	70
		36.6	25.7			0.03	110
<b>Average</b>		<b>44.4</b>	<b>29.7</b>			<b>0.02</b>	<b>90</b>

ganciclovir treatment restored transgene expression (Figure 13C lane 6). In contrast addition of GH during the same time period did not restore transgene expression (Figure 13C lane 8). Ablation of the thyroid gland in ganciclovir treatment mice was complete as indicated by the low HSV1-TK activity of the thyroid rudiments excised from the mice (Table 12A).

The hormonal regulation of the BS6- $\alpha_{2u}$  transgene was also investigated in female mice. Administration of testosterone to female mice induces the expression of the MUP genes (Al-Shawi *et al.*, 1992) presumably by masculinising the GH profile. Administration of T<sub>4</sub> to an ablated female did not induce transgene expression (Figure 13C lane 7). Although testosterone induces transgene expression in normal females (AL-Shawi *et al.*, 1992) testosterone alone did not induce the transgene expression in ablated females (Figure 13C lane 11). However when both testosterone and T<sub>4</sub> were administered to an ablated female expression of the transgene was induced even to levels above those normally found in male transgenic mice (Figure 13C lanes 10 and 2 respectively). The treatment was shown to restore the level of circulating T<sub>3</sub> (Table 12B). Ablation of the thyroid gland was complete as indicated by the low HSV1-TK activity of the thyroid rudiment (Table 12B).

The results presented above demonstrate an absolute requirement for thyroid hormones in the expression of endogenous MUP expression and in the expression of a MUP transgene in male and female transgenic mice. Expression

of the transgene in female mice requires both the presence of  $T_4$  and testosterone indicating that expression requires a "male-like" GH status. Experimental evidence indicates that in addition to the possible role of thyroid hormones in GH transcription, thyroid hormones may be involved in potentiating the GH receptor on the liver membrane (Chernausk *et al.*, 1982, Hochberg *et al.*, 1990). In addition thyroid hormones may also play a role in the GH signal transduction pathway (Meier *et al.*, 1991). These last two points are discussed in further detail in Chapter 6.

## CHAPTER SIX.

### DISCUSSION.

Until now there has been no satisfactory animal model which could be used to examine the effects of complete thyroid hormone deficiency. The genetically hypothyroid mouse retains circulating levels of thyroid hormones (10%, Stein *et al.*, 1989) and suffers from secondary defects possibly due to thyroxine deficiency *in utero* and in the early neonate, while the surgical removal of the thyroid gland results in the loss not only of the thyroxine producing cells (thyrocytes) but also of the parathyroid gland and the C-cells.

The work presented in this thesis demonstrates the use of transgenic ablation to generate an animal model which completely lacks thyroid hormones. The hormone deficiency in this model is inducible and therefore the animal is permitted to develop normally prior to ablation.

The results reported in Chapter 2 revealed that the expression of the thyroglobulin-HSV1-tk transgene was highly restricted to the thyroid gland in female transgenic mice with the site of HSV1-TK expression being confined to the thyroid follicle epithelial cells. In Chapter 3 the effects of ganciclovir treatment on the thyroid gland in transgenic mice are described. Treatment with ganciclovir at a rate of 4.5mg/day for two weeks

resulted in an extensive regression of the thyroid gland while the expression of the transgene was rapidly reduced to zero due to destruction of the follicle epithelial cells. During the second week circulating levels of  $T_4$  and  $T_3$  fell below the limits of detection. The specificity of ablation in the thyroid gland was also assessed. The results indicated that, in the absence of  $T_4$ , circulating levels of parathyroid hormone and calcitonin were normal. This was in agreement with the persistence of an intact parathyroid gland and functional C-cells in the treated mice.

The time course of changes in the morphology of the gland and in the expression of the transgene was examined during ganciclovir treatment (Chapter 4). The changes were rapid with a significant reduction in the expression of the transgene being observed after 1 day of drug treatment. The morphology of the gland also changed rapidly and only a few colloid-containing follicle structures were present 3 days after the start of ganciclovir treatment.

After terminating treatment with ganciclovir the potential of the thyroid gland to regenerate was examined. After a period of four months no recovery of circulating  $T_4$ ,  $T_3$  or regeneration of the thyrocytes was observed suggesting that the thyroid gland does not contain a non-differentiated stem cell population capable of repopulating the gland. The weight gain of the ablated mice was slower than that of age-matched controls. Although the mice showed no distress, they were sluggish

in their movements and their fur was dishevelled in appearance.

The ablation studies were carried out in adult mice where the thyrocyte is essentially non-dividing. Successful ablation of the thyroid gland therefore brings into question the general assumption that phosphorylated ganciclovir kills cells exclusively by being incorporated into DNA. The practical use of the thyroxine-deficient mouse model to examine the role of thyroid hormones in gene expression was examined in Chapter 5. The results indicated that the hepatic expression of the mouse urinary protein genes was dependent on the presence of  $T_4$ . The following section discusses further applications of the thyroxine deficient mouse model.

#### FURTHER APPLICATION OF THE THYROXINE-DEFICIENT MOUSE MODEL.

##### GROWTH HORMONE RECEPTORS.

Hypothyroidism has a profound affect on growth, which may be mediated by one or more different primary effects of the hormone. In the rat, the thyroid hormones stimulate GH transcription. Consequently, due to the regulatory role of GH in IGF-I expression, growth in hypothyroid animals may be reduced by decreased production of IGF-I. Indeed levels are decreased in hypothyroid rats (Chernausk et al., 1982, Mathews et al., 1986). However, the growth-promoting effect of exogenously administered GH is blunted in hypothyroidism (Lewinson et al., 1989). This might be due to an effect



on the cell-surface GH receptor, but published data on the role of thyroid hormones in potentiating the GH receptor is conflicting. Chernausek *et al.*, (1982) reported a doubling of the binding capacity in chronically hypothyroid male rats, whereas Hochberg *et al.*, (1990) showed in mice that the binding of both Prl and GH was inversely correlated with the thyroid hormone level, but was restored by thyroid hormone replacement. Consistent with this, binding was increased in hyperthyroidism.

Thyroid hormones may also be involved in the GH signal transduction pathway in the liver. Although little is known about the pathways activated within a cell, following the binding of GH to its membrane bound receptor, the hepatic GH receptor is associated with tyrosine kinase activity suggesting that the GH receptor may initiate signal transduction by tyrosine phosphorylation (Foster *et al.*, 1988). It has been suggested that thyroid hormones stimulate phosphorylation and dephosphorylation of rat liver cytosolic proteins (Nakamura and DeGroot, 1983). In hypothyroid rats levels of protein kinase C are increased 7.7 fold compared to a euthyroid control (Meier *et al.*, 1991). Increased protein kinase C levels may inhibit transmembrane signalling by down regulating the signals which lead to its activation. For example, in the case of the EGF receptor, protein kinase C leads to phosphorylation of the receptor causing a decrease in EGF-stimulated tyrosine kinase activity (Schlessinger, 1986).

The effect of thyroid hormones in the regulation of the GH gene can be examined in the thyroxine-deficient mouse model by comparing levels of GH mRNA in the pituitary of ablated and non-ablated mice. The effects of thyroid hormones on the hepatic GH receptor can be assessed by determining the levels of GH receptor mRNA in the liver of thyroxine-deficient mice, in conjunction with hepatic membrane GH binding studies. This will give information regarding the role of thyroid hormones in the transcription of the GH receptor gene and in potentiating the GH receptor on the liver cell membrane.

#### RENIN GENE EXPRESSION.

Renin is stored in secretory granules in the convoluted tubular cells of the mouse submaxillary gland. The regulation of the renin-I gene in the submaxillary gland is under the control of testosterone and thyroid hormones (Wilson *et al.*, 1982). Renin-I is expressed at lower levels in the female than in the male mouse. However, male-like levels of expression can be induced in females following treatment with testosterone. Similarly, an increase in the expression levels are observed following T<sub>4</sub> treatment (Wilson *et al.*, 1982). Renin-I levels in hypophysectomised female mice are reduced approximately 300-fold compared to normal females. The administration of either T<sub>4</sub> or testosterone increased renin-I activity 5-fold and 3-fold respectively compared to normal female levels, while the administration of both hormones induced activity approximately 40-fold. The data

suggests that the two hormones act synergistically and that each hormone on its own has an inducing effect. However, since the presence of low levels of residual  $T_4$  was not excluded, it is possible that the action of testosterone would have no effect in the complete absence of  $T_4$ . The action of  $T_4$  on renin expression has been shown not to be mediated through testosterone (Wilson *et al.*, 1982).

The congenitally hypothyroid mouse (*hyt/hyt*) has low basal secretion of renin. Female mice show a blunted response to either  $T_4$  or testosterone when administered separately, but a synergistic increase is observed in both renin activity and renin mRNA when the hormones are present together (McGowan *et al.*, 1988).

It seems possible that the stimulation of renin-I expression by testosterone is mediated by GH.  $T_4$  may be required for GH action (compare MUP expression, Chapter 5). The thyroxine deficient mouse could be used to address this question. GH and testosterone could be administered in the presence or absence of  $T_4$ . In contrast to the *hyt/hyt* mouse the thyroxine-deficiency is inducible and the mouse develops normally prior to ablation.

#### LACTATION.

Lactating dairy cows treated with GH show increased milk yields compared with untreated lactating controls. The mechanism for this lactopoietic response, however, remains to be determined. The role of an intermediate

hormone in mediating the response has been indicated. For example, it has long been recognised that thyroid hormones are important in maintaining lactation. GH-treated lactating dairy cows have been shown to have a tissue-specific increase in the mammary gland activity of the 5'-monodeiodinase enzyme, which catalyses the conversion of the relatively inactive  $T_4$  to the active form of the hormone  $T_3$ . In contrast GH treatment did not alter the activity of the deiodinase enzyme in the kidney or liver (Capuco *et al.*, 1989).

The data, above, indicates that the lactopoietic effect of GH on the mammary gland may involve an increase in the rate of conversion of  $T_4$  to  $T_3$ . This hypothesis can be examined by using the thyroxine-deficient mouse model. 5' monodeiodinase activity in the mammary gland and milk yields from lactating thyroxine deficient female mice could be compared to lactating thyroxine deficient females treated with either GH or  $T_4$ .

#### BRAIN DEVELOPMENT.

Dietary iodine deficiency, which is common in the third world, results in a high incidence of hypothyroidism. Foetal hypothyroidism results in neurological damage which occurs early in pregnancy. This is due to the involvement of thyroid hormones in brain maturation. For example, hypothyroidism results in retardation in nerve fibre myelination, a decrease in the density of the axon network and reduced interactions between the nerves (Eayrs, 1960, Clos *et al.*, 1973). The

hypothyroid state of the neonate can be reversed at birth by thyroid hormone supplements or by the addition of iodine to the water. However the neurological defects are not reversible, indicating that there may be a critical time *in utero* when thyroid hormones must be present. The thyroxine-deficient mouse model could be used successfully to address this question. By treating pregnant thyroxine-deficient transgenic mice with ganciclovir it should be possible to ablate the thyroid glands of the transgenic embryos (the thyroid gland is active in the embryo from day 15 of gestation onwards: Kawaori and Tsuneda, 1985). Replacement of thyroid hormones at different times during the remainder of gestation could reveal the precise time at which thyroid hormones act during brain development.

## MATERIALS AND METHODS.

### MICE.

All manipulations on live animals were carried out according to Home Office regulations (H.A.C.W. license holder).

Mice carrying the thyroglobulin-thymidine kinase transgene (TG-TK) were maintained as heterozygotes on a C57BL/6 x CBA/ca background. Mice carrying the rat  $\alpha_{2u}$  transgene were bred on a C57BL/6 background. F<sub>1</sub> TG/RL transgenics were created by crossing a female TG-TK mouse with a male transgenic mouse carrying the  $\alpha_{2u}$  transgene. Transgenic mice were identified by amplification of part of the transgene in a crude DNA preparation by the polymerase chain reaction (PCR). Briefly, 1cm was removed from the tip of the tail under anaesthetic. The tissue was broken down in 2ml containing 0.3M sodium acetate; 0.5% SDS; 0.01M Tris pH 8.0; 0.02M EDTA pH 8.0; 2mg/ml proteinase K, at 43°C overnight. 2 $\mu$ l of the tail sample was used per reaction which contained 0.2mM of each dATP, dCTP, dGTP and dTTP, 2 units taq polymerase, 10% buffer (100mMol/L Tris-Cl, 15mMol/L gelatine - Boehringer), 10% DMSO, and 20 pMol of each primer. Primers used amplified part of the SupF, tk, and HPRT genes (Al-Shawi *et al.*, 1992). The DNA was denatured at 95°C for 10 minutes, then 30 cycles of PCR were carried out containing 2 steps in

each cycle: 92°C - 0.6 minutes, 65°C - 5 minutes and finally 65°C for 10 minutes at the end. TG/RL transgenic mice were first identified by amplifying part of the tk gene and then part of the  $\alpha_{2u}$ -globulin gene. The  $\alpha_{2u}$ -globulin gene was amplified by PCR at the following conditions: 94°C - 2.5 minutes, 50°C - 0.5 minutes, 72°C - 1 minute, then 35 cycles of PCR containing 3 steps in each cycle: 91°C - 0.5 minutes, 50°C - 0.5 minutes, 72°C - 1 minute and finally 72°C for 10 minutes at the end. Primers used for amplification were supplied by Oswel DNA services, Department of Chemistry, Edinburgh University.

#### ENZYMES AND RADIO-LABELLED NUCLEOTIDES.

All enzymes were purchased from Boehringer and used with the buffers supplied unless otherwise indicated. Radio-labelled nucleotides were purchased from Amersham International.

#### URINE SAMPLES.

Samples were collected by bladder massage. The urine was dialysed against 0.1mM NaCl, 10mM Tris-HCl pH 7.6 for 24 hours at 4°C, then against distilled water for a further 24 hours. The samples were stored at -20°C.

#### SERUM SAMPLES.

Blood samples were collected at the start of the

experiment by tail snip and massage and at the end of the experiment by brachial pouch bleeds. The blood samples were allowed to clot then microfuged at 10,000 rpm for 10 minutes at room temperature. Serum was collected and stored at -20°C.

#### DRUG AND HORMONE TREATMENT.

Female mice were implanted with Alzet osmotic minipumps subcutaneously for up to 7 or 14 days (models 2001 and 2002 respectively). Ganciclovir (Syntex) was dissolved in phosphate buffered saline (PBS) at 188 mg/ml for 2001 pumps and 376 mg/ml for 2002 pumps, and administered at a rate of 4.5 mg/day. Control mice received pumps containing PBS.

L-thyroxine (sigma) was injected intraperitoneally every 24 hours (5µg/20g body weight: Knopf *et al.*, 1983), for the last five days of ganciclovir administration. T<sub>4</sub> was dissolved in 3mM NaOH, 1% NaCl. Bovine growth hormone (bGH - gifted by the National Pituitary and Hormone Agent Programme, Maryland, U.S.A.) was injected intraperitoneally (100µg/20g) every 12 hours for the same time period as T<sub>4</sub>. GH was dissolved at alkaline pH in 900µl distilled H<sub>2</sub>O and 100µl 0.02M NaOH then neutralised with 100µl 0.5M NaPO<sub>4</sub> pH 7.4. 30mg testosterone pellets were implanted subcutaneously.



## HORMONE ASSAYS.

### ELISA

Assays were carried out in duplicate according to the manufactures instructions (Kodak Diagnostics).

Total T<sub>4</sub>: 10µl of standard or serum was added to wells previously coated with a donkey anti-sheep T<sub>4</sub> antibody. 100µl of horseradish peroxidase T<sub>4</sub> conjugate and 100µl of sheep anti-T<sub>4</sub> was added and the mixture was incubated at 37°C for one hour. After washing the wells the amount of bound conjugate was determined by an enhanced luminescence reaction.

Total T<sub>3</sub>: As above, but 25µl of sample or standard was added to well coated with donkey anti-sheep T<sub>3</sub> and using 100µl of horseradish peroxidase T<sub>3</sub> conjugate and 100µl sheep anti-human T<sub>3</sub> antibody.

### Radio-immunoassays (RIA).

Commercial kits were used according to the manufacturer's instructions (Kodak Diagnostics) with the following modifications. Samples were assayed in duplicate.

Calcitonin: 50µl of goat anti-human calcitonin antibody, 50µl of standard or serum and 50µl of iodinated synthetic (human) calcitonin peptide were mixed together in a round bottom plastic tube and incubated overnight at 4°C. 1ml of precipitating reagent containing donkey antiserum against goat gamma-globin was added and the reaction was incubated for a further 30 minutes. The tubes were centrifuged for 20 minutes at >1500g and the supernatant

was decanted. The iodinated calcitonin in the pellet was counted in a gamma counter.

Parathyroid hormone: As above but using guinea pig anti-human (mid-molecule) antiserum, iodinated human PTH and goat anti-guinea pig gamma-globin antiserum.

#### TISSUE PREPARATION AND THYMIDINE KINASE ASSAY.

Tissues were homogenised in 10mM KCl, 2mM MgCl<sub>2</sub>, 10mM Tris-Cl pH 7.5, 1mM ATP, 10mM NaF and 50mM Amino cuproic acid. Extracts were centrifuged at 10,000 rpm for 10 minutes at 4°C and the Supernatants were stored at -70°C. The assay was carried out as described by Brinster *et al*, (1981) but with the following modification, 0.4mM TTP was added to the reaction mix to block endogenous thymidine kinase activity (J. Burke unpublished and Al-Shawi *et al*, 1988). Samples were assayed in duplicate. Briefly 12.2µl of tissue extract was incubated in 72.8µl of reaction mix (10mM MgCl<sub>2</sub>, 150mM Tris-Cl pH 7.5, 10mM ATP, 25mM NaF, 10mM β-mercaptoethanol and 4µCi tritiated thymidine <sup>3</sup>H TMP: 90Ci/mMol) at 37°C for 30 minutes and 60 minutes. At these time points 25µl of sample was spotted onto DEAE-81 paper (Whatman). The discs were washed 3 times for 10 minutes each in 10mM Tris-Cl pH 8.0. After being dried under vacuum the <sup>3</sup>H TTP on the discs was quantified by scintillation counting. Protein concentrations in the tissue extracts were determined by the Bradford method (Bradford, 1976).

## PROTEIN ANALYSIS.

### Western blot analysis.

The tissues were homogenised in the same buffer as described for the thymidine kinase assay but with the addition of a proteinase inhibitor, 10mM Para-hydroxymercuribenzoate (Sigma). Protein concentration in the tissue extracts were determined by the Bradford method (Bradford, 1976). Tissue extracts were run on a 17cm x 14cm x 0.75mm, 12% bis-polyacrylamide laemmli discontinuous gel (Laemmli, 1970), with a 3cm, 4% stacking gel in 0.5M Tris-Cl pH 6.8 and a 14cm resolving gel of 12% polyacrylamide, 1.5M Tris-Cl pH 8.8. Samples were prepared by adding an equal volume of sample buffer (0.15M Tris-Cl pH 8.8, 0.2% (w/v) SDS, 10% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol, 0.001% (w/v) bromophenol blue) and heating at 95°C for 4 minutes. The gel was run for 16 hours at 10mA constant current. The proteins were transferred onto nitrocellulose by the semi-dry blotting method (Khyse-Andersen 1984), using a home made electroblotting apparatus, for 1.5 hours at 4°C. Buffers used were as follows: Anode 1 - 0.3M Tris, 20% methanol, pH 10.4; Anode 2 - 25mM Tris, 20% methanol, pH 10.4; Cathode - 25mM Tris, 20% methanol, 40mM hexanoic acid, pH 9.4. The gel was stained with coomassie blue to check that the proteins had been transfer.

The nitrocellulose membrane was challenged with a 1:250 dilution of rabbit HSV1-TK antiserum overnight at room temperature (a gift of Dr W. Summers), 1:400 dilution biotinylated anti-rabbit Ig (3 hours at room

temperature: Amersham) and a 1:1000 dilution streptavidin alkaline phosphatase (1 hour room temperature: Amersham). After each antibody incubation the filter was washed (2 x 15 minutes) in phosphate buffered saline and 0.1% tween<sub>20</sub>. The immunoreactive product was detected by Nitro blue tetrazolium (sigma)/ Bromo-chloro-indolyl phosphate (Boehringer) substrate (Leary *et al.*, 1983). All antibody dilutions were carried out in "Blotto" (5% "marvel" in Phosphate buffered saline - Johnston *et al.*, 1984).

#### Isoelectric focusing gel.

Agarose IEF gels were cast according to the ampholine manufacturer's instructions (Pharmacia). The gel comprised 1% IEF agarose, 12% sorbitol and 6% ampholines of which 86% were 4.2-4.9 and 14% were 3.5-5.0. The cathode and anode buffers were 1M NaOH and 0.05M H<sub>2</sub>SO<sub>4</sub> respectively. 20µl urine samples were focused for 1.5 hours with limited voltage (1500V). The gel was fixed in 5% sulphosalicylic acid, 10% trichloroacetic acid for 30 minutes and stained in coomassie blue (0.2% (w/v) coomassie brilliant blue R-250; 50% methanol; 10% acetic acid). The gel was destained in 25% ethanol; 8% acetic acid and dried.

#### HISTOLOGY.

Thyroid glands were fixed in Bouins (Sigma) for 24 hours then dehydrated in 70%, 90%, and 95% ethanol for one hour each. Then 100% ethanol for 2 hours and xylene

for a further hour. The gland was embedded in fibrowax (BDH). 5µm sections were cut, fixed, dewaxed and stained with haematoxylin and 1% Eosin (BDH). (haematoxylin:- 0.2% (w/v) haematoxylin, 0.02% (w/v) sodium iodate, 5% (w/v) potassium alum, 2.1% (v/v) acetic acid, 0.5% (v/v) ethanol).

#### Adipose tissue.

Thyroid glands were removed from the mouse and immediately frozen in O.C.T. compound in an isopropanol dry ice bath. 10µm sections were cut and fixed in 4% formaldehyde for 2 minutes. Sections were rinsed in 70% ethanol and stained in sudan black B stain (saturated in 70% ethanol) for 30 minutes. The sections were then rinsed in 70% ethanol and then water prior to being mounted in glycerol.

#### IMMUNOCYTOCHEMISTRY.

##### Calcitonin.

Thyroid glands were fixed in 1% formalin, 0.65% (w/v) Na<sub>2</sub>HPO<sub>4</sub>, and 0.4% (w/v) NaH<sub>2</sub>PO<sub>4</sub> for 24 hours. The glands were embedded as above. 3µm sections were cut, fixed and dewaxed. Endogenous peroxidase activity was blocked with 3% (v/v) H<sub>2</sub>O<sub>2</sub> and 97% (v/v) methanol for 30 minutes. Sections were washed in distilled water then trypsinised for 30 minutes at 37°C (trypsin 0.1% (w/v) in CaCl<sub>2</sub> (0.1% w/v) pH 7.8). Sections were rinsed in water then 0.05M Tris buffered saline pH 7.6 (TBS:- 0.05M Tris-Cl, 0.15M NaCl) and incubated with 1:5 normal swine

serum, diluted in TBS, for 10 minutes. 1:500 calcitonin antibody (Dako), diluted in 1:5 swine serum, was added and incubated for 30 minutes. The sections were rinsed twice in TBS. 1:500 dilution of biotinylated anti-rabbit Ig antibody diluted in 1:5 swine serum (Dako) was incubated for a further 30 minutes. After washing in TBS the sections were incubated with an avidin-biotin complex for 30 minutes (made according to the manufacturer's instructions - Dako). The sections were developed with Diaminobenzidine (DAB - 2.5mg DAB (Sigma) dissolved in 5ml of 0.05M Tris, 0.03M HCl, 0.07% imidazole (w/v, Sigma) pH 7.6, and 0.02% (v/v) H<sub>2</sub>O<sub>2</sub>). The sections were counter stained in haematoxylin.

#### Thymidine kinase.

Thyroid glands were fixed in Bouins solution for 24 hours then embedded in fibrowax as above. 3µm sections were cut, fixed and dewaxed and the sections were treated with H<sub>2</sub>O<sub>2</sub> as above, then 1mM EDTA, 0.05% tween<sub>20</sub> in phosphate buffered saline (PBS) for 10 minutes. Sections were washed in 2% egg albumin in PBS for 30 minutes and incubated with 1:1000 rabbit anti-HSV1-TK serum, diluted in 1% BSA/PBS, over night at 4°C. After washing the sections twice in TBS (see above) for 10 minutes each, the sections were incubated for 1 hour with a secondary antibody:- 1:50 swine anti-rabbit Ig biotinylated (Dako). Again the sections were washed in TBS twice, for 15 minutes each, followed by a further incubation of 30 minutes with the avidin biotin complex (Dako). Sections

were developed using DAB (see above) and counter stained in haematoxylin.

#### Cell division.

Thyroid glands were fixed in Bouins solution for 24 hours then embedded in fibrowax as above. 3µm sections were cut, fixed and dewaxed and the sections were treated with H<sub>2</sub>O<sub>2</sub> as above. Sections were washed in 2% egg albumin in PBS for 30 minutes and incubated with 1:80 dilution of the proliferating cell nuclear antigen (Boehringer) overnight at 4°C. Dilution was in 0.1% BSA/PBS. Sections were then washed in 2% egg albumin in PBS for 30 minutes. 1:900 anti-mouse Ig horseradish peroxidase labelled secondary antibody (diluted in 0.5% BSA/PBS) was incubated for 90 minutes at room temperature. Again the sections were washed in 2% egg albumin for 30 minutes then washed in TBS for 10 minutes. Sections were developed using DAB (see above) and counter stained in haematoxylin.

#### RNA PREPARATION AND NORTHERN BLOTTING.

Total cellular RNA from livers was prepared by the method of Chirgwin *et al.*, (1979). Briefly, approximately 1 gram of liver was homogenised in 4mls of 4M guanidinium thiocyanate, 25mM sodium citrate pH 7.0, 0.5% (w/v) sarkosyl, 0.33% (v/v) sigma antifoam A. The pH of the solution was adjusted to 7.0 and 0.32% (v/v) of β-mercaptoethanol was added prior to homogenisation. The

RNA was purified by centrifugation through a 1.2ml CsCl cushion (5.7M CsCl, 25mM NaAc) using a SW50 rotor 36,000 rpm, 20°C for 12 hours. The RNA pellet was resuspended in 1ml of 7.5M guanidine hydrochloride pH 7.0, 25mM sodium citrate, 125mM DTT. The RNA was precipitated by adding 25µl of 1N acetic acid and 0.5ml ethanol and incubated at -20°C overnight. After centrifuging, in a HB4 rotor at 10,000 rpm 4°C for 30 minutes, the RNA pellet was resuspended in 1ml dH<sub>2</sub>O and precipitated at -20°C with the addition of 1/10<sup>th</sup> volume 2M NaAc pH 5.0. and 3 volumes of ethanol. Following centrifugation for 30 minutes (see above) the RNA pellet was resuspended in dH<sub>2</sub>O.

Thyroid RNA was prepared using the RNazol method of Chomczynski and Sacchi, (1987). Stock solution was made containing 1 volume solution A (0.72% (v/v) of β-mercaptoethanol was added prior to use); 1/10<sup>th</sup> volume solution B; 1 volume water saturated phenol. Solution A:- 47.28% (w/v) guanidium thiocyanate, 25mM sodium citrate pH 7.0, 1% (w/v) sarcosyl. Solution B:- 2M Na Acetate pH 4.0. The tissue was homogenised in 1ml of stock solution, 100µl of chloroform was added and the sample was shaken vigorously for 15 seconds before being left on ice for 15 minutes. After being microfuged for 15 minutes at 4°C the aqueous phase was transferred to a clean tube. The RNA was precipitated with an equal volume of isopropanol for 45 minutes at -20°C. The RNA pellet was washed twice with 75% ethanol and finally dissolved in dH<sub>2</sub>O.

RNA samples were run in a gel containing 1.8% agarose, 6% formaldehyde, 10mM sodium phosphate buffer pH



7.0. Prior to being loaded onto the gel RNA samples were denatured at 60°C for 5 minutes in 24% formamide, 6.3% formaldehyde, 10mM sodium phosphate buffer pH 7.0. The gel was run at 30V for 24 hours. The RNA was transferred to Hybond-N (Amersham) by capillary blotting and covalently bound by U.V. cross-linking as in the manufacturer's instructions.

#### LABELLING OF PROBES.

A 714bp group I MUP gene cDNA, MUP 11, was isolated from pUC by *EcoRI* and *BamHI* digests (A. Chave-Cox, 1986). 30ng of the DNA was labelled as described by Feinberg and Vogelstein (1983). Briefly the DNA was made up to 12µl with water and boiled for 3 minutes to denature the DNA. This was added to 6µl of OLB, 1.2µl of 10mg/ml BSA (enzyme grade), 4µl (40µCi) of  $\alpha$ -<sup>32</sup>P dCTP (> 3000Ci/mMol) and 1µl of klenow (1 unit). The reaction was incubated overnight at 25°C.

OLB; 2:5:3 mixture of A:B:C. A) 625µl 2M Tris-Cl pH 8.0, 25µl 5M MgCl<sub>2</sub>, 350µl H<sub>2</sub>O, 18µl β-mercaptoethanol, 5µl each of dATP, dTTP, dGTP (0.1M in 3mM Tris-Cl, 0.2mM EDTA pH 7.0). B) 2M HEPES pH 6.6. C) Hexadeoxyribonucleotides (Pharmacia) 900D units/ml in 3mM Tris HCl, 0.2mM EDTA pH 7.0.

30ng of an  $\alpha_{2u}$ -globulin specific oligonucleotide (5'-CAAATATAGTTCCCTGCACTCTCCATTTTCCTTAATACGGAAGC-3') was end labelled (Maxam and Gilbert, 1980) in 25µl using 80µCi of gamma <sup>32</sup>P dATP (>5000Ci/mMol), 5 units of T<sub>4</sub>

polynucleotide kinase and 2.5 $\mu$ l of buffer (10mM MgCl<sub>2</sub>, 50mM Tris-Cl pH 7.6). The reaction was incubated at 37°C for 10 minutes then terminated by the addition of 1/5<sup>th</sup> volume 0.2M EDTA.

Unincorporated nucleotides were removed using a NAP-5 column (Pharmacia). The number of radioactive nucleotides incorporated was estimated by quantifying the activity in 1 $\mu$ l of probe by counting Cherenkov counts in a scintillation counter. Routinely > 10<sup>8</sup> counts/ $\mu$ g were obtained.

#### HYBRIDISATION AND WASHING OF FILTERS.

Hybridisations and washes with MUP11 were carried out largely as described by Church and Gilbert, (1984). Filters were prehybridised in 0.5M NaHPO<sub>4</sub> pH 7.2, 7% SDS, 2mM EDTA (68°C 1 hour) and hybridised in the same solution plus labelled, denatured probe at 68°C overnight. The filters were washed in 40mM NaHPO<sub>4</sub>, 5% SDS, 1mM EDTA (68°C, 2 x 10 minutes) and 40mM NaHPO<sub>4</sub>, 1% SDS, 1mM EDTA (68°C, 2 x 20 minutes). The filters were exposed to X-ray film at -70°C for the times indicated in the figure legends.

Pre-hybridisation and hybridisation using the oligonucleotide was as above but the procedure was carried out at 60°C

## SUB-CLONING.

2.5 $\mu$ g of pTG-TK was digested with *MscI* and *EcoRV* (New England Biolabs & Boeringer respectively). A 312bp fragment was isolated by gel electrophoresis in a 5% acrylamide gel. The fragment was eluted from the gel slice in 100 $\mu$ l buffer (0.5M ammonium acetate, 10mM magnesium acetate, 1mM EDTA (pH 8.0) and 1% SDS at 37°C for 2 hours. Following a phenol/chloroform extraction the DNA was precipitated with ethanol.

Ligations were carried out at room temperature for 2 hours in 20 $\mu$ l containing >0.5 pmol/ml of pBluescript II sk<sup>+</sup> (Stratagene LTD) cut with *EcoRV* and >1 pmol/ml of fragment. Ligation buffer was 66mM Tris pH 7.6, 6.6mM MgCl<sub>2</sub>, 10mM DTT, 0.5mM ATP. The ligation mix was used to transform competent *E. coli* HB101 (Bolivar and Backman, 1979). Transformants were selected by ampicillin resistance. Orientation and copy number of the insert was determined by enzyme digests of DNA mini-preparations (lysis was carried out by boiling - Sambrook *et al*, 1989).

Large scale plasmid DNA preparations were prepared as follows: 25mls of an overnight culture containing the plasmid was used to inoculate 250mls of L-Broth and ampicillin (100 $\mu$ g/ml) and incubated overnight at 37°C. The culture was centrifuged at 10,000 rpm for 30 minutes at 4°C. The pellet was resuspended in 20ml of 10mM Tris-Cl, 1mM EDTA pH 7.0 and centrifuged at 10,000 rpm for 3 minutes. The pellet was resuspended in 4ml of sucrose mix

(25% sucrose, 50mM Tris-Cl pH 8.1, 40mM EDTA pH 8.1) and 1.2 ml of 10mg/ml lysozyme in sucrose mix. Following incubation on ice for 15 minutes 1.2ml of 0.5M EDTA pH 8.1 was added and incubation continued for a further 5 minutes. 10.8ml of triton mix (0.2% triton, 125mM EDTA pH 8.1, 0.1M Tris-Cl pH 8.1) was added. Genomic DNA was removed by centrifugation at 20,000 rpm for 60 minutes. To 18ml of supernatant 17.1g of CsCl and 1.8ml of EtBr (10mg/ml) was added. The plasmid DNA was banded by centrifuging in an A50 rotor at 35,000 rpm for 72 hours at 25°C. The plasmid band was withdrawn with a syringe and needle and washed with isopropanol saturated with water and caesium chloride to remove the EtBr. Following dialysis overnight at 4°C against 10mM Tris-Cl pH 8.0 the plasmid DNA was precipitated with 2 volumes of ethanol.

#### IN VITRO TRANSCRIPTION OF RNA.

Purified plasmid (above) was linearised with a *Hind*III digestion. The *in vitro* transcription reaction was in 100µl containing 40mM Tris pH 7.5, 6mM MgCl<sub>2</sub>, 20mM DTT, 2mM spermidine, 10mM NaCl, 100u RNase, 2.5mM each of ATP, GTP, CTP and UTP, 2.5µg linearised plasmid DNA and 50u T<sub>3</sub> RNA polymerase (Promega). This was incubated at 37°C for 1 hour. The DNA template was removed by digestion with RNase free DNase (1u/µg DNA - Promega) at 37°C for 15 minutes. Following a phenol/chloroform extraction the RNA was precipitated with 2 volumes of ethanol. The yield of RNA was estimated by running a

sample against DNA of known concentration on a 1.8% Tris-acetate gel.

#### PRIMER EXTENSION.

Analysis of the transgene transcription start site was carried out using a modification of the method of Ghosh *et al.*, (1980). A 20mer oligonucleotide, complementary to +438 to +419 of the HSV1-tk gene was 5' end-labelled (Maxam and Gilbert, 1980) using gamma-<sup>32</sup>p dATP (>5000Ci/mMol). The RNA was mapped as follows:- *in vitro* transcribed RNA (described above - 10pg, 1pg, 0.2pg mixed with 4µg carrier RNA) or thyroid RNA from either control (5µg) or transgenic (5µg or 1µg) male mice. The RNA and the oligonucleotide were annealed in 10µl, with 0.2ng of end-labelled primer and 2µl of 5x hybridisation buffer (0.2M PIPES pH 6.5, 2M NaCl, 5mM EDTA pH 8.0 and 80% formamide). Samples were denatured at 90°C for 5 minutes and incubated at 42°C over-night. The oligonucleotide was extended with the addition of 90µl buffer per tube (50mM Tris-Cl pH 8.2, 10mM DTT, 6mM MgCl<sub>2</sub>, 2.0% Actinomycin D, 0.5mM each of dCTP, dGTP, dTTP, dUTP and 50u reverse transcriptase), at 42°C for 60 minutes. Following a phenol/chloroform extracted the extension product was ethanol precipitated. After the samples were microfuged for 20 minutes at 10,000 rpm the pellet was dissolved in 3µl sequencing gel loading buffer (United states Biochemical corporation) and denatured at 90°C for 4 minutes prior to being electrophoresed in a 5%

acrylamide-7M urea 1 x TBE gel. The gel was fixed in 10% methanol, 10% acetic acid for 30 minutes and vacuum dried. The gel was exposed to X-ray film at  $-70^{\circ}\text{C}$  for the time indicated in the figure legend.

Sequencing of the M13mp18 was carried out using "Sequenase" according to the manufacturers instructions (United States Biochemicals). Briefly the annealing reaction used 1 $\mu\text{g}$  DNA, made up to 7 $\mu\text{l}$  with water, 2 $\mu\text{l}$  of sequencing buffer and 1 $\mu\text{l}$  primer. This was incubated at  $65^{\circ}\text{C}$  for 2 minutes, then allowed to cool to  $35^{\circ}\text{C}$ . To the annealed DNA mix 1 $\mu\text{l}$  0.1M DTT, 2 $\mu\text{l}$  diluted labelling mix (1:5). 0.5 $\mu\text{l}$  [ $^{35}\text{S}$ ]dATP and 2 $\mu\text{l}$  diluted sequenase (1:8) were added and incubated at room temperature for 5 minutes. 3.5 $\mu\text{l}$  of each reaction was transferred to a termination tube (ddGTP, ddCTP, ddATP, ddTTP), mixed and left to incubate for a further 5 minutes at  $37^{\circ}\text{C}$ . The reaction was stopped by the addition of 4 $\mu\text{l}$  of stop solution, the samples were heated to  $75^{\circ}\text{C}$  for 2 minutes prior to being loaded on a gel as above.

ABBREVIATIONS USED IN TEXT.

ATP	adenosine 5' triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate
bGH	bovine growth hormone
bisacrylamide	N, N,-methlene bis acrylamide
C	cytidine
cAMP	cyclic adenosine (3' -5') monophosphate
CBA/ca	inbred strain of mice
cdNA	complementary deoxyribonucleic acid
C/EBP	transcription factor: CAAT/enhancer binding protein
C-erb	thyroid hormone receptor oncogene
CRE	cAMP response element
CREB	cAMP response element binding protein
CT	calcitonin
C57BL/6	inbred strain of mice
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
ddATP	dideoxyadenosine triphosphate
ddCTP	dideoxycytosine triphosphate
ddGTP	dideoxyguanosine triphosphate
ddTTP	dideoxythymidine triphosphate
DEAE	diethylaminoethyl
dGTP	deoxyguanosine triphosphate
dH <sub>2</sub> O	distilled water
DIT	diiodotyrosine

DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DT-A	diphtheria toxin A subunit
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
dUTP	deoxyuridine triphosphate
EDTA	diaminoethanetetra-acetic acid
EGF	epidermal growth factor
ERE	oestrogen response element
ES	embryonic stem
EtBr	ethidium bromide
FIAU	(1, - (2-deoxy-2-fluoro-β-δ-arabinofuranosyl)-5-iodouracil
<i>fos</i>	nuclear protooncogene
G	guanine
g	grams
ganciclovir	[9-(1,3-dihydroxy-2-propoxymethyl) guanine]
GH	growth hormone
GRE	glucocorticoid response element
GRF	growth hormone releasing factor
Hepes	N-2-Hydroxyethylpiperazine-N'-2-ethane-sulphonic acid
HPRT	hypoxanthine phosphoribosyl transferase
HRE	hormone response element
hsp	heat shock protein
HSV	herpes simplex virus
H <sub>2</sub> O <sub>2</sub>	hydrogen peroxide
IEF	isoelectric focusing



IGF-I	insulin-like growth factor I
IGF II	insulin-like growth factor II
IP <sub>3</sub>	inositol tris-phosphate
<i>Jun</i>	nuclear protooncogene
kb	kilobase pair
kDa	kilodalton
klenow	large fragment Mr 68,000 of <i>E. Coli</i> DNA polymerase I
MHC	myosin heavy chain
MIT	monoiodotyrosine
mRNA	messenger RNA
MUP	major urinary protein
NaF	sodium fluoride
NBT	nitroblue tetrazolium
nGRE	negative glucocorticoid response element
page	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH	-log [H <sup>+</sup> ]
PIP <sub>2</sub>	phosphatidyl inositol 4,5,-biphosphate
PIPES	piperazine-N,N'-bis[2-ethanesulfonic acid]
Pit-1/GHF-1	pituitary specific <i>trans</i> -acting factor
PKA	protein kinase A
PKC	protein kinase C
PRE	progesterone response element
PRL	prolactin
PTH	parathyroid hormone
RNA	ribonucleic acid
RNase	ribonuclease

RNASIN	ribonuclease inhibitor
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
SPI	specific nuclear binding protein I
SV40	simian virus 40
TBE	Tris-borate, EDTA buffer pH 8.3
TBS	Tris buffered saline
Temed	N,N,N',N'-tetremethylethylenediamine
Tg	thyroglobulin gene
TK	thymidine kinase
TMP	thymidine monophosphate
TRE	thyroid hormone response element
TRH	thyrotrophin releasing hormone
Tris	tris-(hydroxymethyl)-aminomethane
TSH	thyroid stimulating hormone
TTF-I	thyroid specific trans-acting factor
TTP	thymidine triphosphate
T <sub>4</sub>	thyroxine
T <sub>3</sub>	3,5,3'-triiodothyronine
rT <sub>3</sub>	reverse T <sub>3</sub>
V	volts
v/v	volume per volume
U.V	ultra-violet
vol.	volume
w/v	weight per volume

## REFERENCES.

Adams, P.M., Stein, S.A., Palnitkar, M., Anthony, A., Gerrity, L., & Shanklin, D.R. (1989). Evaluation and characterization of the hypothyroid *hyt/hyt* mouse 1: Somatic and behavioural studies. *Neuroendocrin.* 49: 138-143

Adler, S., Waterman, M.L., He, X., & Rosenfeld, M.G. (1988). Steroid receptor-mediated inhibition of the rat prolactin gene expression does not require the receptor DNA-binding domain. *Cell* 52: 685-695

Al-Shawi, R., Burke, J., Jones, C.T., Simons, J.P., & Bishop, J.O. (1988). A *MUP* promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol. Cell. Biol.* 8: 4821-4828

Al-Shawi, R., Kinnard, J., Burke, J., & Bishop, J.O. (1990). Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. *Mol. Cell. Biol.* 10: 1192-1198

Al-Shawi,R., Burke,J., Wallace,H., Jones,C.T., Harrison,S., Buxton,D., Maley,S., Chandley,A., & Bishop,J.O. (1991). The herpes simplex type 1 thymidine kinase gene is expressed in the testis of transgenic mice under the control of a cryptic promoter. *Mol.Cell.Biol.* 11: 4207-4216

Al-Shawi,R., Wallace,H., Harrison,S., Jones,C., Johnson,D., & Bishop,J.O. (1992). Sexual dimorphism and growth hormone regulation of a hybrid gene in transgenic mice. *Mol. Endocrin.* 6: 181-190

Andros,G. & Wollman,S.H. (1967). Autoradiographic localization of radioiodide in the thyroid gland of the mouse. *Am. J. Physiol.* 213: 198-208

Arriza,J.L., Weinberger,C., Cerelli,G., Glaser,T.M., Handelin,B.L, Housman,D.E., & Evans,R.M. (1987). Cloning of human mineralocorticoid receptor complementary DNA: Structural and functional kinship with the glucocorticoid receptor. *Science* 237: 268-275

Ashburner,M. (1980). Chromosomal action of ecdysone. *Nature* 285: 435-436

Bakke,J.L., Lawrence,N., Bennett,J., Robinson,J., Bowers,C.Y. (1978). Late endocrine effects of administering monosodium glutamate to neonatal rats. *Neuroendocrin.* 26: 220-228

Beamer,W.G., Elcher,E.M., Maltais,L.J., Southard,J.L. (1981). Inherited primary hypothyroidism in mice. *Science* 212: 61-62

Beato,M. (1989). Gene regulation by steroid hormones. *Cell* 56: 335-344

Bech,K., Feldt-Rasmussen,U., & Madsen,S.N. (1981). Influence of thyroglobulin on basal and stimulated human thyroid adenylate cyclase activity. *J. Clin. Endocrin. Metab.* 53: 264-269

Becker,P.B., Ruppert,S. & Schütz,G. (1987). Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors. *Cell* 51: 435-443

Beckett,G.J., MacDougall,D.A., Nicol,F. & Arthur,J.R. (1989). Inhibition of type I and type II iodothyronine deiodinase activity in rat liver, kidney and brain by selenium deficiency. *Biochem. J.* 259: 887-892

Beckett,G.J., Nicol,F., Proudfoot,D., Dyson,K., Loucaides,G., & Arthur,J.R. (1990). The changes in hepatic enzyme expression caused by selenium deficiency and hypothyroidism in rats are produced by independent mechanisms. *Biochem. J.* 226: 743-747

Behringer,R.R., Mathews,L.S., Palmiter,R.D., & Brinster,R.L. (1988). Dwarf mice produced by genetic ablation of growth hormone-expressing cells. *Genes and development* 2: 453-461

Behringer,R.R., Lewin,T.M., Quaife,C.J., Palmiter,R.D., Brinster,R.L., & D'Ercole,J. (1990). Expression of insulin-like growth factor 1 stimulates normal somatic growth in growth hormone-deficient transgenic mice. *Endocrin.* 127: 1033-1040

Belchetz,P.E., Credley,G., Bird,D., & Himsworth,R.L. (1978). Regulation of thyrotropin secretion by negative feedback on triiodothyrosine on the hypothalamus. *J. Endocrin.* 76: 439-448

Berg,J.M. (1989). DNA binding specificity of steroid receptors. *Cell* 57: 1065-1068

Berk,A.J., & Schmidt,M.C. (1990). How do transcription factors work? *Genes and Development* 4: 151-155

Berry,M.J., Banu,L., & Larsen,P.R. (1991). Type I iodothyronine deiodinase is a selenocysteine-containing enzyme. *Nature* 349: 438-440.

Biggin,M.D., & Tjian, R. (1989). A purified *Drosophila* homeodomain protein represses transcription *in vitro*. *Cell* 58: 433-440

Bishop, J.O., & Al-Shawi, R. (1990). Gene expression in transgenic animals. In: Evolution and animal breeding, C.A.B. International 1989.

Bolivar, F., & Backman, K. (1979). Plasmids of *Escherichia Coli* as cloning vectors. *Methods in Enzymol.* 68: 245-267

Borrelli, E., Heyman, R., Hsi, M., & Evans, R.M. (1988). Targeting of an inducible toxic phenotype in animal cells. *Proc. Natl. Acad. Sci.* 85: 7572-7576

Borrelli, E., Heyman, R.A., Arias, C., Sawchenko, P.E., & Evans, R.M. (1989). Transgenic mice with inducible dwarfism. *Nature* 339: 538-541

Boutin, J-M., Jolicoeur, C., Okamura, H., Gagnon, J., Edery, M., Shiota, M., Banville, D., Dusanter-Fourt, I., Djiane, J. & Kelly, P.A. (1988). Cloning and expression of the rat prolactin receptor, a member of the growth hormone/prolactin receptor gene family. *Cell* 53: 69-77

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72: 248-254

Braun,R.E., Lo,D., Pinkert,C.A., Widera,G., Flaveli,R.A., Palmiter,R.D., & Brinster,R.L. (1990). Infertility in male transgenic mice: Disruption of sperm development by HSV-tk expression in postmeiotic germ cells. *Biology of reproduction* 43: 684-693

Breitman,M.L., Clapoff,S., Rossant,J., Tsui,L.C., Glode,M., Maxwell,I.H., & Bernstein,A. (1987). Genetic ablation: targeted expression of a toxin gene causes microphthalmia in transgenic mice. *Science* 238: 1563-1565

Breitman,M.L., Rombola,H., Maxwell,I.H., Klintworth,G.K., & Bernstein,A. (1990). Genetic ablation in transgenic mice with an attenuated diphtheria toxin A gene. *Mol. Cell. Biol.* 10: 474-479

Brem,G., Wanke,R., Wolf,E., Buchmuller,T., Muller,M., Brenig,B., & Hermanns,W. (1989). Multiple consequences of human growth hormone expression in transgenic mice. *Mol. Biol. Med.* 6: 531-547

Brinster,R.L., Chen,H.Y., & Trumbauer,M. (1981). Somatic expression of the herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* 27: 223-231

Buratowski,S., Hahn,S., Guarente,L., & Sharp P.A.(1989). Five intermediate complexes in transcription initiation by RNA polymerase II. *Cell* 56: 549-561



Camper, S.A., Yao, Y.A.S., & Rottman, F.M. (1985). Hormonal regulation of the bovine prolactin promoter in rat pituitary tumour cells. *J. Biochem.* 260: 12246-12251

Camper, S.A., Saunders, T.L., Katz, R.W., & Reeves, R.H. (1990). The pit-I transcription factor gene is a candidate for the murine snell dwarf mutation. *Genomics* 8: 586-590

Capecchi, M.R. (1989). The new mouse genetics: altering the genome by gene targeting. *Trends in Genetics* 5: 70-76

Capuco, A.V., Keys, J.E. & Smith, J.J. (1989). Somatotrophin increases thyroxine-5'-monodeiodinase activity in lactating mammary tissue of the cow. *J. Endocrin.* 121: 205-211

Castrillo, J.L., Bodner, M., & Karin, M. (1989). Purification of growth hormone-specific transcription factor GHF-1 containing homeobox. *Science* 243: 814-817

Cato, A.C.B., Skroch, P., Weinmann, P., Butkeraitis, P., & Ponta, H. (1988). DNA sequences outside the receptor-binding sites differently modulates the responsiveness of the mouse mammary virus promoter to various steroid hormones. *Embo.J.* 7: 1403-1410

Cella,S.G., Locatelli,V., Mennini,T., Zanini,A., Bendotti,C., Forloni,G.L., Fumagalli,G., Arce,V.M., De Gennaro Colonna,V., Wehrenberg,W.B., & Müller,E.E. (1990). Deprivation of growth hormone-releasing hormone early in the rat's neonatal life permanently affects somatotropic function. *Endocrin.* 127: 1625-1634

Chave-Cox,A.J. PhD Thesis submitted to the University of Edinburgh 1986.

Cheng,T.C., Beamer,W.G., Phillips III,J.A., Bartke,A., Mallonee,R.L., & Dowling,C. (1983). Etiology of growth hormone deficiency in Little, Ames, and Snell dwarf mice. *Endocrin.* 113: 1669-1678

Chernausek,S.D, Underwood,L.E., & Van Wyk,J.J. (1982). Influence of hypothyroidism on growth hormone binding by rat liver. *Endocrin.* 111: 1534-1538

Chirgwin,J.M., Przybyla,A.E., MacDonald,R.J., & Rutter,W.J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochem.* 18: 5294-5299

Chomczynski,P., & Sacchi,N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analy. Biochem.* 162: 156-159

Christophe,D., Gérard,C., Juvenal,G., Bacolla,A., Dumont,J.E., Christophe-Hobertus,C., Ledent,C., & Vassart,G. (1989). Transient expression in primary thyrocytes identifies a cAMP-response element in thyroglobulin gene promoter region. *Mol. Cell. Endocrin.* 64: 5-18

Church,G.M., & Gilbert,W. (1984). Genomic sequencing. *Proc. Nat. Acad. Sci.* 81: 1991-1995

Civitareale,D., Lonigro,R., Sinclair,A.J. & Di Lauro,R. (1989). A thyroid-specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. *EMBO. J.* 8: 2537-2542

Clarke,A.J., Clissold,P.M., Al-Shawi,R., Beattie,P., & Bishop,J.O. (1984). Structure of the mouse major urinary protein genes: different splicing configuration in the 3' non-coding region. *EMBO. J.* 3: 1045-1052

Clissold,P.M., Hainey,S. & Bishop,J.O.,(1984). Messenger RNAs coding for Mouse Major Urinary proteins are differentially induced by testosterone. *Biochem.Genet.* 22: 379-387

Clos,J., Rebiere,A. & Legrand,J. (1973). Differential effects of hypothyroidism and undernutrition on the development of the glial in the rat cerebellum. *Brain Research* 63: 445-449

Costa,R.H., Lai,E., Grayson,D.R., & Darnell Jr,J.E. (1988). The cell-specific enhancer of the mouse transthyretin (prealbumin) gene binds a common factor at one site and a liver-specific factors(s) at two other sites. *Mol. Cell. Biol.* 8: 81-90

Costa,R.H., Grayson,D.R., & Darnell,J.E. (1989). Multiple hepatocyte-enriched nuclear factors function in the regulation of transthyretin and  $\alpha$ 1-antitrypsin genes. *Mol. Cell. Biol.* 9: 1415-1425

Courtois,G., Baumhueter,S. & Crabtree,G.R. (1988). Purified hepatocyte nuclear factor 1 interacts with a family of hepatocyte-specific promoters. *Proc. Natl. Acad. Sci.* 85 7937-7941

Cronmiller,C., Schedl,P., & Cline,T.W. (1988). Molecular characterization of *daughterless*, a *Drosophila* sex determination gene with multiple roles in development. *Genes & Development* 2: 1666-1676

Dalman, F.C., Koenig,R.J., Perdew,G.H., Massa,E., & Pratt,W.B. (1990). In contrast to the glucocorticoid receptor the thyroid receptor is translated in the DNA binding state and is not associated with hsp 90. *J. Biol. Chem.* 265: 3615-3618

Darnell,D., Lodish,H. & Baltimore,D. (1986) *Molecular cell Biology* (first edition). Scientific American Books.

Davis,R.L., Weintraub,H., & Lassar,A.B. (1987).  
Expression of a single transfected cDNA converts  
fibroblasts to myoblasts. *Cell* 51: 987-1000

Dechiara,T.M., Efstratiadis,A., & Robertson,E.J. (1990).  
A growth-deficiency phenotype in heterozygous mice  
carrying an insulin-like growth factor II gene disrupted  
by targeting. *Nature* 345: 78-80

Deléan,A., Ferland,L., Drowin,J., Kelly.P.A. & Labrie,F.  
(1977). Modulation of pituitary thyrotrophin releasing  
hormone receptor levels by estrogen and thyroid hormones.  
*Endocrin.* 100: 1496-1504

Derman,E. (1981). Isolation of a cDNA clone for mouse  
urinary protein: Age- and sex-related expression of mouse  
urinary protein genes is transcriptionally controlled.  
*Proc. Natl. Acad. Sci.* 78: 5425-5429

Derman,E., Krauter,K., Walling,L., Weinberger,C., Ray,M.,  
& Darnell Jr,J.E. (1981). Transcriptional control in the  
production of liver-specific mRNAs. *Cell* 23: 731-739

de Martynoff,G., Pohl,V., Mercken,L., van Ommen,G. &  
Vassart,G. (1987). Structural organization of the bovine  
thyroglobulin gene and of it's 5'-flanking region. *Eur.  
J. Biochem.* 164: 591-599

De Simone,V., Ciliberto,G., Hardon,E., Paonessa,G., Palla,F., Lundberg,L., & Cortese,R. (1987). *Cis-* and *trans-*acting elements responsible for the cell-specific expression of the human  $\alpha$ 1-antitrypsin gene. *Embo. J.* 6: 2759-2766

Desplan,C., Theis,J., & O'Farrell,P.H. (1988). The sequence specificity of homeodomain-DNA interaction. *Cell* 54: 1081-1090

Dumont,J.E. (1971). The action of thyrotrophin on thyroid metabolism. *Vit. Horm.* 29: 287-390

Dumont,J.E., Vassart,G., & Refetoff,S. (1989). In: *The metabolic basis of inherited disease*, edited by McGraw-Hill New York.

Dynan,W.S. (1986). Promoters for house keeping genes. *Trends in Genetics* 2: 196-197

Dynan,W.S. (1989). Understanding the molecular mechanism by which methylation influences gene expression. *Trends in Genetics* 5: 35-36

Eayrs,J.T. (1960). Influence of the thyroid on the central nervous system. *Brit. Med. Bull.* 16: 122-127

Edey, M., Jolicoeur, C., Levi-Meyrueis, C., Dusanter-Fourt, I., Pétridou, B., Boutin, J., Lesueur, L., Kelly, P.A., & Djiane, J. (1989). Identification and sequence analysis of a second form of prolactin receptor by molecular cloning of complementary DNA. *Proc. Natl. Acad. Sci.* 86: 2112-2116

Edlund, T., Walker, M.D., Barr, P.J., & Rutter, W.J. (1985). Cell-specific expression of the rat insulin gene: Evidence for the role of two distinct 5' flanking elements. *Science* 230: 912-916

Eicher, E.V., & Beamer, W. (1976). Inherited ateliotic dwarfism in mice. *J. Heredity* 67: 87-91

Elion, G.B., Furman, P.A., Fyfe, J.A., De Miranda, P., Beauchamp, L., & Schaeffer, H.J. (1977). Selectivity of action of an antiherpetic agent, 9-(2-hydroxyethoxymethyl)guanine. *Proc. Natl. Acad. Sci.* 74: 5716-5720

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* 240: 889-895

Evans, R.M., & Hollenberg, S.M. (1988). Zinc fingers: Gilt by association. *Cell* 52: 1-3

Feinberg, A.P., & Vogelstein, B. (1983). A technique for radiolabelling DNA restriction endonuclease fragments to high specific activity. *Anal. Biochem.* 132: 6-13

Finlayson, J.S., Asofsky, R., Potter, M., & Runner, C.C. (1965). Major urinary protein complex of normal mice: Origin. *Science* 149: 981-982

Flint, D.J., & Gardner, M.J. (1989). Inhibition of neonatal rat growth and circulating concentrations of insulin-like growth factor-1 using an antiserum to rat growth hormone. *J. Endocrin.* 122: 79-86

Foster, C.M., Shafer, J.A., Rozsa, F.W., Wang, X., Lewis, S.D., Renken, D.A., Natale, J.E., Schwartz, J., & Carter-Su, C. (1988). Growth hormone promoted tyrosyl phosphorylation of growth hormone receptor in murine 3T3-F442A fibroblasts and adipocytes. *Biochem.* 27: 326-334

Fowikes, B.J., Edison, L., Mathieson, B.J., & Chused, T.M. (1985). Early T Lymphocytes. *J. Exp. Med.* 162: 802-822

Frain, M., Hardon, H., Ciliberto, G., & Sala-Trepat, J.M. (1990). Binding of a liver-specific factor to the human albumin gene promoter and enhancer. *Mol. Cell. Biol.* 10: 991-999



Friedman, Y., Lang, M., & Burke, G. (1977). Inhibition of thyroid adenylate cyclase by thyroid hormone: a possible locus for the "short-loop" negative feedback phenomenon. *Endocrin.* 101: 858-868

Fyfe, J.A., Keller, P.M., Furman, P.A., Millar, R.L., & Elion, G.B. (1978). Thymidine kinase from herpes simplex virus phosphorylates the new antiviral compound, 9-(2-hydroxyethoxymethyl)guanine. *J. Biol. Chem.* 353: 8721-8727

Gause, I., Eden, S., Jansson, J., & Isaksson, O. (1983). Effects of *in vivo* administration of antiserum to rat growth hormone on body growth and insulin responsiveness in adipose tissue. *Endocrin.* 112: 1559-1566

Gershengam, M.C. (1978). Bihormonal regulation of the TRH receptor in mouse pituitary thyrotropic tumour cells in culture. *J. Clin. Invest.* 62: 937-943

Ghosh, P.K., Reddy, V.B., Piatak, M., Lebowitz, P., & Weissman, S.M. (1980). Determination of RNA sequences by primer directed synthesis and sequencing of their cDNA transcripts. *Meth. Enzymol.* 65: 580-595

Gidioni, D., Dyan, W.S., & Tjian, R. (1984). Multiple specific contacts between a mammalian transcription factor and its cognate promoter. *Nature* 312: 409-413

Gill,G., & Ptashne,M. (1988). Negative effects of the transcriptional activator GAL4. *Nature* 334: 721-724

Gillies,S.D., Morrison,S.L., Oi,V.T., & Tonegawa,S. (1983). A tissue specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. *Cell* 33: 717-728

Glass,C.K., Franco,R., Weinberger,C., Alberts,V.R., Evans,R.M., & Rosenfeld,M.G. (1987). A C-erbA Binding site in the rat growth hormone gene mediates trans-activation by thyroid hormones. *Nature* 329: 738-741

Glass,C.K., Holloway,J.M., Devary,O.V., & Rosenfeld,M.G. (1988). The thyroid hormone receptor binds with opposite transcriptional effects to a common sequence motif in the thyroid hormone and estrogen response element. *Cell* 54: 313-323

Gonzalez,G.A, Yammamoto,K.K., Fisher,W.H., Karr,D., Menzel,P., Biggs III,W., Vale,W.W., & Montminy,M.R. (1989). A cluster of phosphorylation sites on the cyclic AMP-regulated nuclear factor CREB predicted by its sequence. *Nature* 337: 749-752

Gordon,J.W., Scangos,G.A., Plotkin,D.J., Barbosa,J.A., & Ruddle, F.H. (1980). Genetic transformation of mouse embryos by microinjection of purified DNA. *Proc. Natl. Acad. Sci.* 77: 7380-7384

Graupner,G., Wills,K.N., Tzukerman,M., Zhang,X.K., & Pfahl,M. (1989). Dual regulatory role for thyroid-hormone receptors allows control of the retinoic-acid receptor activity. *Nature* 340: 653-656

Grayson,D.R., Costa,R.H., Xanthopoulos,K.G., & Darnell,D.E. (1988). One factor recognizes the liver-specific enhancers in  $\alpha$ 1-antitrypsin and transthyretin genes. *Science* 239: 786-788

Green,S., & Chambon,P. (1987). Oestradiol induction of a glucocorticoid-responsive gene by a chimeric receptor. *Nature* 325: 75-78

Green,S., Kumar,V., Theulaz,I., Wahli,W., & Chambon,P. (1988). The N-terminal DNA-binding "zinc fingers" of the oestrogen and glucocorticoid receptors determines target gene specificity. *Embo. J.* 7: 3037-3044

Grosveld,F., van Assendelft,G.B., Greaves,D.R., & Kollias,G. (1987). Position-independent, high-level expression of the human  $\beta$ -globin gene in transgenic mice. *Cell* 51: 975-985

Hammer,R.E., Brinster,R.L., Rosenfeld,M.G., Evans,R.M., & Mayo,K.E. (1985). Expression of human growth hormone-releasing factor in transgenic mice results in increased somatic growth. *Nature* 315: 413-416

Hammer,R.E., Swift,G., Ornitz,D.M., Quaife,C.J., Palmiter,R.D., Brinster,R.L., & MacDonald,R.J. (1987). The rat elastase 1 regulatory element is an enhancer that directs correct cell specificity and developmental onset of expression in transgenic mice. *Mol. Cell. Biol.* 7: 2956-2967

Han,K., Levine,M.S., & Manley,J.L. (1989). Synergistic activation and repression of the transcription by *Drosophila* homeobox proteins. *Cell* 56: 573-583

Hanahan,D. (1985). Heritable formation of pancreatic  $\beta$ -cell tumours in transgenic mice expressing recombinant insulin/simian virus 40 oncogenes. *Nature* 315: 115-122

Hansen,C., Gerard,C., Vassart,G., Stordeur,P., & Christophe,D. (1988). Thyroid specific and cAMP-dependent hypersensitive regions in thyroglobulin gene chromatin. *Eur. J. Biochem.* 178: 387-393

Harrington,M.A., Jones,P.A., Imagawa,M., & Karin,M. (1988). Cytosine methylation does not affect binding of transcription factor SpI. *Proc. Natl. Acad. Sci.* 85: 2066-2070

Hastie,N.D., Held,W.A., & Toole,J.J. (1979). Multiple genes coding for the androgen-regulated major urinary proteins of the mouse. *Cell* 17: 449-457

Held,W.A., Gallagher,J.F. Hohman,C.M., Kuhn,N.J., Sampsell,B.M., & Hughes Jr,R.G. (1987). Identification and characterization of functional genes encoding the mouse Major Urinary proteins. *Mol.Cell. Biol.* 7: 3705-3712

Herr,W., & Clarke,J. (1986). The SV40 enhancer is composed of multiple functional elements that can compensate for one another. *Cell* 45: 461-470

Heyman,R.A., Borrelli,E., Lesley,J., Anderson,D., Richman,D.D., Baird,S.M., Hyman,R., & Evans,R.M. (1989). Thymidine kinase obliteration: creation of transgenic mice with controlled immune deficiency. *Proc. Natl. Acad. Sci.* 86: 2698-2702

Hinkle,P.M., Pemone,M.H. & Schonbrunn (1981). Mechanism of thyroid hormone inhibition of thyrotrophin-releasing action. *Endocrin.* 108: 199-205

Hinkle,P.M. & Goh,K.G.C. (1982). Regulation of thyrotrophin-releasing hormone receptors and responses by L-triiodothyronine in dispersed rat pituitary cell culture. *Endocrin.* 110: 1725-1731

Hirai,S., Ryseck,R.P., Mechta,F., Brala,R., & Yaniu,M. (1989). Characterization of JunD: A new member of the Jun proto-oncogene family. *Embo. J.* 8: 1433-1439

Hochberg,Z., Bick,T., & Harel,Z. (1990). Alterations of human growth hormone binding by rat liver membranes during hypo- and hyperthyroidism. *Endocrin.* 126: 325-329

Hocquette,J-F., Postel-Vinay,M-C., Djiane,J., Tar,A., & Kelly,P.A. (1990). Human liver growth hormone receptor and plasma binding protein: characterization and partial purification. *Endocrin.* 127: 1665-1672

Hodin,R.A., Lazar,M.A., Wintman,B.I., Darling,D.S., Koenig,R.J., Larsen,P.R., Moore,D.D., & Chin,W.W. (1989). Identification of a thyroid hormone receptor that is pituitary-specific. *Science* 244: 76-79

Hollenberg,S.M., & Evans,R.M. (1988). Multiple and cooperative trans-activation domains of the human glucocorticoid receptor. *Cell* 55: 899-906

Höller,M, Westin,G., Jiricny,J., & Schaffner,W. (1988). SpI transcription factor binds DNA and activates transcription even when binding site is CpG methylated. *Genes and Development* 2: 1127-1135

Horvath,T., Path,T., Nemeth,A., Kadas,I., Hoffmann Traeger,A., Rechenbach,C., & Javor,T. (1989). Drug metabolism in drug-induced liver diseases: pathogenetic role of active metabolites. *Acta Physiol. Hung.* 73: 293-304

Huckably, C.S., Corneely, O.M., Beattie, W.G., Dobson, A.D.W., Tsai, M., & O' Malley, B. (1987). Structure of the chromosomal chicken progesterone receptor gene. *Proc. Natl. Acad. Sci.* 84: 8380-8384

Ingraham, H.A., Chen, R., Mangalam, H.J., Elsholtz, H.P., Flynn, S.E., Lin, C.R., Simmons, D.M., Swanson, L., & Rosenfeld, M.G. (1988). A tissue-specific transcription factor containing a homeodomain specifies a pituitary phenotype. *Cell* 55: 519-529

Izumo, S., Nadal-Ginard, B. & Mahdavi, V. (1986). All members of the MHC multigene family responds to thyroid hormones in a highly tissue-specific manner. *Science* 231: 597-600

Jacob, F., & Monod, J. (1961). Genetic regulatory mechanisms in the synthesis of proteins. *J. Mol. Biol.* 3: 318-356

Jaenisch, R. (1976). Germ line integration and mendelian transmission of the exogenous Moloney leukemia virus. *Proc. Natl. Acad. Sci.* 73: 1260-1264

Jaenisch, R. (1977). Germ line integration of the Moloney leukaemia virus: effect of homozygosity at the M-MuLV locus. *Cell* 12: 691-696

Jansson, J-O., Ekberg, S., Isaksson, O.G., & Eden, S. (1984). Influence of gonadal steroids on age- and sex-related secretory patterns of growth hormone in the rat. *Endocrin.* 114: 1287-1294

Jansson, J-O., Downs, T.R., Beamer, W.G., & Frohman, L.A. (1986). Receptor-associated resistance to growth hormone-releasing factor in the dwarf "little" mice. *Science* 232: 511-512

Jansson, J-O., & Frohman, L.A. (1987). Differential effects of neonatal and adult androgen exposure on the growth hormone secretory pattern in male rats. *Endocrin.* 120: 1551-1557

Jaynes, J.B., & O'Farrell, P.H. (1988). Activation and repression of transcription by homeodomain-containing proteins that bind a common site. *Nature* 336:744-749

Johnson, D.A., Gautsch, J.W., Sportsman, I.R., & Elder, J.H. (1984). Improved technique utilizing nonfat dry milk for analysis of protein and nucleic acids transferred to nitrocellulose. *Gene. Anal. Techn.* 1: 3-8

Kawaori, A. & Tsuneda, M. (1985). Functional development and maturation of the rat thyroid gland in the foetal and newborn periods: an immunohistochemical study. *Acta. Endocrin.* 108: 518-524



Kendall, S.K., Saunders, T.L., Jin, L., Lloyd, R.V., Glode, L.M., Nett, T.M., Keri, R.A., Nilson, J.H. & Camper, S.A. (1991). Targeted ablation of pituitary gonadotrophes in transgenic mice. *Mol. Endocrin.* 5: 2025-2036

Khyse-Andersen, J. (1984). Electrophoretic transfer of multiple gels: a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Meth.* 10: 203-209

Knopf, J.L., Gallagher, J.F., & Held, W.A. (1983). Differential, multihormonal regulation of the Mouse Major Urinary protein gene family in the liver. *Mol. Cell. Biol.* 3: 2232-2240

Koenig, R.J., Brent, G.A., Warne, R.L., Larsen, P.R., & Moore, D.D. (1987). Thyroid hormone receptor binds to a site in the rat growth hormone promoter required for induction by thyroid hormone. *Proc. Natl. Acad. Sci.* 84: 5670-5674

Koenig, R.J., Lazar, M.A., Hodin, R.A., Brent, G.A., Larsen, P.R., Chin, W.W., & Moore, D.D. (1989). Inhibition of thyroid hormone action by a non-hormone binding c-erbA protein generated by alternative mRNA splicing. *Nature* 337: 659-661

Koltunow, A.M., Gregg, K., & Rogers, G.E. (1986). Intron sequences modulate feather keratin gene transcription in *Xenopus* oocytes. *Nucl. Acid. Res* 14: 6375-6392

Krasnow, M.A., Saffman, E.E., Kornfeld, K., & Hogness, D.S. (1989). Transcription activation and repression by *ultrabithorax* proteins in cultured *Drosophila* cells. *Cell* 57: 1031-1043

Krenning, E.P., Docter, R., Bernard, H.F., Visser, T.J., & Hennemann, G. (1978). Active transport of triiodothyronine ( $T_3$ ) into isolated rat liver cells. *FEBS. Letters* 91: 113-116

Krenning, E., Docter, R., Bernard, B., Visser, T. & Hennemann, G. (1980). Regulation of the active transport of 3,3' 5-triiodothyronine ( $T_3$ ) into primary cultured rat hepatocytes by ATP. *FEBS. Letters* 119: 279-282

Krenning, E., Docter, R., Bernard, B., Visser, T. & Hennemann, G. (1981). Characteristics of active transport of the thyroid hormones into rat hepatocytes. *Biochim. Biophysica Acta* 676: 314-320

Kulkarni, A.B., Gubits, R.M., & Feigelson, P. (1985). Developmental and hormonal regulation of  $\alpha_{2u}$ -globulin gene transcription. *Proc. Natl. Acad. Sci.* 82: 2579-2582

Kumar,V., Green,S., Stack,G., Berry,M., Jin,J.R., & Chambon,P. (1987). Functional domains of the human estrogen receptor. *Cell* 51: 941-951

Kurki,P., Ogata,K., & Tan,E.M. (1988). Monoclonal antibodies to proliferating cell nuclear antigen (PCNA)/cyclin as probes for proliferating cells by immunofluorescence microscopy and flow cytometry. *J. Immuno. Methods* 109: 49-59

Laemmli,U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T<sub>4</sub>. *Nature* 227: 680-685

Landel,C.P., Zhao,J., Bok,D., & Evans,G.A. (1988). Lens-specific expression of recombinant ricin induces developmental defects in the eyes of transgenic mice. *Genes and development* 2: 1168-1178

Landschulz,W.H., Johnson,P.F., & McKnight,S.L. (1988). The leucine zipper: A hypothetical structure common to a new class of DNA binding proteins. *Science* 240: 1759-1764

Lang,G., Wotton,D., Owen,M.J., Sewell,W.A., Brown,M.H., Mason,D.Y., Crumpton,M.J., & Kioussis,D. (1988). The structure of the human CD2 gene and its expression in transgenic mice. *Embo. J.* 7: 1675-1682

Larsen, P.R., Harney, J.W., & Moore, D.D. (1986). Repression mediates cell-type-specific expression of the rat growth hormone gene. *Proc. Natl. Acad. Sci.* 83: 8283-8287

Laughon, A., & Scott, M.P. (1984). Sequence of a *Drosophila* segmentation gene: protein structure homology with DNA-binding protein. *Nature* 310: 25-31

Leary, J.J., Brigati, D.J., & Ward, D.C. (1983). Rapid and sensitive colorimetric method for visualising biotin-labelled DNA probes hybridised to DNA or RNA immobilised on nitrocellulose bio-blots. *Proc. Natl. Acad. Sci.* 80: 4045-4049

Ledent, C., Parmentier, M., & Vassart, G. (1990). Tissue-specific expression and methylation of the thyroglobulin-CAT fusion gene in transgenic mice. *Proc. Natl. Acad. Sci.* 87: 6176-6180

Lee, W., Haslinger, A., Karin, M., & Tjian, R. (1987). Activation of transcription by two factors that bind promoter and enhancer sequences of the human metallothionein gene and SV40. *Nature* 325: 368-372

Lewinson, D., Harel, Z., Shenzer, P., Silbermann, M., & Hochberg, Z. (1989). Effect of thyroid hormone and growth hormone on recovery from hypothyroidism of epiphyseal growth plate cartilage and its adjacent bone. *Endocrin.* 124: 937-945

Lichtsteiner, S., Wuarin, J., & Schibler, U. (1987). The interplay of DNA-binding proteins on the promoter of the mouse albumin gene. *Cell* 51: 963-973

Lumpkin, M.D., Mulroney, S.E., & Haramati, A. (1989). Inhibition of pulsatile growth hormone (GH) secretion and somatic growth in immature rats with a synthetic GH-releasing factor antagonist. *Endocrin.* 124: 1154-1159

MacLeod, J.N., Pampori, N.A. & Shapiro, B.H. (1991). Sex differences in the ultradian pattern of plasma growth hormone concentrations in mice. *J. Endocrin.* 131: 395-399

McGowan, R.A., Gross, K.W., & Wilson, C.M. (1988). Effect of androgen and thyroid hormones on renin-1 messenger ribonucleic acid levels in mouse submandibular gland. *Mol. Cell. Biol.* 56: 271-276

McIntosh, I., & Bishop, J.O. (1989). Differential expression in male and female mouse liver of very similar mRNAs specified by two group I Major Urinary protein genes. *Mol. Cell. Biol.* 9: 2202-2207

McKnight, R.A., Shamay, A., Sankaran, L., Wall, R.J., & Hennighausen, L. (1992). Matrix-attachment regions can impart position-independent regulation of a tissue-specific gene in transgenic mice. *Proc. Natl. Acad. Sci.* 89: 6943-6947

Mader,S., Kumar,V., de Verneuil,H., & Chambon,P. (1989). Three amino acid of the oestrogen receptor are essential to its ability to distinguish an oestrogen from a glucocorticoid-response element. *Nature* 338: 271-274

Madon,R.J., Ensor,D.M., Knight,C.H., & Flint,D.J. (1986). Effects of an antiserum to rat growth hormone on lactation in the rat. *J. Endocrin.* 111: 117-123

Mahdavi,V., Chamber,A.P., & Nadal-Ginard,B. (1984). Cardiac  $\alpha$  and  $\beta$  myosin heavy chain genes are organized in tandem. *Proc. Natl. Acad. Sci.* 81: 2626-2630

Mathews,L.S., Norstedt,G., & Palmiter,R.D. (1986). Regulation of insulin-like growth factor 1 gene expression by growth hormone. *Proc. Natl. Acad. Sci.* 83: 9343-9347

Mathews,L.S., Hammer,R.E., Brinster,R.L., & Palmiter,R.D. (1988a). Expression of insulin-like growth factor 1 in transgenic mice with elevated levels of growth hormone is correlated with growth. *Endocrin.* 123: 433-437

Mathews,L.S., Hammer,R.E., Behringer,R.R., D'Ercole,J., Bell,G.I., Brinster,R.L., & Palmiter,R.D. (1988b). Growth enhancement of transgenic mice expressing human insulin-like growth factor 1. *Endocrin.* 123: 2827-2833

Maxam,A.M., & Gilbert,W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. *Meth. Enzymol* 65: 499-560

Maxfield,F.R., Willingham,M.C., Pastan,I., Dragsten,P. & Cheng,J,Y. (1981). Binding and mobility of the cell surface receptor for 3,3',5-triiodo-L-thyronine. *Science* 211: 63-64

Maxwell,F., Maxwell,I.H., & Globe,L.M. (1987). Cloning, sequence determination, and expression in transfected cells of the coding sequence for the *tox* 176 attenuated diphtheria toxin A chain. *Mol. Cell. Biol.* 7: 1576-1579

Mayerhofer,A., Amandor,A.G., Beamer,W.G., & Bartke,A. (1988). Ultrastructural aspect of the goiter in cog/cog mice. *J. Hered.* 79: 200-203

Meehan,R., Lewis,J., McKay,S., Kleiner,E., & Bird,A. (1989). Identification of a mammalian protein that binds specifically to DNA containing methylated CpGs. *Cell* 58: 499-507

Meier,C.A., Fabbro,D., Meyhack,I., Hemmings,B., Olbrecht,U., Jakob.A., & Walter,P. (1991). Effect of hypothyroidism and thyroid hormones replacement on the level of protein kinase C and protein kinase A in the rat liver. *FEBS Letters* 282: 397-400

Miksicek,R., Borgmeyer,U., & Nowock,J. (1987). Interaction of the TGGCA-binding protein with upstream sequences is required for efficient transcription of mouse mammary tumor virus. *Embo. J.* 6: 1355-1360

Miller,J., McLachlan,A.D., & Klug,A. (1985). Repetitive zinc-binding domains in the protein transcription factor IIIA from *xenopus* oocytes. *Embo. J.* 4: 1609-1614

Moffat,K.G., Gould,J.H., Smith,H.K., & O'Kane,C.J. (1992). Inducible cell ablation in *Drosophila* by cold-sensitive ricin A chain. *Development* 114: 681-687

Monaci,P., Nicosia,A., & Cortese,R. (1988). Two different liver-specific factors stimulate *in vitro* transcription from the human  $\alpha$ 1-antitrypsin promoter. *Embo. J.* 7: 2075-2087

Moore,W.V., & Wolff,J. (1974). Thyroid-stimulating hormone binding to beef thyroid membranes. *J. Biol. Chem* 249: 6255-6263

Muglia,L., & Rothman-Denes,L.B. (1986). Cell type-specific negative regulatory element in the control region of the rat  $\alpha$ -fetoprotein gene. *Proc. Natl. Acad. Sci.* 83: 7653-7657



Nakamura, H., and DeGroot, L.J. (1983). Thyroid hormone stimulation of phosphorylation and dephosphorylation of rat liver cytosolic proteins. *Endocrin.* 112: 670-680

Narayan, P., & Towle, H.C. (1985). Stabilization of a specific nuclear mRNA precursor by thyroid hormone. *Mol. Cell. Biol.* 5: 2642-2646

Nelson, C., Crenshaw III, E.B., Franco, R., Lira, S.A., Albert, V.R., Evans, R.M., & Rosenfeld, M.G. (1986). Discrete *cis*-active genomic sequences dictates the pituitary cell type-specific expression of the rat prolactin and growth hormone genes. *Nature* 322: 557-562

Nelson, C., Albert, V.R., Elsholtz, H.P., Lu, L.I.W., & Rosenfeld, M.G. (1988). Activation of cell-specific expression of rat growth hormone and prolactin genes by a common transcription factor. *Science* 239: 1400-1405

Norstedt, G., & Palmiter, R. (1984). Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* 36: 805-812

Nunez, K. (1988). In: *Hormones and their actions, part 1*, edited by Elsevier science publishers (biomedical division)

Nunez, J. & Pommier, J. (1982). Formation of thyroid hormones. *Vit. Horm.* 39: 175-229

O'Riordan, J.L.H., Malan, P.G., & Gould, R.P. (1988). Essentials of endocrinology (second edition). Blackwell scientific publication.

Orlowski, J. & Lingrel, J.B. (1990). Thyroid and glucocorticoid hormones regulate the expression of multiple Na,K-ATPase genes in cultured neonatal rat cardiac myocytes. *J. Biol. Chem.* 265: 3462-3470

Ornitz, D.M., Palmiter, R.D., Hammer, R.E., Brinster, R.L., Swift, G.H., & MacDonald, R.J. (1985). Specific expression of an elastase-human growth hormone fusion gene in pancreatic acinar cells of transgenic mice. *Nature* 313: 600-602

Pabo, C.O., & Sauer, R.T. (1984). Protein-DNA recognition. *Ann. Rev. Biochem.* 53: 293-321

Palmiter, R.D., Brinster, R.L., Hammer, R.E., Trumbauer, M.E., Rosenfeld, M.G., Birnberg, N.C., & Evans, R.M. (1982). Dramatic growth of mice from eggs microinjected with metallothionein-growth hormone fusion genes. *Nature* 300: 611-615

Palmiter, R.D., Norstedt, G., Gelin, R.E., Hammer, R.E., & Brinster, R.L. (1983). Metallothionein-human GH fusion gene stimulate growth of mice. *Science* 222: 809-814

Palmiter, R.D., Behringer, R.R., Quaife, C.J., Maxwell, F., Maxwell, I.H., & Brinster, R.L. (1987). Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell* 50: 435-443

Palumbo, G. (1987). Thyroid hormonogenesis. *J. Biol. Chem.* 262: 17182-17188

Pelham, H.R.B. & Bienz, M. (1982). A synthetic heat shock promoter element confers heat-inducibility of the herpes simplex virus thymidine kinase gene. *Embo. J.* 1: 1473-1477

Perlino, E., Cortese, R., & Ciliberto. (1987). The human  $\alpha$ 1-antitrypsin gene is transcribed from two different promoters in macrophages and hepatocytes. *Embo. J.* 6: 2767-2771

Phillips III, J.A., Beamer, W.G., & Bartke, A. (1982). Analysis of growth hormone genes in mice with genetic defects of growth hormone expression. *J. Endocrin.* 92: 405-407

Picard, D., & Yamamoto, K.R. (1987). Two signals mediate hormone dependent nuclear localisation of the glucocorticoid receptor. *Embo. J.* 6: 3333-3340

Pierce, J.G., & Parsons, T.F. (1981). Glycoprotein hormones: Structure and function. *Ann. Rev. Biochem.* 50: 465-495

Pierce, J.W., Gifford, A.M., & Baltimore, D. (1991). Silencing of the expression of the immunoglobulin kappa gene in non-B cells. *Mol. Cell. Biol.* 11: 1431-1437

Pinkert, C.A., Ornitz, D.M., Brinster, R.L., & Palmiter, R.D. (1987). An albumin enhancer located 10kb upstream functions along with its promoter to direct efficient, liver-specific expression in transgenic mice. *Genes and Development* 1: 268-276

Ptashne, M. (1986). A Genetic switch. Palo Alto California and Cambridge, Massachusetts: Blackwell Scientific publication and Cell press.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. *Nature* 335: 683-689

Renkawitz, R., Schütz, G., Von der Ahe, D., & Beato, M. (1984). Sequences in the promoter region of the chicken lysozyme gene required for steroid regulation and receptor binding. *Cell* 37: 503-510

Rimoin, D.L., & Richmond, L. (1972). The pygmy (pg) mutant of the mouse- a model of the human pygmy. *J. Clin. Endocrinol. Metab.* 35: 467-468

Roberts,C.T., Brown,A.L., Graham,D.E., Seelig,S., Berry,S., Gabbay,K.H., & Rechler, M.M. (1986). Growth hormone regulates the abundance of insulin-like growth factor 1 RNA in adult rat liver. *J. Biol. Chem.* 261: 10025-10028

Robinson,I.C.A.F., & Clark,R.G. (1987). The secretory pattern of GH and its significance for growth in the rat. Elsevier science publishers B.V. (Biomedical Division). Growth hormone-Basic and clinical aspects.

Ross,S.R., & Solter,D. (1985). Glucocorticoid regulation of mouse mammary tumour virus sequences in transgenic mice. *Proc. Natl. Acad. Sci.* 82: 5880-5884

Rozengurt,E. (1986). Early signals in the mitogenic response. *Science* 234: 161-166

Ryan,R., Shupnik,M.A., & Gorski,J. (1979). Effect of estrogen on preprolactin messenger ribonucleic acid sequences. *Biochem.* 18: 2045-2048

Ryan,T.M., Behringer,R.D., Martin,N.C., Townes,T.M., Palmiter,R.D., & Brinster,R.L. (1989). A single erythroid-specific DNase 1 super-hypersensitive site activates high levels of human  $\beta$ -globin gene expression in transgenic mice. *Genes and Development* 3: 314-323

Sakai, D.D., Helms, S., Carlstedt-Duke, J., Gustafsson, J.A., Rottman, F.M., & Yamamoto, K.R. (1988). Hormone-mediated repression: a negative glucocorticoid response element from the bovine prolactin gene. *Genes & Development* 2: 1144-1154

Saluz, H.P., Feavers, I.M., Jiriary, J., & Post, J.P. (1988). Genomic sequencing and *in vivo* footprinting of an expression specific DNase 1 hypersensitive site of avian vitellogenin II promoter reveals a demethylation of a mCpG and a change in specific interactions of protein with DNA. *Proc. Natl. Acad. Sci.* 85: 6697-6700

Sambrook, J., Fritsch, E.F., & Maniatis, T. (1989). *Molecular cloning: A laboratory manual.*

Santisteban, P., Kohn, L.D. & Di Lauro, R. (1987). Thyroglobulin gene expression is regulated by insulin and insulin-like growth factor I, as well as thyrotrophin, in FRTL-5 thyroid cells. *J. Biol. Chem.* 262: 4048-4052

Santler, J.E. (1957). Growth in the cell populations of the thyroid gland of rats treated with thiouracil. *J. Endocrin.* 15: 151-161

Sap, J., de Magistris, L., Stunnenberg, H., & Vennström, B. (1990). A major thyroid hormone response element in the third intron of the rat growth hormone gene. *Embo.J* 9: 887-896

Sarnecki, D.H., & Degroot, L.J. (1989). In: *Endocrinology* (2nd Ed). edited by Degroot, L.J. W.B. Saunders Company U.S.A.

Savadogo, M., & Roeder, R.G. (1985). Interaction of a gene-specific transcription factor with the adenovirus major late promoter upstream of the TATA box region. *Cell* 43: 165-175

Schlessinger, J. (1986). Allosteric regulation of the epidermal growth factor receptor kinase. *J. Cell. Biol.* 103: 2067-2072

Schüle, R., Müller, M., Kaltshmidt, C., & Renkawitz, R. (1988). Many transcription factors interact synergistically with steroid receptors. *Science* 242: 1418-1420

Schwabe, J.W.R., & Rhodes, D. (1991). Beyond zinc fingers: steroid hormone receptors have a novel structural motif for DNA recognition. *Trends. Biochem.* 16: 291-296

Shaw, P.H., Held, W.A., & Hastie, N.D. (1983). The gene family for major urinary proteins: expression in several secretory tissues of the mouse. *Cell* 32: 755-761

Shepherd, J.C.W., McGinnis, W., Carrasco, A.E., DeRobertis, E.M., & Gehring, W.J. (1984). Fly and frog homeodomains show homologies with yeast mating type regulatory proteins. *Nature* 310: 70-71

Shivji, M.K.K., Kenny, M.K., & Wood, R.D. (1992). Proliferating cell nuclear antigen is required for DNA excision repair. *Cell* 69: 367-374

Shupnik, M.A., Chin, W.W., Habener, J.F. & Ridgeway, E.C. (1985). Transcriptional regulation of the thyrotrophin subunit genes by thyroid hormone. *J. Biol. Chem.* 260: 2900-2903

Sinha, Y.N., Wolff, G.L., Baxter, S.R., & Domon, O.E. (1979). Serum and pituitary concentrations of growth hormone and prolactin in pygmy mice. *Proc. Soc. Exp. Biol. Med.* 162: 221-223

Smale, S.T., & Baltimore, D. (1989). The "initiator" as a transcription control element. *Cell* 57: 103-113

Smith, W.C., & Talamantes, L.F. (1987). Identification and characterisation of a heterologous population of growth hormone receptors in mouse hepatic membranes. *J. Biochem.* 262: 2213-2219

Spiegelberg, T., & Bishop, J.O. (1988). Tissue specific gene expression in mouse hepatocytes cultured in growth-restricted medium. *Mol. Cell. Biol.* 8: 3338-3344



St. Clair, M., Lambe, C.U., & Furman, P.A. (1987). Inhibition by ganciclovir of cell growth and DNA synthesis of cells Biochemically transformed with herpes virus genetic information. *Antimicro. Agents and chemotherapy* 31: 844-849

Stein, S.A., Shanklin, D.R., Krulich, L., Roth, M.G., Chubb, C.M., Adams, P.M. (1989). Evaluation and characterisation of the *hyt/hyt* hypothyroid mouse. *Neuroendocrin.* 49: 509-519

Steiner, C., Muller, M., Baniahmad, A., & Renawitz, R. (1987). Lysozyme gene activity in chicken macrophages is controlled by positive and negative regulatory elements. *Nucl. Acid. Research.* 15: 4163-4178

Sterling, K., & Lazarus, J.H. (1977). The thyroid and its control. *Ann. Rev. Physiol.* 39: 349-371

Stief, A., Winter, D.M., Strätling, W.H., & Sippel, A.E. (1989). A nuclear DNA attachment element mediates elevated and position-independent gene activity. *Nature* 341: 343-345

Szoka, P.R., & Paigen, K., (1978). Regulation of mouse major urinary production by the MUP-A gene. *Genetics* 90: 597-612

Tannenbaum, G.S., & Martin, J.B. (1976). Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrin.* 98: 562-570

Thisse, B., Stoetzel, C., Gorostiza-Thisse, C., & Perrin-Schmitt, F. (1988). Sequence of the *twist* gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *Embo. J.* 7: 2175-2183

Uchida, T., Pappenheimer Jr, A.M., & Greany, R. (1973). Diphtheria toxin and related proteins. *J. Bio. Chem.* 218: 3838-3844

Umesono, K., & Evans, R.M. (1989). Determinants of target gene specificity for steroid/thyroid hormone receptors. *Cell* 57: 1139-1146

Van Heuverswyn, B., Streydio, C., Brocas, H., Refetoff, S., Dumont, J. & Vassart, G. (1984). Thyrotrophin controls transcription of the thyroglobulin gene. *Proc. Natl. Acad. Sci.* 81: 5941-5945

Van Heuverswyn, B., Leriche, A., Van Sande, J., Dumont, J.E. & Vassart, G. (1985). Transcriptional control of the thyroglobulin gene expression by cyclic AMP. *FEBS letters.* 188: 192-196

Van Sande, J., Perret, E.R.J., Lejeune, C., Maenhaut, C., Vassart, G. & Dumont, J.E. (1990). Thyrotrophin activates both the cyclic AMP and the PIP<sub>2</sub> cascades in CHO cells expressing the human cDNA of the TSH receptor. *Mol. Cell. Endocrin.* 74: R1-R6

Walker, M.D., Edlund, T., Boulet, A.M., & Rutter, W.J. (1983). Cell-specific expression controlled by the 5'-flanking region of the insulin and chymotrypsin genes. *Nature* 306: 557-561

Wass, J.A.H. (1988). In: *Endocrinology* (2nd Ed). edited by Degroot, L.J. W.B. Saunders Company U.S.A.

Waxman, D.J., Morrissey, J.J., Macleod, J.N., & Shapiro, B.H. (1990). Depletion of serum growth hormone in adult female rats by neonatal monosodium glutamate treatment without loss of female-specific hepatic enzyme p450 2d(11c12) and steroid 5 $\alpha$ -reductase. *Endocrin.* 126: 712-720

Whitaker, J. PhD Thesis submitted to the University of Edinburgh 1989.

Whitelaw, C.B.A., Harris, S., McClenaghan, M., Simons, J.P., & Clarke, A.J. (1992). Position-independent expression of the ovine  $\beta$ -lactoglobulin gene in transgenic mice. *Biochem. J.* 286: 31-39

Wilson,C.M., Myhre,M.J., Reynolds,R.C., & Wilson,J.D. (1982). Regulation of the mouse submaxillary gland renin by thyroxine. *Endocrin.* 110: 982-989

Wilson,R., McKillop,J.H., Travers,M., Smith,J., Smith,E., Thomson,J.A. (1990). The effect of antithyroid drugs on intercellular mediators. *Acta Endocrinol. (Copenh)* 122: 605-609

Wollman,S.H., Andros,G., Cannon,G.B., & Eagleton,G.B. (1968). Production and involution of the hyperplastic thyroid gland. In: Young S, Inman DR (eds) *Thyroid Neoplasia*. Academic Press, New York, p 201-209

Yamaizumi,M., Mekada,E., Uchida,T. & Okada,Y. (1978). One molecule of diphtheria toxin fragment A introduced into a cell can kill the cell. *Cell* 15: 245-250

Yamamoto,K.R. (1985). Steroid receptor regulated transcription of specific genes and gene networks. *Ann. Rev. Genet.* 19:209-252

Yamamoto,K.K., Gonzalez,G.A., Biggs III,W.H., & Montminy,M.R. (1988). Phosphorylation-induced binding and transcriptional efficacy of nuclear factor CREB. *Nature* 334: 494-498

# 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,<sup>1,2</sup> JOANNE BURKE,<sup>1</sup> HELEN WALLACE,<sup>1</sup> CHERYL JONES,<sup>1</sup> STEPHEN HARRISON,<sup>2</sup> DAVID BUXTON,<sup>3</sup> STEPHEN MALEY,<sup>3</sup> ANN CHANDLEY,<sup>4</sup> AND JOHN O. BISHOP<sup>1,2,5\*</sup>

*Institute of Cell and Population Biology, Division of Biology,<sup>1</sup> and AFRC Centre for Genome Research,<sup>2</sup> University of Edinburgh, Moredun Research Institute,<sup>3</sup> and Medical Research Council Human Genetics Unit, Western General Hospital,<sup>4</sup> Edinburgh, United Kingdom, and Department of Biological Sciences, University of Maryland, Baltimore, Maryland 21228<sup>5</sup>*

Received 7 March 1991/Accepted 14 May 1991

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 Met<sup>46</sup> and Met<sup>60</sup>, 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 spermatogonia.

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 (ablating) 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 antiherpetic agent to the transgenic mouse. Several potentially useful antiherpetic 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 *Bgl*II site in the 5' noncoding region and a *Bst*EII site 430 bp downstream of the polyadenylation signal (Fig. 1). The *Bgl*II 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 *Bst*EII site as described previously (1). The 1,887-bp *Eco*RI-*Bst*EII 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.

\* Corresponding author.

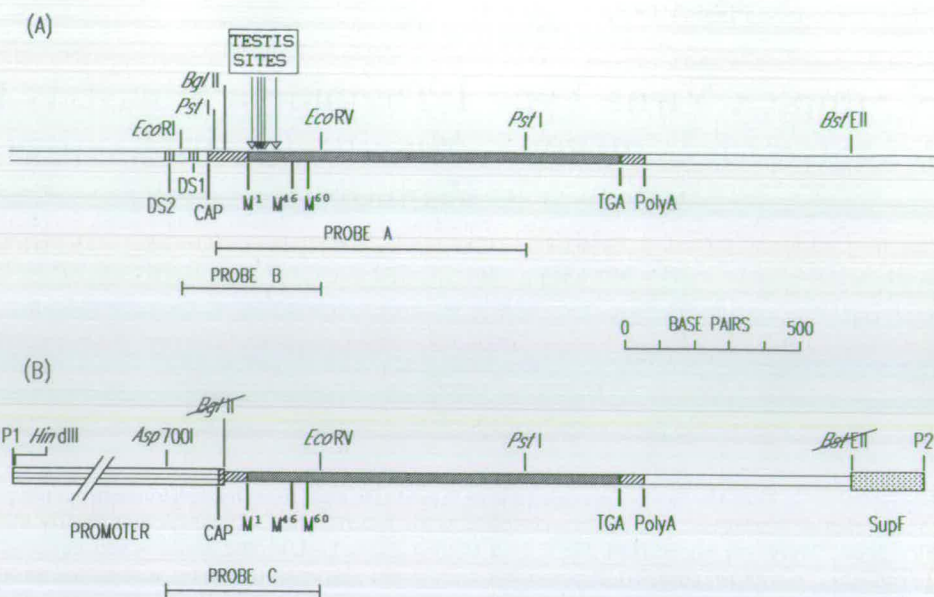


FIG. 1. (A) Diagram of the HSV-1 *tk* gene. DS1 and DS2 are the transcriptional control regions (38); M<sup>1</sup>, M<sup>46</sup>, and M<sup>60</sup> are the first three ATG codons of the reading frame. The testis transcription initiation sites (TESTIS SITES) are described in the text. The fragment used as a reporter sequence with cellular promoters was always the same *Bgl*II-to-*Bst*EII fragment, with the promoter attached at the *Bgl*II site and the bacterial *supF* gene at the *Bst*EII site. Both sites were eliminated during construction. (B) The MUP BS6-*tk-supF* gene. The reporter sequence is attached to the HSV *tk* gene at the *Bgl*II site, which was first blunted with DNA polymerase I. P1 and P2 are polylinkers at the junctions with plasmid vector 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 (*Bgl*II site to polyadenylation site), (ii) probe A (Fig. 1), an 841-bp *Pst*I fragment of pTK1, and (iii) probe B, RNA transcribed from a 419-bp *Eco*RI-*Eco*RV fragment of HSV-1 *tk* (-80 to +339) cloned in pT7-2 (U.S. Biochemicals). Primer extension analysis was carried out as described previously (16), using as primer a 5'-end-labelled (32) 23-nucleotide oligonucleotide complementary to the region from +369 to +347 of HSV-1 *tk*. RNase protection assays were carried out as described previously (28) with use of probe C (Fig. 1), RNA transcribed from a 450-bp *Asp*700I-*Eco*RV fragment of BS6-*tk-supF* (-142 to +348) cloned in pBluescript II SK+ (Stratagene Ltd.). Approximately  $5 \times 10^5$  dpm of antisense probe was hybridized overnight at 45°C with 5  $\mu$ g of total testis RNA. Digestion was with 40  $\mu$ g of RNase A and 650 U of RNase T<sub>1</sub> 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-dichloroisocoumarin (DCI) or tosyl phenyl chloroketone (TPC) and *p*-hydroxymercuribenzoate (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- $\mu$ m 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- $\mu$ m sections were treated with 1% H<sub>2</sub>O<sub>2</sub> 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 BS6*Sau*2-*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

Construct	Fertility <sup>a</sup>	n <sup>b</sup>	TK activity (pmol/min/mg of protein)					
			Testis		Liver		Preputial gland	
			Mean ± SE <sup>c</sup>	Range <sup>d</sup>	Mean ± SE	Range	Mean ± SE	Range
BS6- <i>tk-supF</i>	S	5	84.0 ± 20.7	31–140	4.39 ± 2.04	0.05–12.7	27.4 ± 13.3	0.04–83
BS6- <i>tk-supF</i> (TR)	S	4	195 ± 40.8	68–286	5.86 ± 3.68	0.62–18.5	70.9 ± 37.6	14.4–200
	F	2	2.52	1.77–3.26				
BS2- <i>tk-supF</i>	S	3	60.7 ± 9.2	47–83	0.47 ± .005	0.46–0.48	0.01 ± 0.01	0.0–0.04
BS6 <i>Sau2-tk-supF</i>	S	6	60.6 ± 10.9	17.4–91	0.09 ± 0.03	0.01–0.16	3.66 ± 1.29	0.0–7.8
	F	1	2.0		0.06		1.53	
TG- <i>tk-supF</i>	S	3	54.5 ± 9.0	40.4–73.3	0.01 <sup>e</sup>		0.35 <sup>e</sup>	

<sup>a</sup> S, sterile males; F, fertile males.

<sup>b</sup> Number of transgenic lines studied.

<sup>c</sup> Mean of line means.

<sup>d</sup> Range of line means.

<sup>e</sup> Mean of two lines. Values typically observed in extracts from nontransgenic mice are shown in Table 3.

globulin (TG) gene (13). BS6-*tk-supF* (TR) contains two copies of BS6-*tk-supF*, each lacking a short 5' region and separated by a 500-bp fragment of mouse DNA, which were integrated at a single chromosomal site in a line of transgenic mice (2, 27a). The complete tandem dimer together with flanking DNA sequences was recovered from two bacteriophage lambda clones and reintroduced into the mouse genome (2). These constructs displayed different patterns of tissue-specific expression (Table 1). In addition, TG-*tk-SupF* was expressed in the thyroid gland (44a).

Expression in the testis was remarkably consistent: the male offspring of every transgenic G<sub>0</sub> female expressed HSV-1 TK in the testis. Furthermore, most of the lines were male sterile, and sterility was invariably associated with a higher level of expression in the testis. Altogether, 21 transgenic lines representing five constructs expressed HSV-1 TK at a high level and were male sterile, while three transgenic lines expressed the enzyme at a low level and were fertile. When the expression of each construct was averaged over all transgenic lines for which data were available, the level of expression of the reporter gene in the testis was nearly the same in most cases. The single exception was BS6-*tk-supF* (TR), which had a level of expression roughly twice that of the other lines. This may relate to the fact that BS6-*tk-supF* (TR) was microinjected as a tandem dimer (see above). The relatively uniform expression of HSV-1 *tk* when coupled with a variety of promoters suggests that expression in the testis is a function of the *tk* sequence, which was the same in all cases.

Some lines of each series were extensively screened for expression in other tissues. Low-level expression of MUP promoter constructs was very occasionally found in the lachrymal gland. Other tissues were negative. Although all of the constructs carried the bacterial *supF* gene, this was not the cause of their expression in the testis: a construct identical to BS6-*tk-supF* but without the *supF* gene was expressed in the testis at similar levels (1). The data thus confirm our conclusion (1) that sterility correlates with expression of HSV-1 TK in the testis, not with expression in the liver (constructs BS6*Sau2-tk-supF* and TG-*tk-supF*) or in the preputial gland (constructs BS2-*tk-supF* and TG-*tk-supF*).

**Truncated forms of HSV-1 *tk* 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 (39), 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-bp transcript expected if transcription was initiated at the cap site of the BS6 gene.

Primer extension (not shown) and nuclease 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* ( $M^1$ ; Fig. 1) and the second methionine codon ( $M^{46}$ ; 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  $M_r$ -43,000 (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  $M^{46}$  and  $M^{60}$ . They were produced in increased amounts when  $M^1$  was deleted (20) or if a stop codon was introduced between  $M^1$  and  $M^{46}$  (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,  $M^{85}$ . 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  $ATG^1$  and  $ATG^2$ . 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  $ATG^1$ . 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).

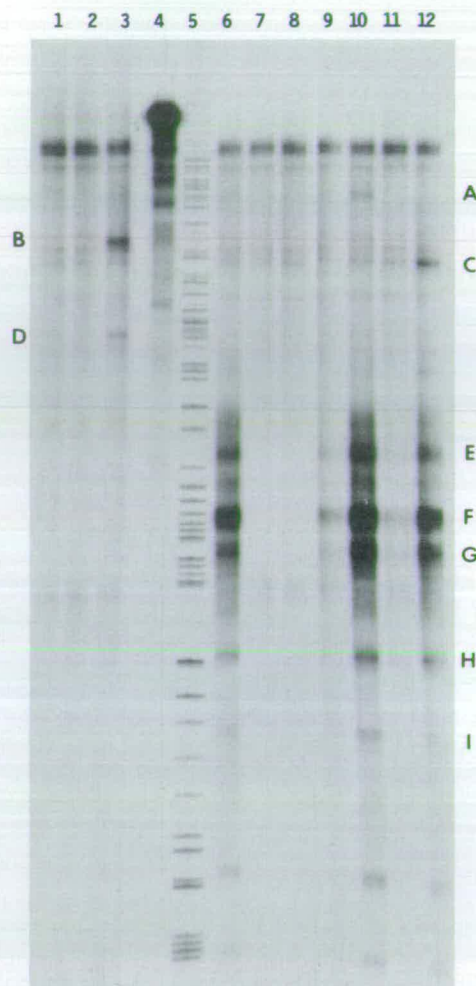


FIG. 3. Mapping of 5' ends of HSV-1 *tk*-specific RNA in livers and testes of transgenic mice. The RNA riboprobe was transcribed from a fragment of BS6-*tk-supF* which overlaps the MUP gene cap site (see Materials and Methods). Lanes: 1 and 7, tRNA controls; 2, RNA from nontransgenic liver; 3, RNA from line 78 (BS6-*tk-supF*) liver; 4, undigested probe; 5, sequencing ladder; 8, RNA from nontransgenic testis; 6 and 9 to 12, RNA from transgenic testis from line 78 (lane 6), line 40 (BS6-*tk-supF*) (lane 9), line S87 (BS6-*Sau2-tk-supF*) (lane 10), line TK16 (HSV-1 *tkDS2*-) (lane 11), and line TG66 (TG-*tk-supF*) (lane 12). Note that the 3' end of the riboprobe is not complementary to the region around the cap sites of constructs HSV-1 *tkDS2*- and TG-*tk-supF*; the furthest 5' position of these constructs that could be mapped with this probe is shown as marker C.

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,



TABLE 2. Transcription initiation sites in liver and testis of line L78 transgenic mice<sup>a</sup>

Tissue	Transcription initiation sites as determined by:	
	Primer extension	Nuclease protection
Liver		-1
		+73
	+129	+133
	+144	+153
Testis	+159	+160
	+169	+168
	+208	+203

<sup>a</sup> Methionine initiation codons are as follows: M<sup>1</sup>, +79; M<sup>46</sup>, +214; M<sup>60</sup>, +256.

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 *tkDS2*-) 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 G<sub>0</sub> transgenic

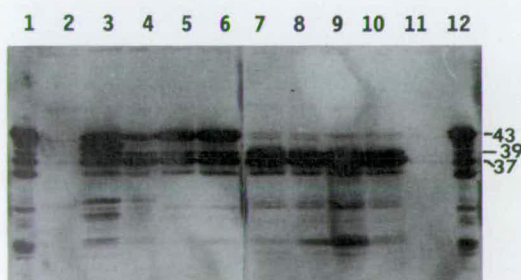


FIG. 4. Effect of protease inhibitors on HSV-1 TK polypeptides in extracts of transgenic preputial glands and testes. Each lane was loaded with 50  $\mu$ g of protein. Proteins were transferred to a filter and developed with anti-HSV-1 TK antibody as described in Materials and Methods. The positions of the 43K, 39K, and 37K polypeptides are marked. Lanes: 1 and 12, HSV-1-infected cells; 2 to 6, preputial gland extracts from a nontransgenic control mouse (lane 2), and from line 40 mice (HSV-1 *tk-supF* construct) (lanes 3 to 6), without added protease inhibitor (lane 3), with 20 mM DCI (lane 4), with 10 mM TPC plus 10 mM PHMB (lane 5), and with 20 mM TPC plus 20 mM PHMB (lane 6); 7 to 11, testis extracts from a nontransgenic control mouse (lane 11), and from line 40 mice (lanes 7 to 10) with 20 mM TPC plus 20 mM PHMB (lane 7), with 10 mM TPC plus 10 mM PHMB (lane 8), with 20 mM DCI (lane 9), and without added protease inhibitor (lane 10).



FIG. 5. HSV-1 TK-specific polypeptides in testis extracts from different lines of transgenic mice. The positions of the 39K and 37K polypeptides are marked. Lanes contain testis extracts as follows: 1, nontransgenic control; 2, line 64 (BS6-*tk-supF*); 3, line 78 (BS6-*tk-supF*); 4, line 46 (BS6-*tk-supF*); 5, line TK16 (HSV-1 *tkDS2*-); 6, line TG66 (TG-*tk-supF*); 7, line S87 (BS6Sau2-*tk-supF*).

males carrying the HSV-1 *tkDS2*- fragment and in male transgenic offspring of a G<sub>0</sub> female (founder of line TK16). Three of the G<sub>0</sub> 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 G<sub>0</sub> males carrying *tk* constructs (1). Such males are probably mosaics with nontransgenic precursor cells in their germ line (see below). The third expressing G<sub>0</sub> 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 testes 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 spermatozoan 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

Tissue	TK activity (pmol/min/mg of protein)					
	Nontransgenic control	$G_0$ transgenic males				Line TK16 male, sterile
		TK10, fertile <sup>a</sup>	TK22, fertile	TK39, sterile	TK40, fertile <sup>a</sup>	
Testis	0.11	23.15	0.16	37.75	4.25	97.23
Liver	0.06	0.46	0.00	0.04	0.00	0.07
Preputial gland	0.16	0.00	0.00	0.00	0.00	0.28
Kidney	0.08	0.27	0.20	0.00	0.00	0.08
Brain	0.20	ND <sup>b</sup>	ND	0.35	0.07	2.99
Muscle	0.00	ND	ND	0.00	0.08	0.00
Lachrymal gland	0.07	ND	ND	0.03	0.00	8.01
Submaxillary gland	0.03	ND	ND	0.00	0.00	1.02

<sup>a</sup> Fertile nontransmitting.<sup>b</sup> 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 spermio-

genesis-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).

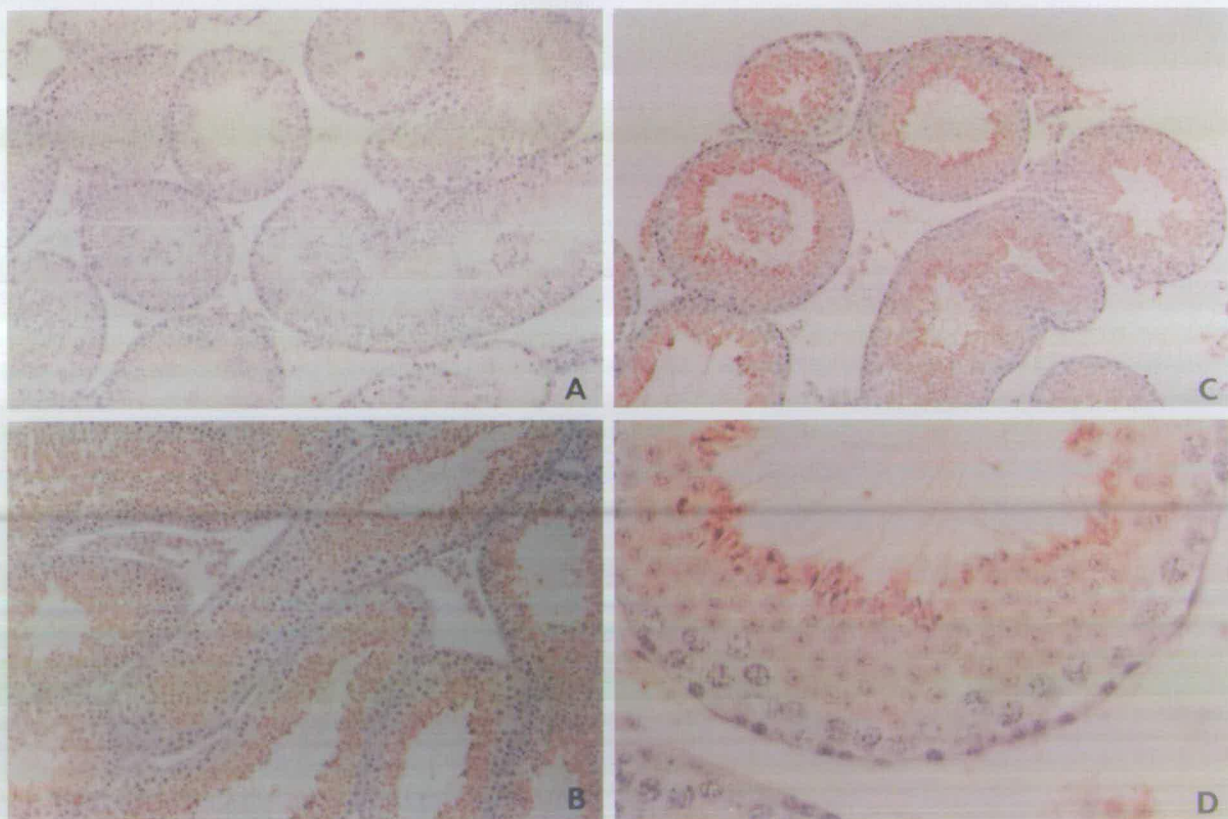


FIG. 6. Testes of BS6-*tk-supF* transgenic mice immunostained with anti-HSV-1 TK serum. (A) Nontransgenic testis; (B) transgenic testis from line TG66 (TG-*tk-supF*); (C and D) transgenic testis from line 78 (BS6-*tk-supF*). Magnifications: A, B, and C,  $\times 70$ ; D,  $\times 280$ .

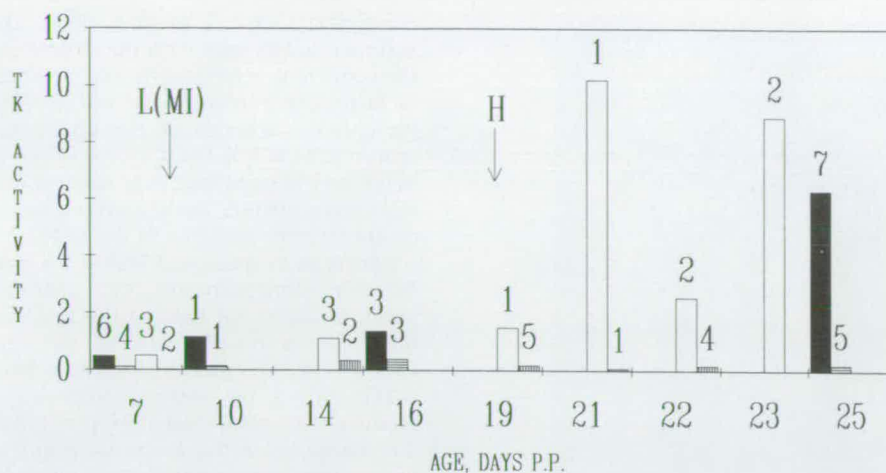


FIG. 7. Time course of HSV-1 TK expression in testis during sexual maturation. Testes of pups from crosses of line 40 and line 46 females with nontransgenic males were assayed as described prior to determining their transgenic status. Ordinate, HSV-1 TK activity, expressed as picomoles of TMP synthesized per minute per milligram of protein. P.P., after birth. The bars show the average HSV-1 TK activity for cases in which more than one pup was measured. The numbers of transgenic L40 (■) and L46 (□) and nontransgenic L40 (▨) and L46 (▩) pups are shown above the bars. The times at which meiotic figures [leptotene of first meiosis, L(MI)] and haploid meiotic products (spermatids, H) are first observed are indicated. These are the stages to which the most advanced tubules have progressed at that time.

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  $G_0$  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  $G_0$  transgenic males were obtained with each of the constructs listed in Table 1. Taken together, 47% of the transgenic  $G_0$  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 insertional event occurred prior to DNA replication at the one-cell stage, leading to a uniformly heterozygous individual  $G_0$  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		Line 46	
		Mouse 1	Mouse 2	Mouse 1	Mouse 2
Sperm weight (mg)	91, 95	72	74	80	63
Epididymal sperm	$2-3 \times 10^6$	$10^5$	$5 \times 10^3$	$10^5$	$5 \times 10^4$
Contents of vas deferens	Numerous sperm 80% normal Motility normal	Very few sperm None with normal morphology Nonmotile			
HSV-1 TK activity <sup>a</sup> (line mean $\pm$ SE)		140 $\pm$ 6.7		139 $\pm$ 17.6	

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

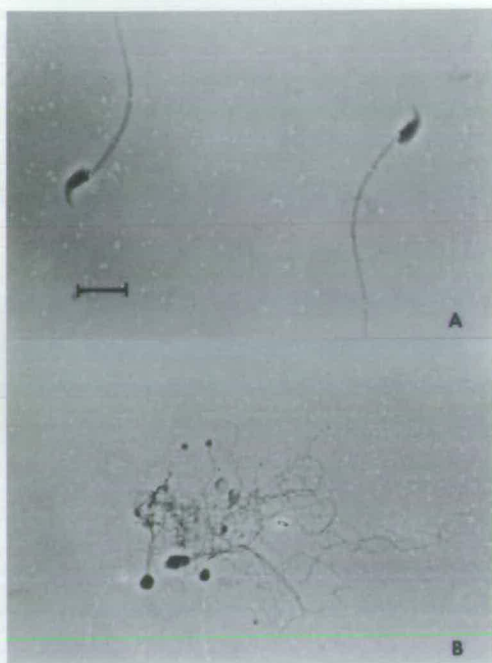


FIG. 8. Normal and defective spermatozoa. The contents of the vas deferens of nontransgenic (A) and line 40 (B) mice were stained with eosin. The bar represents 10  $\mu$ m.

erozygous and, by analogy with the heterozygous male offspring of transgenic females, are assumed to generate only defective spermatozoa. The nontransgenic germ cells presumably develop separate syncytia which generate competent spermatozoa.

## 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 (*Hpa*II tiny fragment) island (3) in containing many potential methylation sites. However it is clearly not a true housekeeping promoter since it is consistently expressed 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- $\beta$ -interferon-*tk* construct, Iwakura et al. (24) observed minority transcripts (about 20%) which hybridized with both

$\beta$ -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 completely) 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 normally 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 polypeptide 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<sup>60</sup>, the third methionine of the reading frame, which lies within the strongest translational initiation consensus of the first three ATG codons. M<sup>60</sup> 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 assays. 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 *tkDS2-* 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 neutralized 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 cosmid containing the major histocompatibility complex class II E<sub>a</sub><sup>d</sup> gene was at

first attributed to the expression of  $E_{\alpha}^d$  (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- $\beta$ -interferon-*tk* foreign gene caused male sterility, which was attributed to the expression of  $\beta$ -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  $\beta$ -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 higher levels of expression but not in lines with intermediate expression levels.

#### ACKNOWLEDGMENTS

We thank B. Roizman and W. C. Summers for generous gifts of anti-HSV-1 TK serum and B. Clements and H. Marsden for gifts of cells infected with wild-type and mutant HSV-1. Melville Richardson, Helen McIlroy, Gaz Brown, and Mark Lawson provided assistance.

The work was supported by the Medical Research Council, the Agricultural and Food Research Council, and the AFRC Centre for Genome Research.

#### REFERENCES

1. Al-Shawi, R., J. Burke, C. T. Jones, J. P. Simons, and J. O. Bishop. 1988. A Mup promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol. Cell. Biol.* **8**:4821-4828.
2. Al-Shawi, R., J. Kinnaird, J. Burke, and J. O. Bishop. 1990. Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. *Mol. Cell. Biol.* **10**:1192-1198.
3. Bird, A. P. 1986. CpG-rich islands and the function of DNA methylation. *Nature (London)* **321**:209-213.
4. Borrelli, E., R. Heyman, M. Hsi, and R. M. Evans. 1988. Targeting of an inducible phenotype to mammalian cells. *Proc. Natl. Acad. Sci. USA* **85**:7572-7576.
5. Borrelli, E., R. A. Heyman, C. Adrias, P. E. Sawchenko, and R. M. Evans. 1989. Transgenic mice with inducible dwarfism. *Nature (London)* **339**:538-541.
6. Braun, R. E., R. R. Behringer, J. J. Peschon, R. L. Brinster, and R. D. Palmiter. 1989. Genetically haploid spermatids are genetically diploid. *Nature (London)* **337**:373-376.
7. Braun, R. E., D. Lo, C. A. Pinkert, G. Widera, R. A. Flavell, R. D. Palmiter, and R. L. Brinster. 1990. Infertility in male transgenic mice: disruption of sperm development by HSV-tk expression in postmeiotic germ cells. *Biol. Reprod.* **43**:684-693.
8. Brinster, R. L., H. Y. Chen, M. Trumbauer, A. W. Senear, R. Warren, and R. D. Palmiter. 1981. Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* **27**:223-231.
9. Carey, J. E., P. O. Clarke, and B. T. Storey. 1981. Oxidative metabolism of spermatozoa from inbred and random bred mice. *J. Exp. Zool.* **216**:285-292.
10. Cheng, M. S., and W. H. Prusoff. 1978. Association of thymidylate kinase activity with pyrimidine deoxyribonucleoside kinase induced by herpes simplex virus. *J. Biol. Chem.* **253**:1325-1327.
11. Cheng, Y. C., and M. Ostrander. 1976. Deoxythymidine kinase induced in HeLa tk- cells by herpes simplex virus type I and type II. *J. Biol. Chem.* **251**:2605-2610.
12. Coen, D. M., A. F. Irmieri, J. G. Jacobson, and K. M. Kerns. 1989. Low levels of herpes simplex virus thymidine-thymidylate kinase are not limiting for sensitivity to certain antiviral drugs or for latency in a mouse model. *Virology* **168**:221-231.
13. de Martynoff, G., V. Pohl, L. Mercken, G. J. van Ommen, and G. Vassart. 1987. Structural organization of the bovine thyroglobulin gene and of its 5'-flanking region. *Eur. J. Biochem.* **164**:591-599.
14. Eisenberg, S. P., D. M. Coen, and S. L. McKnight. 1985. Promoter domains required for expression of plasmid-borne copies of the herpes simplex virus thymidine kinase gene in virus-infected mouse fibroblasts and microinjected frog oocytes. *Mol. Cell. Biol.* **5**:1940-1947.
15. Garrity, P. A., and B. J. Wold. 1990. Tissue-specific expression from a compound TATA-dependent and TATA-independent promoter. *Mol. Cell. Biol.* **10**:5646-5654.
16. Ghosh, P. K., V. B. Reddy, M. Piatak, P. Lebowitz, and S. M. Weissman. 1980. Determination of RNA sequences by primer directed synthesis and sequencing of their cDNA transcripts. *Methods Enzymol.* **65**:580-595.
17. Goldman, D. S., A. A. Kiessling, C. F. Millette, and G. M. Cooper. 1987. Expression of c-mos in germ cells of male and female mice. *Proc. Natl. Acad. Sci. USA* **84**:4509-4513.
18. Gompels, U., and A. Minson. 1986. The properties and sequence of glycoprotein H of herpes simplex virus type I. *Virology* **153**:230-247.
19. Haarr, L., H. S. Marsden, C. M. Preston, J. R. Smiley, W. C. Summers, and W. P. Summers. 1985. Utilization of internal AUG codons for initiation of protein synthesis directed by mRNAs from normal and mutant genes encoding herpes simplex virus-specified thymidine kinase. *J. Virol.* **56**:512-519.
20. Halpern, M. E., and J. R. Smiley. 1984. Effects of deletions on expression of the herpes simplex virus thymidine kinase gene from the intact viral genome: the amino terminus of the enzyme is dispensable for catalytic activity. *J. Virol.* **50**:733-738.
21. Hecht, N. B., P. A. Bower, S. H. Waters, P. C. Yelick, and R. J. Distel. 1986. Evidence for haploid expression of mouse testicular genes. *Exp. Cell. Res.* **164**:183-190.
22. Heyman, R. A., E. Borrelli, J. Lesley, D. Anderson, D. D. Richman, S. M. Baird, R. Hyman, and R. M. Evans. 1989. Thymidine kinase obliteration: creation of transgenic mice with controlled immune deficiency. *Proc. Natl. Acad. Sci. USA* **86**:2698-2702.
23. Irmieri, A. F., M. M. Manos, J. G. Jacobson, J. S. Gibbs, and D. M. Coen. 1989. Effect of an amber mutation in the herpes simplex virus thymidine kinase gene on polypeptide synthesis

- and stability. *Virology* **168**:210-220.
24. Iwakura, Y., M. Asano, Y. Nishimune, and Y. Kawade. 1988. Male sterility of transgenic mice carrying exogenous mouse interferon-beta gene under the control of the metallothionein enhancer-promoter. *EMBO J.* **7**:3757-3762.
  25. Jacobson, J. G., S. L. Martin, and D. M. Coen. 1989. A conserved open reading frame that overlaps the herpes simplex virus thymidine kinase gene is important for viral growth in cell culture. *J. Virol.* **63**:1839-1843.
  26. Jamieson, A. T., and J. H. Subak-Sharpe. 1974. Biochemical studies on the herpes simplex virus-specified deoxypyrimidine kinase activity. *J. Gen. Virol.* **24**:481-492.
  27. Johnson, D. A., J. W. Gautsch, J. R. Sportsman, and J. H. Elder. 1984. Improved technique utilizing non-fat dried milk for analysis of proteins and nucleic acids transferred to nitrocellulose. *Gene Anal. Tech.* **1**:3-8.
  - 27a. Kinnaird, J., C. Heinlein, R. Al-Shawi, and J. O. Bishop. Unpublished data.
  28. Krieg, P. A., and D. A. Melton. 1987. In vitro RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* **155**:397-415.
  29. Liu, Q. Y., and W. C. Summers. 1988. Site-directed mutagenesis of a nucleotide-binding domain in HSV-1 thymidine kinase: effects on catalytic activity. *Virology* **163**:638-642.
  30. Maitland, N. J., and J. K. McDougall. 1977. Biochemical transformation of mouse cells by fragments of herpes simplex virus DNA. *Cell* **11**:233-241.
  31. Marsden, H. S., L. Haarr, and C. M. Preston. 1983. Processing of herpes simplex virus proteins and evidence that translation of thymidine kinase mRNA is initiated at three separate AUG codons. *J. Virol.* **46**:434-445.
  32. Maxam, A. M., and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. *Methods Enzymol.* **65**:499-560.
  33. McKnight, S. L., E. R. Gavis, R. Kingsbury, and R. Axel. 1981. Analysis of transcriptional regulatory signals of the HSV thymidine kinase gene: identification of an upstream coding region. *Cell* **25**:385-398.
  34. McKnight, S. L., R. C. Kingsbury, A. Spence, and M. Smith. 1984. The distal control signals of the herpesvirus tk gene share a common hexanucleotide control sequence. *Cell* **37**:253-262.
  - 34a. Mueller, I. Unpublished data.
  35. Nebel, B. R., A. P. Amarose, and E. M. Hackett. 1961. Calendar of gametogenic development in the prepubertal male mouse. *Science* **134**:832-833.
  36. Peschon, J. J., R. R. Behringer, R. L. Brinster, and R. D. Palmiter. 1987. Spermatid-specific expression of protamine 1 in transgenic mice. *Proc. Natl. Acad. Sci. USA* **84**:5316-5319.
  37. Pinkert, C., G. Widera, C. Cowing, E. Heber-Katz, R. D. Palmiter, R. A. Flavell, and R. L. Brinster. 1985. Tissue-specific, inducible and functional expression of the Ead MHC class II gene in transgenic mice. *EMBO J.* **4**:2225-2230.
  38. Richardson, W. D., B. L. Roberts, and A. E. Smith. 1980. Nuclear location signals in polyoma virus large-T. *Cell* **44**:77-85.
  39. Roberts, J. M., and R. Axel. 1982. Gene amplification and gene correction in somatic cells. *Cell* **29**:109-119.
  40. Shackelford, G. M., and H. E. Varmus. 1987. Expression of the proto-oncogene int-1 is restricted to postmeiotic male germ cells and the neural tube of mid-gestational embryos. *Cell* **50**:89-95.
  41. Veerisetty, V., and G. A. Gentry. 1985. HSV1-specific thymidylate kinase in infected cells. *Intervirology* **24**:42-49.
  42. Virbasius, J. V., and R. C. Scarpulla. 1990. The rat cytochrome c oxidase subunit IV gene family: tissue-specific and hormonal differences in subunit IV and cytochrome c mRNA expression. *Nucleic Acids Res.* **18**:6581-6586.
  43. Wagner, E. F., T. A. Stewart, and B. Mintz. 1981. The human beta-globin gene and a functional viral thymidine kinase gene in developing mice. *Proc. Natl. Acad. Sci. USA* **78**:5016-5020.
  44. Wagner, T. E., P. C. Hoppe, J. D. Jollick, D. R. Scholl, R. L. Hodinka, and J. B. Gault. 1981. Microinjection of a rabbit beta-globin gene into zygotes and its subsequent expression in adult mice and their offspring. *Proc. Natl. Acad. Sci. USA* **78**:6376-6380.
  - 44a. Wallace, H., C. Ledent, G. Vassart, J. O. Bishop, and R. Al-Shawi. Submitted for publication.
  - 44b. Whitaker, J., R. Al-Shawi, L. Smith, and J. O. Bishop. Submitted for publication.
  45. Wigler, M., S. Silverstein, L. S. Lee, A. Pellicer, Y. C. Cheng, and R. Axel. 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. *Cell* **11**:223-232.
  46. Wilkie, N. M., J. B. Clements, W. Boll, N. Mantei, and C. Weissmann. 1979. Hybrid plasmids containing an active thymidine kinase gene of herpes simplex virus 1. *Nucleic Acids Res.* **7**:859-877.

# Specific Ablation of Thyroid Follicle Cells in Adult Transgenic Mice\*

ELEN WALLACE, CATHERINE LEDENT, GILBERT VASSART, JOHN O. BISHOP,  
D RAYA AL-SHAWI

*Institute of Cell and Population Biology, Division of Biology, University of Edinburgh (H.W., R.A., J.O.B.),  
Edinburgh EH9 3JN, and the Agricultural and Food Research Council Centre for Genome Research (J.O.B.,  
A.), Edinburgh EH9 3JQ, United Kingdom; Institut de Recherche Interdisciplinaire, Faculté de Médecine  
L., G.V.), 1070 Brussels, Belgium; and the Department of Biological Sciences, University of Maryland  
O.B.), Baltimore, Maryland 21228*

**ABSTRACT.** The coding region of the herpes simplex type 1 virus thymidine kinase gene was coupled to the promoter of the bovine thyroglobulin gene and introduced into the genome of mice. The viral thymidine kinase (HSV1-TK) was expressed mainly in the thyroid glands and testis. Upon treatment of transgenic females with the antiherpetic agent Ganciclovir the thyroid regressed, while the parathyroid gland was unaffected. The number of thyroid follicle cells was greatly reduced after 3 days, and they were completely absent after 7 days of treatment. After 14 days, the levels of circulating  $T_4$  and  $T_3$  were below the limits of detection, total soluble protein recovered from the thyroid and parathyroid glands together was 10% of the control value, and the level of thyroid HSV1-TK was more than 100-fold lower than that in transgenic controls. Levels of circulating

PTH and calcitonin remained normal. At the time of treatment the mice were adults. Thus, the thyroid follicle cells were selectively ablated after normal development with a functional thyroid gland. When treatment with Ganciclovir was terminated after 14 days, no circulating  $T_4$  or  $T_3$  or other indications of thyroid regeneration were detected for a subsequent period of 90 days. During this time the mice gained weight more slowly than controls, at a rate consistent with the suppression of GH synthesis by thyroid deficiency. The production of mouse major urinary protein (MUP) ceased in the treated mice and was completely restored by the administration of  $T_4$ . MUP production was not restored by GH, demonstrating that the expression of the *Mup* genes requires  $T_4$  in addition to GH. (*Endocrinology* 129: 3217-3226, 1991)

SEVERAL hormones are produced by specialized cells that have no other known function than to produce a single hormone and secrete it appropriately. Nevertheless, because cells with different specialized functions are often physically close to and interspersed with each other, it is usually impossible to produce by surgery an experimental animal with a single hormonal deficiency. An example is the anterior pituitary, in which GH, PRL, LH, SH, gonadotropin, and other hormones are all produced by different specialized secretory cells. This problem has been successfully addressed by the technique of transgenic ablation. For example, expression of the diphtheria toxin A-protein was targeted to the pancreas with the elastase promoter (1) and to the pituitary with the GH promoter (2). In the first instance the pancreatic exocrine cells and in the second the pituitary somatotrophs and lactotrophs were specifically destroyed. A disadvantage

of this method, however, is that the toxin is produced and takes its effect as soon as the tissue-specific promoter becomes active, thus eliminating any possibility of selecting the time of hormone deprivation in relation to experimental requirements. An alternative method that potentially allows some control of the time of ablation is based on the special properties of herpes virus type 1 thymidine kinase (HSV1-TK). When the expression of the herpesvirus gene (*HSV1-tk*) was targeted to the pituitary by means of the GH promoter, treatment of the transgenic mice with 1-(2-deoxy-2-fluoro- $\beta$ -D-arabino-furanosyl)5-iodouracil (FIAU) led to complete ablation of the somatotrophs and lactotrophs without impairment of other specialized secretory pituitary cells, such as thyrotrophs and gonadotrophs (3). In these experiments somatotrophs and lactotrophs regenerated when FIAU administration was discontinued.

The potential of inducible transgenic ablation is considerable. The main requirements are 1) that the foreign protein (HSV1-TK, above) should not affect the cells in the absence of the agent (FIAU, above), 2) that the agent should not be toxic in its own right or be converted to a toxic product by cellular enzymes in the absence of

Received July 16, 1991.

Address all correspondence and requests for reprints to: Dr. John Bishop, Department of Biological Sciences, University of Maryland, 701 Wilkens Avenue, Baltimore, Maryland 21228.

\* This work was supported by AFRC Grant TAP2 and the AFRC Centre for Genome Research.

HSV1-TK, 3) that the foreign protein should convert the agent to a toxic product (in this case phosphorylated FIAU), and 4) that the toxic product should not affect nearby cells. FIAU and Ganciclovir (9-[1,3-dihydroxy-2-propoxy(methyl)]guanine; DHPG) were developed, like Acyclovir, to combat herpes virus infections. They are effective first, because they are nontoxic in their unaltered state but become lethally toxic when phosphorylated, and second, because they are efficiently phosphorylated by HSV1-TK, but less efficiently or not at all by cellular nucleoside kinases (4, 5).

Side-effects of HSV1-TK expression in the targetted cells have not been reported. However, transgenic *HSV1-tk* reporter genes are expressed in the testis in addition to the target tissue regardless of the nature of the promoter to which they are linked, and the transgenic males are almost invariably sterile (6-8). While this is inconvenient from the viewpoint of husbandry and means that homozygotes cannot be obtained, possible complications due to the expression of HSV1-TK in the testis can be avoided by restricting the studies to transgenic females.

Nucleotides are not taken up by cells. Consequently, the phosphorylated antiherpetic agents are unlikely to damage nearby cells when released by the death of the affected cells. However, nucleotides do pass between cells through gap junctions, and the selectivity of HSV1-TK-mediated cell ablation is likely to depend on whether the target cells communicate with other cell types through gap junctions.

Previously, HSV1-TK-mediated transgenic ablation was deliberately targeted to dividing cells (3, 9, 10), leaving open the question of whether nondividing cells can also be ablated in this way. However, the potential of the method would be greatly increased if nondividing differentiated cells could be killed. An ablation system capable of selectively killing differentiated target cells could be applied to mature animals that have developed normally and, consequently, lack defects brought about by congenital or neonatal hormonal deficiency.

The thyroid is a suitable organ with which to explore the potential of transgenic ablation. It contains two known types of secretory cell, follicle cells which produce  $T_4$  and  $T_3$ , and C-cells which secrete calcitonin (CT), and it is in intimate contact with the parathyroid gland which secretes PTH. By targeting HSV1-TK to the follicle cells, the effects of follicle cell ablation on C-cells and on the parathyroid can be determined. Furthermore, the follicle cells provide a means to test the efficacy of ablation of nondividing cells; in normal adult rats and mice, the follicle cells are essentially nondividing, with mitotic indices of less than  $10^{-3}$  (11, 12), although they can be stimulated to divide both *in vivo* and *in vitro* by supranormal levels of TSH (13).

We have targeted HSV1-TK expression to the thyroid

follicle cells of transgenic mice by coupling the *HSV1-tk* gene to the bovine thyroglobulin promoter (14). Expression was largely confined to the thyroid follicle cells of female mice. Mature (up to 18-week-old) female mice infused with DHPG by minipump suffered a complete loss of thyroid hormone production. The thyroid glands regressed, follicle cells were destroyed, and follicle lumens were no longer present. The levels of circulating PTH and CT were unaffected, showing that ablation was specific to the follicle cells.

## Materials and Methods

### Transgenic and other mice

The fragments used to assemble plasmid *ptgTK* were derived from the following sources (sites that were made blunt-ended with DNA polymerase-I Klenow fragment before ligation are marked with an asterisk): 1, a 2297-basepair (bp) *EcoRI-PvuII* fragment of pBR322 containing the replication origin and  $\beta$ -lactamase gene; 2, a 342-bp *PvuII-HindIII\** fragment of simian virus-40 containing the replication origin, enhancer, and early promoter were taken together from pSV2gpt (15); 3, an 8-bp *BamHI\** to *HindIII* polylinker fragment derived from pPOLYIII-D (16) and pUC8 (17), containing the *XhoI* site as shown in Fig. 1; 4, a 3050-bp *EcoRI-AluI* fragment of the bovine thyroglobulin gene containing the cap site, part of the noncoding region, and more than 3 kilobases (kb) of 5'-flanking sequence (14); 5, a 1765-bp *BglIII\*-BstEI\** fragment from pTK (18) containing the *HSV-tk* coding sequence, part of the noncoding region, the polyadenylation site, and some 3'-flanking sequence; 6, a 10-bp *Sall\*-BamHI\** fragment from the M13tg130 polylinker (19); 7, a 408-bp *AluI-XbaI* fragment of the  $\pi$ VX containing the *SupF* gene (20); and 8, a 36-bp *XbaI-EcoRI* fragment of the M13tg130 polylinker containing the *KpnI* site as shown in Fig. 1. A DNA fragment containing the hybrid *TG-tk* gene and the *SupF* gene was removed by restriction in *XbaI* polylinkers (*XhoI-KpnI*) and purified as previously described (6), and approximately 2 pl were injected at a concentration of 1.5  $\mu$ g/ml into either pronucleus of C57BL/6  $\times$  CBA/Ca embryos (6). Transgenic offspring were identified by polym

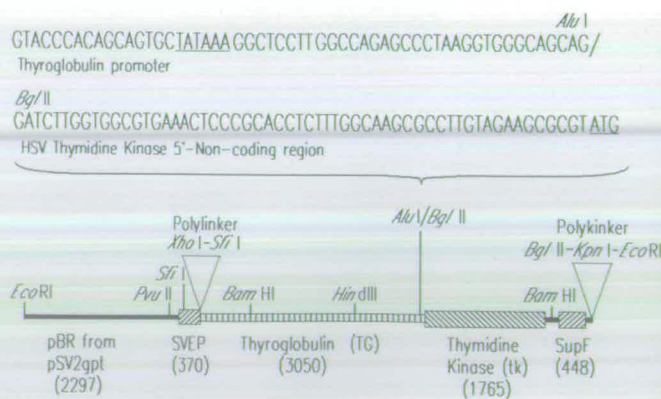


FIG. 1. Diagram of the thyroglobulin promoter-HSV-thymidine kinase reporter construct (*TG-tk*). The sequence of the *AluI-BglIII* junction between the TATA box and the *HSV-tk* initiation codon is shown.



chain reaction analysis of tail-cut DNA. Two hundred and five embryos were transferred to pseudopregnant recipients, 90 pups were born, 15 transgenics were identified, and 4 lines were established. One of these generated the high expression transgenic line TG66.19, which was employed in the experiments described here. Thyroidectomy was performed by surgery, and mice were taken 7 days later. Mice were maintained and bred in accordance with European Economic Community guidelines, endorsed and implemented by the UK Home Office.

#### Drug administration

Transgenic female mice, all from line TG66.19, were 8–18 weeks of age when minipumps (Alzet model 2002, Alza Corp., Fremont, CA) were implanted sc under general anesthesia. DHPG was administered at a rate of 4.5 mg/day for up to 14 days. Control mice received saline-containing minipumps.  $T_4$  was injected daily (5  $\mu$ g/20 g BW, ip) (21) for the last 5 days of 4-day DHPG administration. Controls were injected with  $T_4$  diluent (3 mM NaOH and 1% NaCl; 50  $\mu$ l/20 g BW, ip). The hormone GH (bGH; 100  $\mu$ g/20 g) was injected ip at 12-h intervals for 5 days.

#### Hormone, protein, and enzyme determinations

$T_4$  and  $T_3$  levels were determined using Amerlite competitive enzyme-linked immunosorbent assays (Amersham, Amersham, UK; TT4 and TT3), which employ enhanced luminescence to measure peroxidase-conjugated hormone with increased sensitivity (22). The sensitivities of these assays (2 SD of a zero standard) are reported by the manufacturer to be greater than 0.15 nmol/liter, respectively. PTH and CT were determined by RIA. PTH was determined with Amersham kit R3.800, which employs guinea pig antihuman (midmolecule) antiserum, radioiodinated human PTH, and goat antiserum against guinea pig  $\gamma$ -globulin. CT was determined with Amersham kit IMB.400, employing goat antihuman CT, radioiodinated synthetic (human) CT, and donkey antiserum against human  $\gamma$ -globulin. Thymidine kinase assays were carried out as previously described (23), but 0.2 mM thymidine-5'-triphosphate was added to inhibit endogenous thymidine kinase (24). Protein was determined by the Bradford method (25).

#### Immunohistochemistry

Thyroid glands were removed, fixed in Bouin's solution, embedded in paraffin wax, and stained with hematoxylin and eosin.

#### Analysis of urinary protein

Urine was collected by bladder massage and dialyzed first against 0.3 M NaCl-10 mM Tris-HCl (pH 7.5) and then against water. Twenty microliters of urine were applied to each lane of agarose gel containing pH 3.5–4.9 ampholines (Pharmacia, Little Chalfont, UK). After focusing (15 kilovolt-amperes; 90 min), the gel was stained with Coomassie blue.

## Results

### Tissue-specific expression of HSV1-TK directed by a thyroglobulin promoter

Thyroglobulin, the protein from which  $T_4$  is generated, is synthesized exclusively in thyroid follicle cells, and the

promoter of the thyroglobulin gene directs the expression of the chloramphenicol acetyl transferase reporter gene to these cells (26). We attached the coding, 3'-noncoding, and 3'-flanking regions of the HSV1-*tk* gene to a 3.1-kb fragment containing the promoter and 5'-flanking regions of the bovine thyroglobulin gene through a junction in the 5' noncoding regions of the two genes (Fig. 1) and introduced the hybrid gene into the germline of mice by direct microinjection. Four lines of transgenic mice were established, three of which expressed HSV1-TK in the thyroid gland at different levels (Table 1). In females, high level expression was confined to the thyroid glands, although there was consistently a very low level of expression in the submaxillary glands. In males there was high level expression in both thyroid glands and testis (data not shown). Expression in the testis is a common (probably universal) feature of hybrid genes that contain the HSV1-*tk* reporter (6, 7) and is due to the presence of a sequence, probably within the HSV1-*tk*-coding region, which acts as a testis-specific housekeeping-type promoter (8). The experiments described here were performed with females of a line (TG66.19) established from the  $G_0$  female which showed the highest level of HSV1-TK expression in the thyroid (Table 1). The high expression level has been transmitted through several subsequent generations. The level of expression in the submaxillary glands of this line, relative to that in the thyroid, was about 0.2% (Table 1).

### Ablation of thyroid follicle cells by DHPG

When transgenic female mice of line TG66.19 were treated with DHPG by minipump for 2 weeks, serum  $T_4$  and  $T_3$  levels fell to below the sensitivity limits of the assays (see *Materials and Methods*), and the luminometer readings were close to zero (Tables 2 and 3). Regular injection of  $T_4$  throughout the last 5 days of DHPG treatment restored the level of circulating  $T_3$  to about half the normal level, that is to a level similar to the

TABLE 1. Thymidine kinase activities in tissues of female transgenic and control mice

Tissue	Transgenic line				
	TG14	TG26	TG62	TG66.19	Control
Thyroid	10.6	11.4	1.2	178.8	0.3
Liver	0.05	0.04	0.03	0.04	0.03
Brain	0.28	0.17	0.05	0.37	0.27
Muscle	0.03	ND	ND	0.01	0.02
Submaxillary gland	0.17	0.11	0.11	0.32	0.01
Kidney	0.05	ND	ND	0.05	0.03
Heart	0.07	ND	ND	0.11	0.08
Ovary	0.51	0.29	0.3	0.43	0.33

Thymidine kinase activity is expressed as picomoles per min/mg protein. Control, Nontransgenic  $F_2$  mice from a C57BL  $\times$  CBA/Ca cross. ND, Not determined.

TABLE 2. Effect of DHPG treatment on levels of circulating T<sub>4</sub> and T<sub>3</sub> and on thyroid protein and HSV1-TK in TG66.19 transgenic female mice

Transgenic status	Treatment	Total protein (μg): Before After	Total TK units: Before After	T <sub>4</sub> (nmol/liter)		T <sub>3</sub> (nmol/liter)	
				Before	After	Before	After
Transgenic	PBS	144	17.50	44.1	39.5		0.58
	DHPG	10	0.02	29.8	0.0		ND
	DHPG + D	20	0.00				
	DHPG + T <sub>4</sub>	14 3	0.03 0.01	50.1	64.2	0.95	0.33
	DHPG + GH	10 7	0.03 0.02	20.1	0.0		0.00
	Control	DHPG	100	0.01	66.4	40.4	0.73

Controls were F<sub>2</sub> mice from a C57BL × CBA/Ca cross, equivalent in background to the transgenic mice. Treatments (see *Materials and Methods*): PBS, implanted with a minipump containing saline; DHPG, with a minipump containing DHPG; D, injected with T<sub>4</sub> diluent; T<sub>4</sub>, injected with T<sub>4</sub>; GH, injected with GH. Before, Before treatment; After, after treatment. Serum samples were pooled for hormone assays indicated. ND, Not determined. Total protein and total HSV1-TK units (picomoles of thymidine 5'-monophosphate synthesized per min) refer to total thyroid extracts.

TABLE 3. Effect of DHPG treatment on levels of circulating T<sub>4</sub> in control and TG66.19 transgenic female mice

Transgenic status	Treatment	T <sub>4</sub> (nmol/liter)		n
		Before	After	
Transgenic	PBS	40.2 ± 11.2	25.7 ± 9.0	5
	DHPG	52.1 ± 3.5	0.0	5
Control	PBS	44	24	1
	DHPG	45.5 ± 22.9	28.3 ± 15.0	3
	None	45.8 ± 19.0		20

Values are the mean and SD. PBS and DHPG, Implanted with minipumps containing saline and DHPG, respectively. n, Number of estimates. Untreated controls (Control, None), Five mice were sampled on four occasions. Before, Before treatment; After, after treatment.

controls. As would be expected, administration of GH during the second week of treatment had no effect on circulating T<sub>4</sub> or T<sub>3</sub> levels.

When nontransgenic controls were treated with DHPG in the same way, the level of T<sub>4</sub> fell by about 40%, on the average (Table 3), and the T<sub>3</sub> level also fell (Table 2). This effect seems to be due to surgery or to the effect of carrying the minipumps, since transgenic and control mice treated with saline showed a similar depression of circulating T<sub>4</sub> (Table 3). The levels of T<sub>4</sub> and T<sub>3</sub> in untreated control mice sampled at the same time of day on different days were also quite variable (see T<sub>4</sub> values, Table 3). However in these mice, which were not implanted with minipumps, the variance between samples and within mice was similar to the variance between mice, signifying that differences in T<sub>4</sub> (and T<sub>3</sub>) levels did not follow any obvious pattern. This contrasts with the consistent difference observed before and after minipump implantation.

The thyroid glands of DHPG-treated transgenic mice were greatly reduced in size (Fig. 2), and this was reflected in the yield of protein obtained from them; the total soluble protein obtained from treated transgenic thyroids was less than 10% that of untreated transgenic or DHPG-treated nontransgenic mice (Table 2). We experienced difficulty in dissecting out the regressed thyroids cleanly, and these values probably overestimate the residual thyroid protein. The total HSV1-TK activity in the thyroid glands of untreated line TG66.19 mice ranged between 6 and 17.5 U/thyroid. Any thyrocytes not destroyed by DHPG treatment may be expected to contain HSV1-TK, and we provisionally take the level of HSV1-TK activity as a measure of the success of DHPG ablation. HSV1-TK activity varied between 0.03 and 0.01 U/thyroid in the residual thyroid tissue from DHPG-treated transgenic animals (Table 2). Since the sensitivity limit of the HSV1-TK assay is below 0.01 U/thyroid, the results suggest that the administration of DHPG for 14 days destroyed 99.5–99.8% of follicle cells.

The destruction of follicle cells was confirmed by examining stained sections of thyroid glands. Thyroids from nontransgenic mice and untreated line TG66.19 mice contain typical thyroid follicles filled with pink-staining thyroglobulin-containing colloid and surrounded by cuboidal follicle cells with large nuclei (Fig. 3, A, B, D, and E). In the thyroids of transgenic mice treated with DHPG, no follicles with included colloid were present after 3 days. From day 7 onward, no follicle cells could be recognized (data not shown). Figure 3, C and F, show a typical thyroid section made after 14 days of treatment. Some of the sparsely distributed cells with moderately sized nuclei can be identified as C-cells by specific immunostaining, but the origin of the others is uncertain.

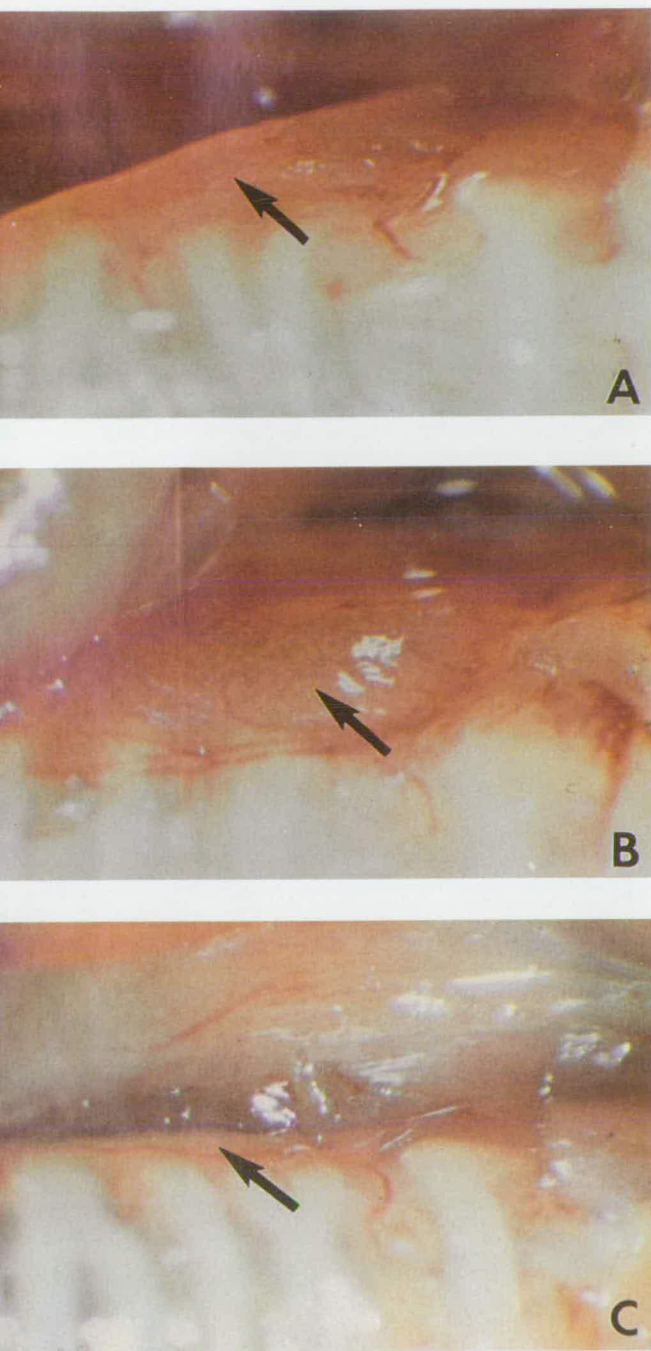


FIG. 2. Thyroid glands of normal female (A), untreated transgenic female (B), and transgenic female treated with 4.5 mg/day DHPG for 14 days (C).

At this time, the glands contained structures that resembled empty follicle residues. However, these appeared *de novo* between days 7–14, that is after the initial disappearance of the follicles and of typical follicle cells. They are, therefore, unlikely to be follicles. The flattened cells with condensed nuclei that surround the margins of the empty structures (Fig. 3, C and F) may be endothelial

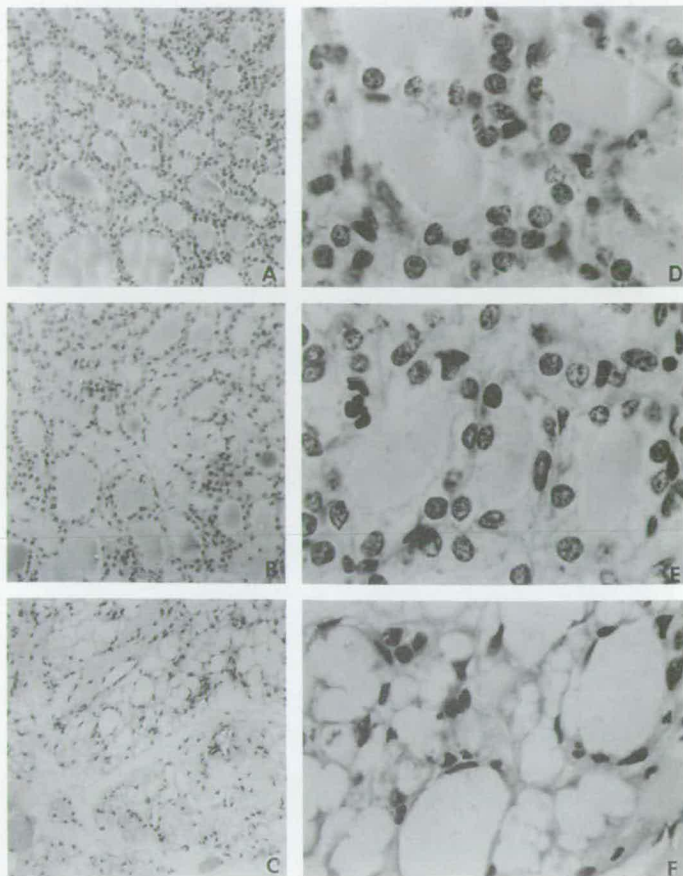


FIG. 3. Thyroid follicles of normal females (A and D), untreated transgenic females (B and E), and transgenic females treated for 14 days with DHPG (C and F). Magnifications: A–C,  $\times 100$ ; D–F,  $\times 400$ .

cells. Endothelial cells associated with thyroid adipose tissue are known to multiply during  $T_4$  deprivation (27). Whatever the origin of the residual cells, they are present in very much lower numbers than the follicle cells in a euthyroid animal. Thus, the physical destruction of follicle cells is the main and probably the sole cause of the loss of circulating  $T_4$  and  $T_3$ .

#### *Levels of PTH and CT are maintained in DHPG-treated line TG66.19 mice*

The parathyroid gland is closely associated with the thyroid. In sections of thyroid rudiments from ablated animals, the parathyroids appeared to be unaffected (Fig. 4). In agreement with this observation, no significant change in the level of PTH was observed in either transgenic or nontransgenic animals treated with DHPG (Table 4). The production and secretion of PTH clearly do not depend upon circulating  $T_4$  or  $T_3$ . Furthermore, the products of DHPG phosphorylation or any other putative agents of ablation had not passed to the parathyroid in amounts sufficient to impair PTH secretion.

The C-cells lie in localized clusters between the thyroid follicles. In the DHPG-treated transgenic mice, the level

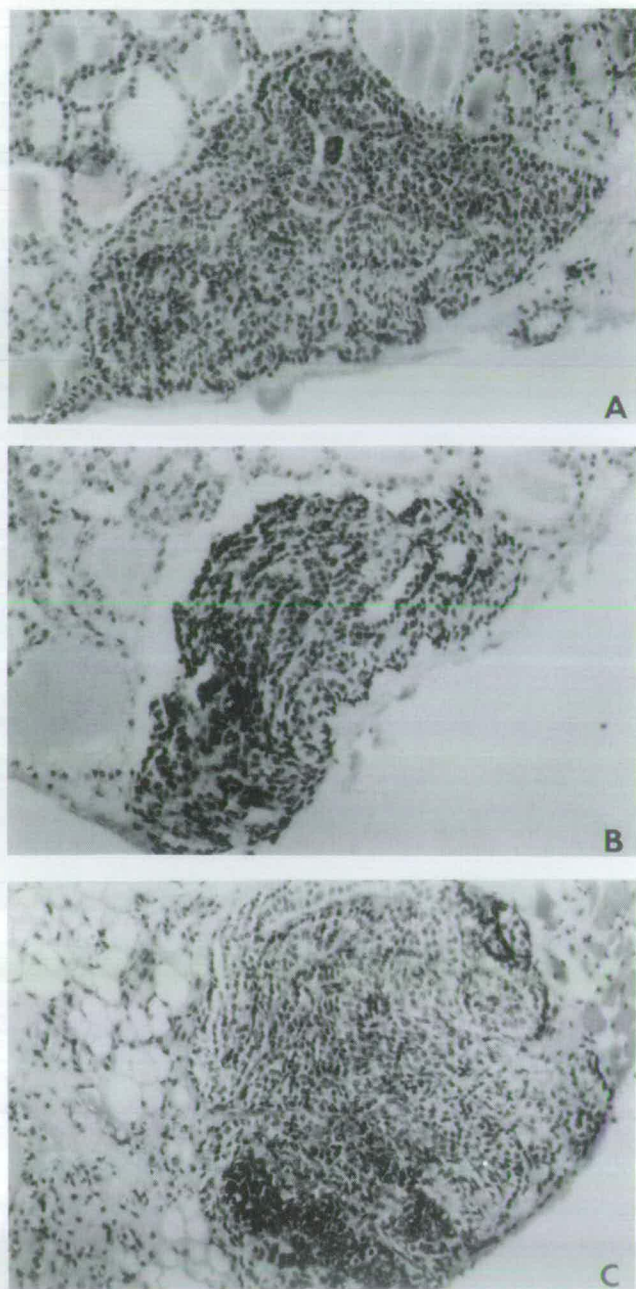


FIG. 4. Parathyroid glands of normal females (A), untreated transgenic females (B), and transgenic females treated for 14 days with DHPG (C).

of CT was effectively unchanged (Table 5). A thyroidectomized control mouse showed no circulating CT. In view of the overall structural changes that must accompany the shrinkage of the gland, it is perhaps surprising that CT levels did not fall during ablation. The results are consistent with the identification of apparently unaffected C-cells in transgenic thyroid rudiments after 14 days of DHPG treatment (see above) and suggest that there is little or no transfer of phosphorylated DHPG between follicle cells and C-cells across the epithelial

TABLE 4. Effect of DHPG treatment on levels of circulating  $T_4$  and PTH in control and TG66.19 transgenic female mice

Transgenic status	Treatment	$T_4$ (nmol/liter)		PTH (nmol/liter)	
		Before	After	Before	After
Transgenic	PBS	44.1	39.5	54	49
		44.9	15.8	ND	36
	DHPG	29.8	0.0	71	55
		51.4	0.0	ND	48
Control	DHPG	21.0	10.8	63	84

PBS and DHPG, Implanted with minipumps containing saline or DHPG, respectively. ND, Not determined. Before, before treatment; After, after treatment.

TABLE 5. Effect of DHPG treatment on levels of circulating  $T_4$  and CT in control and TG66.19 transgenic female mice

Transgenic status	Treatment		$T_4$ (nmol/liter)		CT (ng/liter)	
			Before	After	Before	After
Transgenic	PBS	P	20.3	20.6	36	29
		P	47.5	28.5	25	28
	DHPG	P	48.3	0.0	24	26
		P	57.7	0.0	30	27
Control	DHPG	P	49.1	33.6	42	39
Thyroidectomized	None		56.5	8.1	27	0

PBS and DHPG, Implanted with minipumps containing saline or DHPG, respectively. P, Pooled samples (two mice). Before, Before treatment; After, after treatment.

basement membrane.

The thyroidectomized mouse showed a residual 1% level of  $T_4$  (Table 5). This may be due to incomplete thyroidectomy. We have observed that the C-cells are localized within the thyroid, so that in principle, most of the C-cells might be removed without complete removal of follicle cells. The objective of the thyroidectomy was to generate a CT-negative mouse to validate the  $T_4$  assay.

#### *Expression of Mup genes in the liver as an indicator of reduced level of circulating $T_4$*

Transcription of the mouse major urinary protein (MUP) genes in the liver is dependent on both  $T_4$  and GH (21). The genes are expressed at much higher levels in males than in females, and this sex difference is mediated by the different temporal patterns of circulating GH in the two sexes (28). The pulsatile male pattern can be mimicked by administering GH according to a regular injection regimen.

One of the many effects of  $T_4$  is to stimulate transcription of the GH gene (29, 30). Since  $T_4$  deprivation leads

marked decline in GH synthesis, thyroidectomized mice lack both thyroid hormones and GH (31, 32). Consequently,  $T_4$  deprivation influences *Mup* expression not only directly, but also via GH. The administration of  $T_4$  could restore circulating GH, while the administration of GH does not restore circulating  $T_4$  in the absence of functional thyroid (Table 2). The administration of  $T_4$  to thyroidectomized mice was reported to increase *Mup* expression, although not to maximal levels, and it is therefore suggested that the  $T_4$  requirement is not absolute, but rather that GH and  $T_4$  act synergistically. However, an alternative interpretation would be that the requirement for  $T_4$  is absolute, but that the mice have a residual low level of  $T_4$  due to incomplete thyroidectomy. We therefore reexamined the regulation of *Mup* expression by  $T_4$  and GH in transgenically ablated TG66.19 mice. Since the MUPs (proteins) are secreted into the plasma and rapidly excreted into the urine, we initially estimated *Mup* expression from the level of urinary MUP.

After 14 days of DHPG treatment the level of MUP in the urine of TG66.19 mice was very low (Fig. 5, lanes 3 and 4). The levels of MUP were normal in urine from control transgenic mice given saline by minipump and in control nontransgenic mice treated with DHPG (Fig. 5, lanes 1, 2, 11, and 12). When  $T_4$  was administered to the TG66.19 mice during the second week of DHPG treatment, the thyroid follicle cells were again ablated, as shown by the low protein and zero HSV1-TK content of the residual thyroid tissue (Table 2). However, the serum contained near-normal levels of both  $T_4$  and  $T_3$  (Table 2) and the urine contained normal levels of MUP (Fig. 5, lanes 9 and 10). Thus,  $T_4$  administration completely reversed the suppression of *Mup* expression by thyroid

ablation.

The administration of GH during the second week of DHPG treatment did not inhibit ablation of thyroid follicle cells; the level of protein recovered from the residual thyroid tissue was low, the thyroid contained no HSV1-TK activity, and  $T_4$  and  $T_3$  were undetectable in the plasma (Table 2). Unlike  $T_4$  treatment, GH administration did not increase the level of urinary MUP above the very low level brought about by thyroid ablation (Fig. 5, lanes 7 and 8). In an experiment to confirm its biological activity, treatment of normal female mice with the same GH preparation and according to the same protocol raised MUP excretion to male levels (data not shown). Thus, biologically active GH did not induce *Mup* expression in the absence of  $T_4$ . This result demonstrates for the first time that  $T_4$  is absolutely required for *Mup* expression.

The levels of urinary MUP accurately reflected the  $T_4$  status of the control and experimental animals. The levels of *Mup* mRNA in liver correlated closely with the levels of urinary MUP (data not shown). Thus, the urinary MUP level could be employed as a noninterventionist indicator of the success of thyroid ablation.

#### *Lack of thyroid regeneration after withdrawal of DHPG*

In the transgenic ablation of lymphocytes (10) and somatotrophs (3), repopulation of the tissue was observed after withdrawal of the antiherpetic agent. On the contrary, when DHPG was withdrawn from treated TG66.19 mice, there was no recovery of circulating  $T_3$  or  $T_4$  and no evident reappearance of thyroid tissue over a period of 90 days (data not shown). Thus, it seems that young adult mice do not contain a nondifferentiated stem cell population capable of regenerating the follicle cells, and this is consistent with the view that when follicle cell numbers increase as a result of disease, the increase occurs through slow division of the follicle cells themselves. We cannot exclude alternative explanations at this time, for example the existence of a stem cell population that expresses HSV1-TK (in the transgenic mice) and is consequently sensitive to DHPG, but such explanations are less likely.

#### *Effect of $T_4$ deprivation on body weight*

Thyroid deficiency can be induced by treatment with methimazole (MMI) or propylthiouracil (PTU), which prevent the iodination of thyroglobulin by inhibiting the oxidation of iodide (33). Successful treatment of young rodents results in the absence of normal weight gain. The effect of DHPG ablation was to reduce, rather than abolish, weight gain in 8-week-old mice (Fig. 6). During the first 2 weeks of treatment, weight gain did not differ from that of control mice infused with saline. Thereafter, however, the weight gain of the treated mice fell behind



Fig. 5. Isoelectric focusing gel of mouse urine samples collected before and after DHPG or saline treatments supplemented by injection of GH and  $T_4$ . Odd-numbered lanes, Before treatment; even-numbered lanes, after treatment. Lanes 1-10, TG66.19 transgenic female mice as follows: lanes 1 and 2, saline infusion; lanes 3 and 4, DHPG infusion; lanes 5 and 6, DHPG infusion, injection with  $T_4$  diluent; lanes 7 and 8, DHPG infusion, injection with GH; lanes 9 and 10, DHPG infusion, injection with  $T_4$ ; lanes 11 and 12, nontransgenic control infused with DHPG. Variation in the MUP pattern between individual mice is due to heterozygosity at the *Mup* locus in the C57/BL  $\times$  CBA cross.

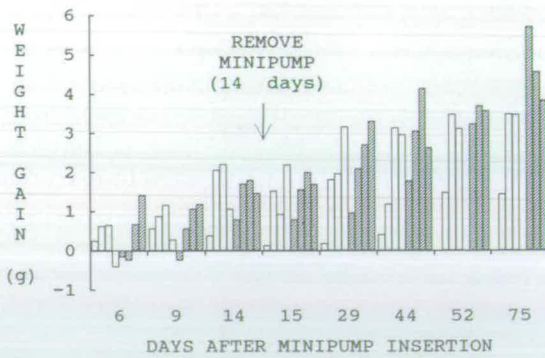


FIG. 6. Weight gain in transgenic females during and after DHPG treatment. Data were gathered from eight mice, implanted at 8 weeks of age, two of which were killed on day 44. Open and shaded columns show mice implanted with minipumps containing DHPG and saline, respectively.

that of the controls. Shivering, which frequently follows MMI treatment, was not observed after DHPG treatment. These observations suggest that the complete lack of weight gain observed after MMI or PTU treatment may result from the inhibition of oxidation reactions other than the oxidation of iodide (34–39).

$T_3$  has a profound stimulatory effect on the expression of the GH gene (29, 30, 40–42). GH deprivation reduces growth by about 50% up to at least 8.5 weeks after birth (2, 43, 44), due partly to GH deficiency and partly to consequential deficiency of insulin-like growth factor-I (44). Thus, GH and insulin-like growth factor-I deficiency probably contribute to the slower growth observed in the ablated mice and may account for it entirely.

### Discussion

Up to the present there has been no satisfactory animal model with which to explore the consequences of complete thyroid deprivation. Surgical removal of the thyroid results in loss of the parathyroid and thyroid C-cells as well as the follicle cells. Drugs such as MMI and PTU inhibit  $T_4$  production inconsistently and often have side-effects (34–39), while the genetically hypothyroid mouse suffers from secondary defects due to  $T_4$  deficiency during development (45, 46) and retains a significant level (10%) of circulating  $T_4$  (47). Although the administration of  $^{131}I$  is commonly used to ablate the thyroid gland in rats, it is seldom administered to mice and only in conjunction with thyroidectomy. This may be because in the mouse, minimal thyroid-lethal doses of radioiodine cause damage to nearby organs (tracheae and thymus). The same minimal doses also affect remote organs, causing a very high incidence of pituitary tumors and female sterilization (48), presumably because the administration of radioiodine must inevitably result in its delivery to the chromosomes of cells that carry thyroid receptors.

Our results demonstrate the complete ablation of thy-

roid follicle cells in a line of transgenic mice. The levels of circulating  $T_3$  and  $T_4$  fell below the limits of detection during 14 days of treatment with DHPG by minipump. Total thyroid protein fell by 90–95%, and the level of thyroid transgenic HSV1-TK activity fell by at least 99.5%. At the same time, there was no effect on circulating levels of PTH and CT. Ablation was, thus, autoradiographically demonstrable in the cells that express HSV1-TK, with the implication that follicle cells and C-cells do not communicate directly or indirectly across the epithelial basement membrane.

The experiments were carried out with adult mice that had presumptively normal thyroids up to the time that DHPG was administered. Consequently, they should not have incurred defects induced by  $T_4$  deprivation during development (45, 46). In contrast to the use of inhibitors of iodination, the ability to synthesize  $T_4$  was not recovered when DHPG treatment was terminated. No regeneration of follicle cells or recovery of circulating  $T_3$  or  $T_4$  was observed up to at least 90 days after removal of the minipumps. In addition, the mice showed no evidence of side-effects, such as weight loss, which might accompany treatment with MMI or PTU. During the 75 days after minipump removal that have so far been examined, the mice gained weight at about half the rate of controls and showed no distress. Any trauma due to minipump implantation and replacement may be assumed to have subsided. Thus, the postoperative mice provide an ideal model of total  $T_3$  and  $T_4$  deprivation against a background of normal development.

It is to be expected that the health of the thyroid-ablated mice will deteriorate in the longer term. Thyroid deprivation is known to lead to a large increase in circulating TSH (49, 50) and a large decrease in GH (34, 32) and to affect positively or negatively the transcription of various genes in the liver and myocardium (51–56). A longer term assessment of the mice is now required.

At 18 weeks of age, the thyroid gland is essentially nondividing tissue, and mitotic figures are extremely rare in thyroid follicle cells of normal mice (11). The successful ablation of the follicle cells, therefore, calls in question the assumption generally made that phosphorylated DHPG kills cells exclusively by being incorporated into DNA and preventing further extension of the polynucleotide chain. Since DHPG is an analog of guanosine and different guanosine phosphate derivatives play several key roles in cellular metabolism, it is possible that more than one mechanism comes into play. Our preliminary results indicate that the rate of mitosis is not elevated in the follicle cells of untreated TG66.19 mice. The frequency of mitosis seems to increase during the first 2 days of DHPG treatment, but not sufficiently to account for the approximately 50% death of follicle cells estimated to occur during that time. Experiments are

gress to further evaluate the involvement of cell division in the ablation of follicle cells.

### Acknowledgments

We thank Melville Richardson, Gary Brown, Mark Lawson, Ann McIlroy, Ann Walker, Frank Johnstone, Colin Nicolson, Armand Sanchez for assistance, Syntex for providing pUC, and the U.S. National Hormone and Pituitary Agency for support.

### References

- Palmiter RD, Behringer RR, Quaife JC, Maxwell F, Maxwell IH, Brinster RL 1987 Cell lineage ablation in transgenic mice by cell-specific expression of a toxin gene. *Cell* 50:435-443
- Behringer RR, Mathews LS, Palmiter RD, Brinster RL 1988 Dwarf mice produced by genetic ablation of growth-hormone-expressing cells. *Genes Dev* 2:453-461
- Borrelli E, Heyman RA, Arias C, Sawchenko PE, Evans RM 1989 Transgenic mice with inducible dwarfism. *Nature* 339:538-541
- Cheng YC, Huang ES, Lin JC, Mar EC, Pagano JS, Dutschman GE, Grill SP 1983 Unique spectrum of activity of 9-[(1,3-dihydroxy-2-propoxy)methyl]-guanine against herpesviruses *in vitro* and its mode of action against herpes simplex virus type 1. *Proc Natl Acad Sci USA* 80:2767-2770
- Martin JC, Dvorak CA, Smeets DF, Matthews TR, Verheyden JP 1983 9-[(1,3-Dihydroxy-2-propoxy)methyl]guanine: a new potent and selective antiherpes agent. *J Med Chem* 26:759-761
- Al-Shawi R, Burke J, Jones CT, Simons JP, Bishop JO 1988 A *Mu*p promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol Cell Biol* 8:4821-4828
- Braun RE, Lo D, Pinkert CA, Widera G, Flavell RA, Palmiter RD, Brinster RL 1990 Infertility in male transgenic mice: disruption of sperm development by HSV-tk expression in postmeiotic germ cells. *Biol Reprod* 43:684-693
- Al-Shawi R, Burke J, Wallace H, Jones C, Harrison S, Buxton D, Maley S, Chandley A, Bishop JO 1991 The herpes simplex type 1 virus thymidine kinase is expressed in the testis of transgenic mice under the control of an internal promoter. *Mol Cell Biol* 11:4207-4216
- Borrelli E, Heyman R, Hsi M, Evans RM 1988 Targeting of an inducible phenotype to mammalian cells. *Proc Natl Acad Sci USA* 85:7572-7576
- Heyman RA, Borrelli E, Lesley J, Anderson D, Richman DD, Baird SM, Hyman R, Evans RM 1989 Thymidine kinase obliteration: creation of transgenic mice with controlled immune deficiency. *Proc Natl Acad Sci USA* 86:2698-2702
- Wollman SH, Andros G, Cannon GB, Eagleton GB 1968 Production and involution of the hyperplastic thyroid gland. In: Young S, Inman DR (eds) *Thyroid Neoplasia*. Academic Press, New York, pp 201-209
- Santler JE 1957 Growth in the cell populations of the thyroid gland of rats treated with thiouracil. *J Endocrinol* 15:151-161
- Dumont JE, Vassart G, Refetoff S 1989 Thyroid disorders. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) *The Metabolic Basis of Inherited Disease*. McGraw-Hill, New York, pp 1843-1880
- de Martynoff G, Pohl V, Mercken L, van Ommen GJ, Vassart G 1987 Structural organization of the bovine thyroglobulin gene and of its 5'-flanking region. *Eur J Biochem* 164:591-599
- Mulligan RC, Berg P 1980 Expression of a bacterial gene in mammalian cells. *Science* 209:1422-1427
- Lathe R, Vilotte JL, Clark AJ 1987 Plasmid and bacteriophage vectors for excision of intact inserts. *Gene* 57:193-201
- Vieira J, Messing J 1982 The pUC plasmids, an M13 mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* 19:259-268
- Wilkie NM, Clements JB, Boll W, Mantei N, Weissmann C 1979 Hybrid plasmids containing an active thymidine kinase gene of herpes simplex virus 1. *Nucleic Acids Res* 7:859-877
19. Kiény MP, Lathe R, Lecocq JP 1983 New versatile cloning and sequencing vectors based on bacteriophage M13. *Gene* 26:91-99
20. Seed B 1983 Purification of genomic sequences from bacteriophage libraries by recombination and selection *in vivo*. *Nucleic Acids Res* 11:2427-2445
21. Knopf JL, Gallagher JF, Held WA 1983 Differential multihormone regulation of the mouse urinary protein gene family in the liver. *Mol Cell Biol* 3:2232-2240
22. Whitehead TP, Thorpe GHG, Carter TJN, Groucutt C, Kricka LJ 1983 Enhanced luminescence procedure for sensitive determination of peroxidase-labelled conjugates in immunoassay. *Nature* 305:158-159
23. Brinster RL, Chen HY, Trumbauer M, Senear AW, Warren R, Palmiter RD 1981 Somatic expression of herpes thymidine kinase in mice following injection of a fusion gene into eggs. *Cell* 27:223-231
24. Jamieson AT, Subak-Sharpe JH 1974 Biochemical studies on the herpes simplex virus-specified deoxythymidine kinase activity. *J Gen Virol* 24:481-492
25. Bradford M 1976 A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248-254
26. Ledent C, Parmentier M, Vassart G 1990 Tissue-specific expression and methylation of a thyroglobulin-chloramphenicol acetyltransferase fusion gene in transgenic mice. *Proc Natl Acad Sci USA* 87:6176-6180
27. Smeds S, Wollman SH 1983 Capillary endothelial cell multiplication in adipose tissue pads on the thyroid during the feeding of thiouracil. *Endocrinology* 112:1718-1722
28. Norstedt G, Palmiter R 1984 Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* 36:805-812
29. Evans RM, Birnberg NC, Rosenfeld MG 1982 Glucocorticoid and thyroid hormones transcriptionally regulate growth hormone gene expression. *Proc Natl Acad Sci USA* 79:7659-7663
30. Spindler SR, Mellon SH, Baxter JD 1982 Growth hormone gene transcription is regulated by thyroid and glucocorticoid hormones in cultured rat pituitary cells. *J Biol Chem* 257:11627-11634
31. Hervas F, Morreale de Escobar G, Escobar del Rey F 1975 Rapid effects of single small doses of L-thyroxine and triiodo-L-thyronine on growth hormone, as studied in the rat by radioimmunoassay. *Endocrinology* 97:91-101
32. Martial JA, Seeburg PH, Guenzi D, Goodman HM, Baxter JD 1977 Regulation of growth hormone gene expression: synergistic effects of thyroid and glucocorticoid hormones. *Proc Natl Acad Sci USA* 74:4293-4295
33. DeGroot LJ, Davis AM 1962 Studies on the biosynthesis of iodotyrosines: a soluble thyroidal iodide-peroxidase tyrosine-iodinase system. *Endocrinology* 70:492-510
34. Horvath T, Past T, Nemeth A, Kadas I, Hoffmann Traeger A, Rechenbach C, Javor T 1989 Drug metabolism in drug-induced liver diseases: pathogenetic role of active metabolites. *Acta Physiol Hung* 73:293-304
35. Rutgers M, Heusdens FA, Bonthuis F, Rooda SJ, Visser TJ 1990 Identification of 3,3',5-triiodothyroacetic acid sulfate: a major metabolite of 3,3',5-triiodothyronine in propylthiouracil-treated rats. *Endocrinology* 127:1617-1624
36. Yamamoto T, Pierce WM, Hurst HE, Chen D, Waddell WJ 1990 Ethyl carbamate metabolism: *in vivo* inhibitors and *in vitro* enzymatic systems. *Drug Metab Dispos* 18:276-280
37. Chiba K, Horii H, Kubota E, Ishizaki T, Kato Y 1990 Effects of N-methylmercaptoimidazole on the disposition of MPTP and its metabolites in mice. *Eur J Pharmacol* 180:59-67
38. Wilson R, McKillop JH, Travers M, Smith J, Smith E, Thomson JA 1990 The effects of antithyroid drugs on intercellular mediators. *Acta Endocrinol (Copenh)* 122:605-609
39. Kirkland JL 1990 Propylthiouracil-induced hepatic failure and encephalopathy in a child. *DICP* 24:470-471
40. Flug F, Copp RP, Casanova J, Horowitz ZD, Janocko L, Plotnick M, Samuels HH 1987 *Cis*-acting elements of the rat growth hormone gene which mediate basal and regulated expression by thyroid

- hormone. *J Biol Chem* 262:6373-6382
41. Glass CK, Franco R, Weinberger C, Albert VR, Evans RM, Rosenfeld MG 1987 A *c-erb-A* binding site in rat growth hormone gene mediates *trans*-activation by thyroid hormone. *Nature* 329:738-741
  42. Sap J, de Magistris L, Stunnenberg H, Vennstrom B 1990 A major thyroid hormone response element in the third intron of the rat growth hormone gene. *EMBO J* 9:887-896
  43. Beamer WH, Eicher EM 1976 Stimulation of growth in the little mouse. *J Endocrinol* 71:37-45
  44. Behringer RR, Lewin TM, Quaife CJ, Palmiter RD, Brinster RL, D'Ercole AJ 1990 Expression of insulin-like growth factor I stimulates normal somatic growth in growth hormone-deficient transgenic mice. *Endocrinology* 127:1033-1040
  45. Noguchi T, Sugasaki T 1984 Hypomyelination in the cerebrum of the congenitally hypothyroid mouse. *J Neurochem* 42:891-893
  46. Adams PM, Stein SA, Palnitkar M, Anthony A, Gerrity L, Shanklin DR 1989 Evaluation and characterization of the hypothyroid *hyt/hyt* mouse. I. Somatic and behavioural studies. *Neuroendocrinology* 49:138-143
  47. Stein SA, Shanklin DR, Krulich L, Roth MG, Chubb CM, Adams PM 1989 Evaluation and characterization of the *hyt/hyt* hypothyroid mouse. II. Abnormalities of TSH and the thyroid gland. *Neuroendocrinology* 49:509-519
  48. Gorbman A 1950 Functional and structural changes consequent to high dosages of radioactive iodine. *J Clin Endocrinol Metab* 10:1177-1191
  49. Bowers CY, Schally AV, Reynolds GA, Hawley WD 1967 Interactions of L-thyroxine or L-triiodothyronine and thyrotropin-releasing factor on the release and synthesis of thyrotropin from the anterior pituitary gland of mice. *Endocrinology* 81:741-747
  50. Francis CM 1972 Effect of graded doses of thyroxine on the plasma TSH levels in mice. *Ind J Physiol Pharmacol* 16:215-218
  51. Towle HC, Mariash CN, Schwartz HL, Oppenheimer JH 1971 Quantitation of rat liver messenger ribonucleic acid for malic enzyme during induction by thyroid hormone. *Biochemistry* 20:3486-3492
  52. Magnuson MA, Nikodem VM 1983 Molecular cloning of a cDNA sequence for rat malic enzyme. Direct evidence for induction *in vivo* of rat liver malic enzyme mRNA by thyroid hormone. *J Biol Chem* 258:12712-12717
  53. Sinha AM, Umeda PK, Kavinsky CJ, Rajamanickam C, Hsu J, Jakovcic S, Rabinowitz M 1982 Molecular cloning of mRNA sequences for cardiac alpha- and beta-form myosin heavy chain expression in ventricles of normal, hypothyroid, and thyrotoxic rabbits. *Proc Natl Acad Sci USA* 79:5847-5851
  54. Everett AW, Clark WA, Chizzonite RA, Zak R 1983 Change in synthesis rates of alpha- and beta-myosin heavy chains in rabbit heart after treatment with thyroid hormone. *J Biol Chem* 258:2425-2425
  55. Lompre AM, Nadal Ginard B, Mahdavi V 1984 Expression of cardiac ventricular alpha- and beta-myosin heavy chain genes developmentally and hormonally regulated. *J Biol Chem* 259:6446-6446
  56. Izumo S, Nadal Ginard B, Mahdavi V 1986 All members of the MHC multigene family respond to thyroid hormone in a highly tissue-specific manner. *Science* 231:597-600



# Sexual Dimorphism and Growth Hormone Regulation of a Hybrid Gene in Transgenic Mice

Raya Al-Shawi, Helen Wallace, Stephen Harrison, Cheryl Jones, Deborah Johnson, and John O. Bishop

Agricultural and Food Research Council Centre for Genome Research  
(R.A., S.H., D.J., J.O.B.)

Institute of Cell and Molecular Biology, Division of Biology (H.W., J.O.B.)

University of Edinburgh  
Edinburgh, EH9 3JQ, EH9 3JN, United Kingdom

Medical Research Council Human Genetics Unit (C.J.)  
Western General Hospital  
Edinburgh, EH4 2XV, United Kingdom

Department of Biological Sciences (J.O.B.)  
University of Maryland, Baltimore County  
Baltimore, Maryland 21228

The sexually dimorphic expression of the urinary protein genes of mice (*Mup* genes) in the liver is mediated by the different male and female temporal patterns of circulating GH. Normal females were induced to male levels when GH was administered by injection to mimic the male GH pattern, showing that expression at the male level does not require a male sex steroid status in addition to intermittent GH. Two *Mup*- $\alpha_{2u}$ -globulin hybrid transgenes with different *Mup* gene promoters showed sexually dimorphic expression, and their expression in females increased to male levels upon testosterone treatment. GH-deficient (*lit/lit*) mice did not express these transgenes, and GH-deficient females did not respond to testosterone treatment, showing that GH was required for induction. Both normal and GH-deficient females were induced to male levels when GH was administered by injection. This is the first report of a transgene responsive to GH. A transgene consisting of a *Mup* promoter fused to a Herpes simplex virus thymidine kinase reporter sequence also showed sexual dimorphism, although to a lesser degree. It was expressed at the same level in normal females and GH-deficient mice of both sexes and was induced when GH-deficient mice were treated with GH. We propose that this transgene has a basal constitutive expression, possibly due to the absence of any rodent DNA downstream of the promoter. Since expression of the transgene was significantly induced by GH, the GH response

is due at least in part to sequences in the promoter region. (*Molecular Endocrinology* 6: 181-190, 1992)

## INTRODUCTION

The level of circulating GH changes with time. In rats the GH profile is quite different in the two sexes. Males are characterized by a low or zero basal level punctuated by high-level spikes which occur at fairly regular intervals, while in females the basal level is higher and the spikes are more frequent and less pronounced (1, 2). We will refer to these as male and female GH patterns. The different GH patterns are believed to result from different patterns of secretion (3). The synthesis of GH is regulated, among other factors, by hydrocortisone and thyroxine (4). However, its secretion is mainly directed by the action of GRF and somatostatin on the pituitary somatotrophs (5). The GRF and somatostatin which impinge on the somatotrophs are secreted by hypothalamic neurones. Consequently it is believed that the secretory pattern of these neurones is different in males and females, and that this dictates the male and female GH patterns. Like other secondary sexual characteristics, the pattern of GRF secretion by the hypothalamus in rats seems to be regulated by the sex steroids (6, 7).

Several genes are expressed differently in male and female liver, and it has been proposed that the male and female GH patterns are responsible for establishing and maintaining the sexual dimorphism of this tissue (8-10). Recently, induction of a male-specific form of rat liver cytochrome P450 was shown to depend cru-

cially upon the length of the troughs between the peaks of circulating GH (11). The mouse major urinary protein (MUP) genes (*Mup*) are among those that are expressed differently in the two sexes. MUP is encoded by a gene family, and the bulk of liver MUP synthesis is due to the expression of a subset of about 12 genes known as the group 1 genes (12, 13). Relative to total liver mRNA the level of *Mup* mRNA is 5–10 times higher in males than in females (14), and females can be induced to express male-like levels by treatment with testosterone (15). The different mRNA levels reflect different rates of transcription of the *Mup* genes in the two sexes (16, 17), and masculinization of females by treatment with testosterone increases transcription to the male rate (17). Several observations suggest that the effect of testosterone on MUP expression is not due to a direct effect on the liver, but rather to the fact that it induces the male GH pattern. The most persuasive evidence comes from studying the expression of the *Mup* genes in GH-deficient mutant mice. Mice homozygous for the recessive mutation *little* (*lit*) have a low level of circulating GH (18) and small body size, while the levels of other pituitary hormones are normal (19). The mutation gives rise to a lesion which makes the pituitary refractory to stimulation by GRF (20), and GH therapy restores normal growth (21, 22). Despite having a normal level of circulating testosterone (23), *lit/lit* mice express MUP at a very low level (10, 24), demonstrating a requirement for GH. The link with sexual dimorphism was established by administering GH to GH-deficient mice in different ways. When GH was administered by injection to mimic the male GH pattern, the normal male level of MUP expression was restored (10, 24). In contrast, GH did not induce male expression when administered by infusion to mimic the female GH pattern. Similarly, male mice with chronically high nonpulsatile levels of circulating GH expressed MUP at a lower level (10). These experiments leave open the possibility that, in addition to their GH-mediated effect, sex steroids also affect MUP expression by a direct effect on the liver. We have eliminated that possibility by showing that MUP expression is masculinized in normal female mice when they are treated with GH by injection.

An important step in elucidating a transcriptional control mechanism is to define the *cis*-acting DNA elements which mediate the process. No liver cell culture system in which *Mup* genes are expressed has yet been described, and there is no recognized method for simulating GH pulsatility in cell culture systems. We therefore performed experiments with transgenic mice as a first step to identifying *cis*-acting DNA sequences that respond to different GH patterns. *Mup* hybrid genes were introduced into the mouse genome, and normal and GH-deficient (*lit/lit*) transgenic mice were generated by matings. The results show that expression of the transgenes is extremely sensitive to the GH status of the animals and that full induction of the transgenes is dependent on a male pattern of circulating GH.

## RESULTS

### Tissue-Specific Expression of *Mup*- $\alpha_{2u}$ -Globulin Hybrid Genes in Transgenic Mice

The mouse *Mup* genes and the rat  $\alpha_{2u}$ -globulin genes are homologous. In order to produce a gene with RNA and protein products that can be distinguished from those of the endogenous mouse genes, we constructed two *Mup*- $\alpha_{2u}$ -globulin hybrid genes (Fig. 1; also see *Materials and Methods*). BS6- $\alpha_{207}$  is a group 1 *Mup* gene (BS6), with 2.2 kilobases (kb) of 5'-flanking sequence and 1.8 kb of 3'-flanking sequence, and with a region extending from a site in exon 2 to a site in exon 6 replaced by the homologous region of  $\alpha_{2u}$ -globulin gene  $\alpha_{207}$ . The second hybrid gene, BL1- $\alpha_{207}$ , differs from BS6- $\alpha_{207}$  only in that the promoter and 5'-flanking sequence of a second group 1 *Mup* gene (BL1) were substituted for the homologous BS6 region. The BS6- $\alpha_{207}$  and BL1- $\alpha_{207}$  hybrid genes were injected separately into the pronuclei of C57BL/6 embryos heterozygous for the *little* mutation (*lit/+*). Transgenic GH-deficient (*lit/lit*) and normal (*lit/+* or *+/+*) offspring were generated for study by mating *lit/+* transgenic mice with *lit/+* or *lit/lit* partners.

Transgenic lines were established and examined by Northern blot analysis of RNA extracted from eight tissues of normal males. In 9 out of 11 lines expression was observed in male liver (Fig. 2A) at levels between 10% and 110% (average 54%) of the level of expression of  $\alpha_{2u}$ -globulin mRNA in male rat liver (Table 1). Very low levels of expression were observed in the preputial glands of two lines (e.g. Fig 2A, lane 3), while in one

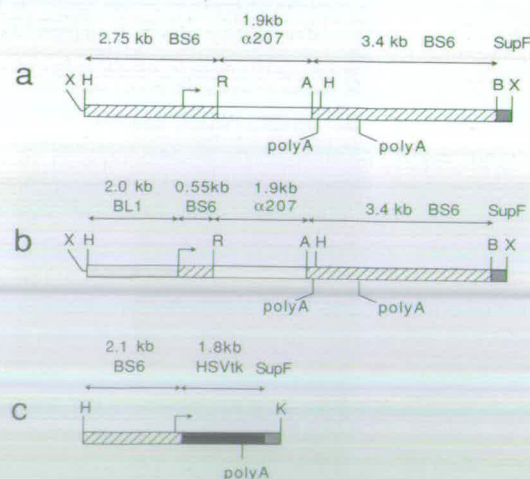
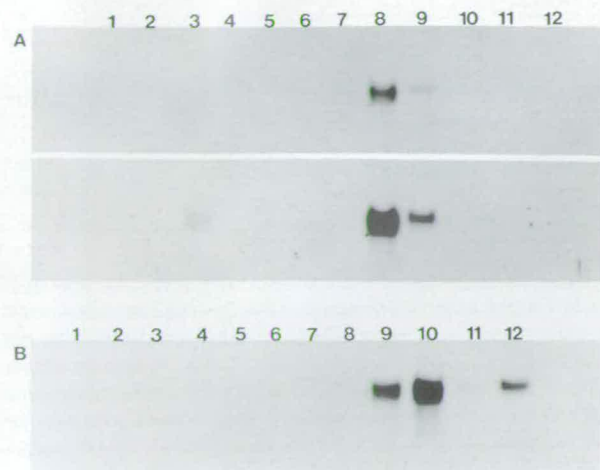


Fig. 1. Hybrid Genes

Genes were constructed as described in *Materials and Methods*. a, BS6- $\alpha_{207}$ ; b, BL1- $\alpha_{207}$ ; c, BS6-tk.  $\square$ , MUP gene BS6 sequence;  $\square$ , a, b,  $\alpha_{2u}$ -globulin gene  $\alpha_{207}$  sequence;  $\square$ , b, MUP gene BL1 promoter;  $\blacksquare$ , HSV-tk reporter;  $\blacksquare$ , bacterial SupF gene. The positions of the cap sites are indicated by arrows. The positions of polyA sites are also indicated. Key restriction sites are abbreviated as follows: X, XbaI; H, HindIII; R, EcoRI; A, Aval; K, KpnI.



**Fig. 2.** Tissue-Specific Expression of Hybrid Genes BS6- $\alpha$ 207 and BL1- $\alpha$ 207 in Male Transgenic Mice

Unless stated otherwise each lane contained 30  $\mu$ g total cellular RNA. Filters were probed with a labeled  $\alpha$ <sub>2u</sub>-globulin-specific oligonucleotide (see *Materials and Methods*). A, Line BS6-24 (BS6- $\alpha$ 207). The autoradiograph was exposed for 24 h (top) and 72 h (bottom). Lanes 1-8, Tissues from a line BS6-24 transgenic mouse: 1, lachrymal gland; 2, salivary gland; 3, preputial gland; 4, striated muscle; 5, brain; 6, testis; 7, kidney; 8, liver. Lane 9, Two micrograms of total adult male rat liver RNA. Lanes 10-12, Tissues from a nontransgenic mouse: 10, liver; 11, testis; 12, preputial gland. B, Line BL1-39 (BL1- $\alpha$ 207). Lanes 1-10, Tissues from a line BL1-39 transgenic mouse: 1, lachrymal gland; 2, submaxillary gland; 3, parotid gland; 4, striated muscle; 5, preputial gland; 6, brain; 7, testis; 8, kidney; 9 and 10, liver (10  $\mu$ g and 30  $\mu$ g). Lanes 11 and 12, Total adult male rat liver RNA (0.5  $\mu$ g and 2  $\mu$ g).

line (BL1-5) the level of expression in the preputial gland was higher than in the liver. Since it occurred in only 1 out of 9 lines, high level expression in the preputial gland may be assumed to be due to mutation or rearrangement of the foreign DNA integrated in line BL1-5 or to a chromosomal position effect (25). No expression was observed in other tissues. Thus, to a large extent, expression of the transgenes was restricted to the liver.

In Northern blots of liver RNA from mice carrying either transgene two mRNAs were consistently observed, both close to 800 base pairs (bp) in length and in approximately equal amounts. A single major primer extension product was observed (Fig. 3B), initiated at the *Mup* cap site described previously (26). The 3' ends of the transgenes were derived from *Mup* gene BS6, and *Mup* gene transcripts are known to have at least three different splice configurations in the 3' noncoding region and also several alternative polyadenylation sites (27-29). Thus the two size classes of mRNA probably arise through differential processing of a single mRNA precursor.

#### Sexually Dimorphic Expression of the *Mup*- $\alpha$ <sub>2u</sub>-Globulin Hybrid Genes

In 8 of the 9 expressing lines (Table 1) normal females did not express the transgene (e.g. compare lanes 3

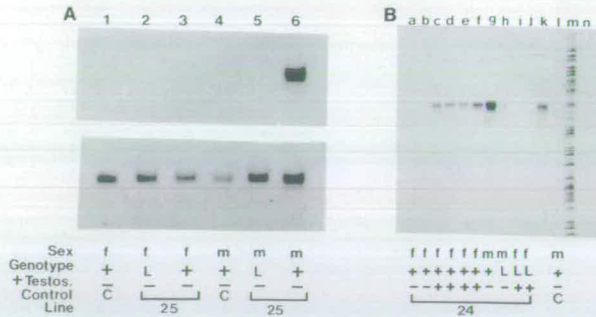
**Table 1.** Sexually Dimorphic Expression of BS6- $\alpha$ 207 and BL1- $\alpha$ 207 Hybrid Genes

Line	Relative expression level		
	Liver		Preputial gland (male)
	Male	Female	
BS6-3	0.2	0.0	ND
BS6-6	0.0	0.0	0.0
BS6-10	0.9	0.0	0.0
BS6-24	0.5	0.0	0.0*
BS6-25	0.7	0.0	0.0*
BS6-26	0.2	0.0	0.0
BS6-36	0.2	0.0	ND
BL1-5	1.1	0.0	2.4
BL1-35	0.1	0.0	0.0
BL1-39	1.0	0.4	0.0
BL1-41	0.0	0.0	0.0

Northern blots were probed with an  $\alpha$ <sub>2u</sub>-globulin-specific oligonucleotide (see *Materials and Methods*), except that in the case of BL1-39 the same oligonucleotide was used as primer in a primer extension assay. Autoradiographs were scanned densitometrically and related in each case to the signal obtained from the same quantity of adult rat liver RNA (taken as 1.0); 0.0\* indicates a faint band which could be detected by eye but was not registered by the densitometer; ND, not determined.

and 6 of Fig. 3A). Females of the ninth line (line BL1-39) expressed the transgene at a level lower than males of the same line (Table 1). The general result (6 out of 6 lines) is that expression of BS6- $\alpha$ 207 was extremely dimorphic, and that the data obtained with BL1- $\alpha$ 207 do not allow a distinction to be drawn between the two constructs. Hepatic *Mup* gene expression is on average 5-10 times higher in males than in females. However, different *Mup* genes show different degrees of sexually dimorphic expression (14, 15, 24), and the expression ratio of the BS6 gene in the two sexes is not known because its products cannot be distinguished from those of approximately 5 other identical or near-identical group 1 *Mup* genes. The rat  $\alpha$ <sub>2u</sub>-globulin genes are highly expressed in male but not at all in female liver (30). Thus it is not possible at this time to distinguish the contributions of mouse and rat sequences to the expression of the hybrid transgenes. This does not detract from the value of the hybrid genes as constructs which show a large response to GH as transgenes.

The effect of testosterone treatment on transgene expression was examined by Northern blot (not shown) and primer extension analysis (Fig. 3B) of hepatic RNA from line BS6-24. Testosterone treatment of normal females induced the expression of the transgene, and the mRNA had the normal cap site. The extent of induction was lower than that of normal males of the same line (Fig. 3B, lane g), even though the endogenous *Mup* genes were fully induced (data not shown). However, prolonged (5-week) treatment of female mice with testosterone induced male-like levels of hybrid gene expression (data not shown). The results show that, like the endogenous genes, the transgene is induced



**Fig. 3.** Sexually Dimorphic Expression, GH Dependency, and Testosterone Induction of Hybrid Gene BS6- $\alpha$ 207 in Liver of Transgenic Mice

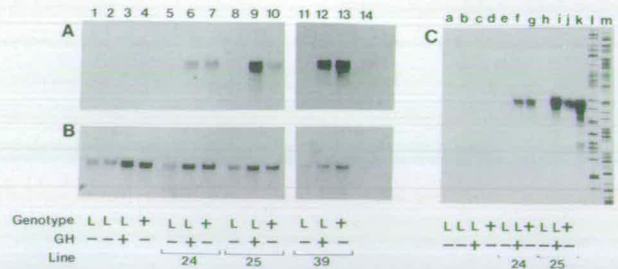
Sex: f, female; m, male. Genotype: +, *lit/+* or *+/+*; L, *lit/lit*. Mice treated or untreated with testosterone (Testos.): + and -, respectively. Nontransgenic control: C. A, Lack of expression of BS6- $\alpha$ 207 in the livers of females and GH-deficient males from line BS6-25. Northern blot analysis. Total liver RNA (30  $\mu$ g) was applied to each lane, and the filter was probed with the  $\alpha$ <sub>2u</sub>-globulin-specific oligonucleotide (*top*), stripped, and probed with an apolipoprotein-A1 cDNA clone (*bottom*). Lanes 1-3, Female: 1, nontransgenic; 2, GH-deficient transgenic; 3, normal transgenic. Lanes 4-6, Male: 4, nontransgenic; 5 GH-deficient transgenic; 6, normal transgenic. B, Effects of testosterone induction on expression of BS6- $\alpha$ 207 in normal and GH-deficient females. All transgenic mice were from line BS6-24. Primer extension analysis was carried out as described using 10  $\mu$ g total liver RNA in each case, except where stated otherwise. Lanes a-f, Normal females: a and b, untreated; c-f, individual females treated with testosterone. Lanes g and h, Male controls: g, normal male; h, GH-deficient male. Lanes i and j, GH-deficient females treated with testosterone. Lanes k, l, and n, Controls: k, male rat liver (1  $\mu$ g); l, nontransgenic male mouse; n, no RNA. Lane m, Sequencing ladder (C-track).

by testosterone, although there is a difference in the level of its response.

### GH Regulation of the *Mup*- $\alpha$ <sub>2u</sub>-Globulin Hybrid Genes

The *Mup* genes were expressed at very low levels in both GH-deficient males and GH-deficient females (see Fig. 5A, lanes a and k). Similarly, the level of BS6- $\alpha$ 207 and BL1- $\alpha$ 207 mRNA in GH-deficient males and females was usually below the limit of detection (Fig. 3A). mRNA was detected at a very low level in a few GH-deficient males, but the incidence of such males was not a feature of a particular transgenic line. Unlike normal females (with normal pituitary glands), GH-deficient females were not induced by testosterone (Fig. 3B, lanes i and j), consistent with the view that the effect of testosterone stimulation is mediated by GH.

To confirm the central role of GH in inducing the expression of the transgenes, GH-deficient males were treated with GH by injection. The treatment induced transgene mRNA to levels similar to those present in transgenic normal males (Fig. 4A, lanes 6, 9, and 12), and the mRNA invariably had the expected cap site



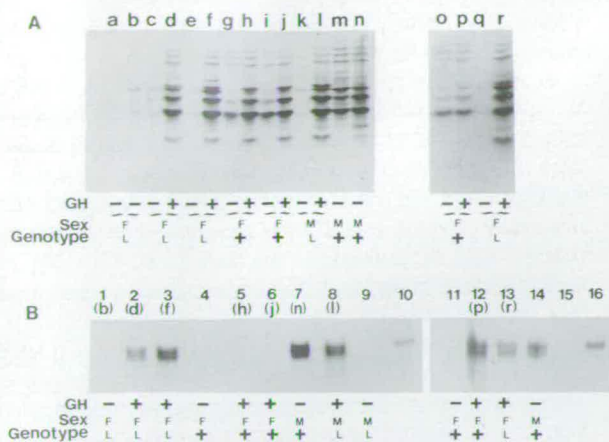
**Fig. 4.** Effect of GH on the Expression of BS6- $\alpha$ 207 and BL1- $\alpha$ 207 in the Liver of Transgenic Male GH-Deficient Mice

Genotype: +, *lit/+* or *+/+*; L, *lit/lit*. Mice treated or not treated with GH: + and -, respectively. A and B, Thirty micrograms of total liver RNA was applied to each lane, and filters were probed with the  $\alpha$ <sub>2u</sub>-globulin-specific oligonucleotide (A), stripped, and probed with an apolipoprotein-A1 cDNA clone (B). Lanes 1-4, Nontransgenic controls: 1, mock-injected; 2, GH-deficient, untreated; 3, GH-deficient, injected with GH; 4, normal, untreated. Lanes 5-7, Line BS6-24 (BS6- $\alpha$ 207): 5, GH-deficient, untreated; 6, GH-deficient, injected with GH; 7, normal, untreated. Lanes 8-10, Line BS6-25 (BS6- $\alpha$ 207): 8, GH-deficient, untreated; 9, GH-deficient, injected with GH; 10, normal, untreated. Lanes 11-13, Line BL1-39 (BL1- $\alpha$ 207): 11, GH-deficient, untreated; 12, GH-deficient, injected with GH; 13, normal, untreated. Lane 14, Male rat liver RNA (2  $\mu$ g). C, Primer extension analysis was carried out as described using 10  $\mu$ g total liver RNA in each case. Lane a, No RNA. Lanes b-d, Nontransgenic controls: b, GH-deficient mock-injected; c, GH-deficient injected with GH; d, normal, untreated. Lanes e-g, Transgenic mice, line BS6-24 (BS6- $\alpha$ 207): e, GH-deficient, mock injected; f, GH-deficient, injected with GH; g, normal, untreated. Lanes h-j, Transgenic mice, line BS6-25 (BS6- $\alpha$ 207): h, GH-deficient mock-injected; i, GH-deficient, injected with GH; j, normal, untreated. Lane k, Male rat liver. Lanes l and m, Sequencing tracks: l, A-track; m, C-track.

(Fig. 4C). These results demonstrate that, in common with the endogenous *Mup* genes, the hybrid transgenes contain sequences that respond to GH induction.

Normal females express the endogenous *Mup* genes at a higher level than GH-deficient females, indicating that the female GH pattern also induces the *Mup* genes. Males and females express individual *Mup* genes at different relative levels (14, 15, 24), suggesting that the inducing effects of the male and female GH patterns are different. When induced with testosterone, normal females express individual *Mup* genes at male levels (15), presumably because their pituitaries develop the male pattern of circulating GH. Isoelectric focusing (IEF) analysis of urinary proteins showed that the resident *Mup* genes were consistently induced by GH in GH-deficient females (Fig. 5A, lanes d and f). Thus the female liver can be masculinized by exposure to pulsatile GH.

In GH-deficient females there is no prior exposure to normal levels of GH and very little GH secretion, while normal females have prior exposure to the female GH pattern, and any injected GH supplements GH produced by ongoing pituitary secretion. Like GH-deficient females, normal females were induced to express the



**Fig. 5.** Effect of GH on the Expression of the BS6- $\alpha$ 207 Hybrid Gene in Liver of Female Transgenic Mice

Urine was collected from mice before and after treatment. RNA was extracted from the livers of the same mice after treatment. Sex: F, female; M, male. Genotype: +, *lit*/*+* or +/+; L, *lit*/*lit*. Mice treated or untreated with GH: + and -, respectively. A, Urinary MUPs resolved on an IEF gel and stained with Coomassie blue. Lanes a-j, Line BS6-24 females: a and b, GH-deficient, before and after mock injection; c and d, GH-deficient, before and after GH injection; e and f, same; g and h, normal, before and after GH injection; i and j, same. Lanes k-n, Male controls: k and l, GH-deficient, before and after GH injection; m and n, untreated normal males. Lanes o-r, Line BS6-26 females: o and p, normal, before and after GH injection; q and r, GH-deficient, before and after GH injection. The patterns shown are characteristic MUP patterns, identical to those observed with nontransgenic mice. The protein products of the hybrid genes cannot be visualized with Coomassie stain (see text). B, Northern blots of total liver RNA (30  $\mu$ g) probed with the  $\alpha$ 2u-globulin-specific oligonucleotide probe. Letters in parentheses indicate the corresponding MUP samples shown in A. Lanes 1-6, Line BS6-24 females: 1, GH-deficient, mock-injected; 2 and 3, GH-deficient, injected with GH; 4, normal, mock-injected; 5 and 6, normal, injected with GH. Lanes 7-9, Line BS6-24 male controls: 7, normal, untreated; 8, GH-deficient, injected with GH; 9, GH-deficient, untreated. Lane 10, Male rat liver RNA (2  $\mu$ g). Lanes 11-13, Line BS6-26 females: 11, normal; 12, normal, injected with GH; 13, GH-deficient, injected with GH. Lanes 14-16, Controls: 14, normal male; 15, nontransgenic normal male; 16, male rat liver RNA (2  $\mu$ g).

*Mup* genes at male levels by GH (Fig. 5A, lanes h and j). Thus, GH administered by injection to normal females overcomes any effects due to prior exposure and either overrides the female GH pattern or entrains the secretion of GH, or both.

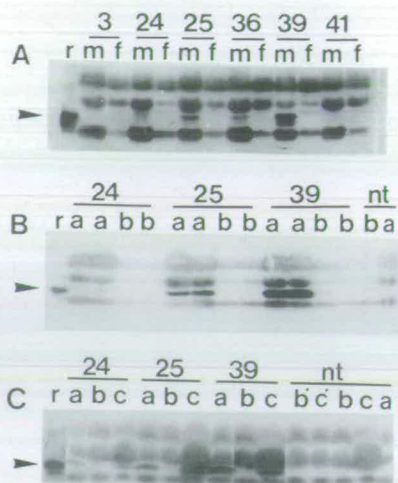
The response of the BS6- $\alpha$ 207 gene to GH injection was investigated in females of lines BS6-24 and BS6-26. In both GH-deficient and normal line BS6-26 females the transgene responded in the same way as the endogenous genes and was induced by GH (Fig. 5B, lanes 12 and 13). In contrast, although transgene expression was again induced in GH-deficient line BS6-24 females (Fig. 5B, lanes 2 and 3), it was not induced in normal females of this line (Fig. 5B, lanes 5 and 6), although the endogenous *Mup* genes were induced in

the same individuals (Fig. 5A, lanes h and j). Thus, although there was essentially no difference in the response of the endogenous *Mup* genes, and none in the response of the BS6- $\alpha$ 207 gene present in line BS6-26, the BS6- $\alpha$ 207 gene present in line BS6-24 responded differently to GH treatment depending on whether or not the female mouse carrying it was GH-deficient. The probable cause of the lack of response of normal line BS6-24 females is the dissimilar levels of endogenous GH in GH-deficient and normal mice. According to this view, although GH injection can override or entrain the pattern of endogenous GH secretion, either the new GH pattern does not conform closely enough to the male type, or else the response of the liver to the new GH pattern is incomplete. The former explanation is the more likely in view of the recent demonstration that the sexually dimorphic expression of rat liver cytochrome P450 depends crucially on the occurrence of periods between the GH peaks when the GH level is low or zero (11). In addition, it is necessary to assume that the transgenic locus in line BS6-24 is less responsive to the incomplete change, whatever it is, than either the endogenous *Mup* genes or the transgenic locus in line BS6-26. Since all the mice employed were bred on the C57BL background, there should be no effect of segregating or linked modifier genes, and the most likely cause of the different responses of the BS6-24 and BS6-26 loci is that one of the two is influenced by a chromosomal position effect.

#### Expression of MUP- $\alpha$ 2u-Globulin Hybrid Protein

BS6- $\alpha$ 207 and BL1- $\alpha$ 207 code for the same urinary protein, which can be identified by challenging Western blots with anti- $\alpha$ 2u-globulin serum. The antiserum cross-reacts at a low level with MUP, although, presumably because it has different affinities for different MUP proteins, the relative intensities of different MUP components in the immunostained gels (Fig. 6, B and C, nt) were strikingly different from those obtained by Coomassie staining (data not shown). A novel antibody-reactive protein which comigrated with  $\alpha$ 2u-globulin was detected in the urine of most transgenic normal males but not in that of females or GH-deficient males, thus accurately reflecting the incidence of hybrid gene mRNA in the liver (Fig. 6, A and B). Similarly, treatment of GH-deficient transgenic males with GH led to the secretion of the antibody-reactive protein (Fig. 6C, lane c).

No protein with the same mobility as the antibody-reactive protein could be detected in Coomassie-stained agarose-IEF or sodium dodecyl sulfate polyacrylamide gels, showing that urinary levels of the hybrid protein were very low. In contrast, the level of hybrid gene mRNA in the liver was on the same order as the level of MUP mRNA. The relative deficiency of the hybrid protein in urine presumably relates to the structure of either the mRNA or the protein and may be due to any of a number of causes, for example, a low rate of translation or of renal clearance.



**Fig. 6.** Urinary Secretion of the MUP- $\alpha_{2u}$ -Globulin Protein in Transgenic Mice Carrying the BS6- $\alpha_{207}$  and BL1- $\alpha_{207}$  Hybrid Genes

The Western blots were developed using anti- $\alpha_{2u}$ -globulin serum as primary antibody (see *Materials and Methods*). Lines are denoted with the following abbreviations: 3, BS6-3; 24, BS6-24; 25, BS6-25; 36, BS6-36; 39, BL1-39; 41, BL1-41, nt, Nontransgenic mice; r, 0.1  $\mu$ g male rat urinary protein. A, Lack of expression in the urine of female mice. Thirty micrograms of mouse urinary protein collected from individual normal mice were applied to each lane; m, male; f, female. An additional band present in most female samples is also present in nontransgenic female urine (not shown). B, Lack of expression in the urine of GH-deficient mice. Forty micrograms of mouse urinary protein collected from individual male mice were applied to each lane; a, normal mouse; b, GH-deficient mouse. C, Expression in the urine of GH-treated GH-deficient transgenic male mice. Urine from individual normal or GH-deficient transgenic (and nontransgenic control) mice was mixed with an equal volume of urine from GH-deficient or normal nontransgenic mice, respectively, before dialysis to equalize the protein concentrations. Forty microliters of urine were applied to each lane. a, Normal mouse; b and c, GH-deficient mouse before and after GH injection; b' and c', GH-deficient mouse before and after mock injection.

### Expression of a BS6 Promoter-Thymidine Kinase (TK) Reporter Gene in GH-Deficient Mice

We previously described a hybrid gene (BS6-tk) in which a 2.2-kb fragment containing the promoter and 5'-flanking sequence of *Mup* gene BS6 was joined to the coding region of the HSV-1 thymidine kinase gene (17). Between its 5' end and the junction (in the 5'-noncoding region) with the tk gene, BS6-tk is identical to BS6- $\alpha_{207}$  and BL1- $\alpha_{207}$  (Fig. 1). BS6-tk was introduced into the genome of C57BL/6  $\times$  CBA/Ca F<sub>2</sub> embryos, and transgenic lines were maintained by matings with C57BL/6  $\times$  CBA/Ca F<sub>1</sub> offspring. In three out of four lines the hybrid gene was expressed in a sexually dimorphic manner in the liver as determined by TK assays, Northern blot analysis, and run-on transcription (17). Expression in males was about five times higher than in females, a ratio similar to that shown by endog-

enous *Mup* genes, but much less than that shown by BS6- $\alpha_{207}$  and BL1- $\alpha_{207}$ .

In view of the sexually dimorphic expression of BS6-tk, it was important to determine whether it would respond to GH. If so, the conclusion could be drawn that the response is due to the promoter and/or 5'-flanking region of the *Mup* gene. GH-deficient and normal mice carrying the BS6-tk gene were produced by mating BS6-tk transgenic females [line 78 (17)] to C57BL/6 *lit*<sup>+</sup> males and then back-crossing transgenic *lit*<sup>+</sup> female offspring to *lit*<sup>+</sup> males. As determined by TK assays (Table 2), the male/female expression ratio of BS6-tk in normal back-cross offspring was about 2. This is lower than the ratio previously observed in mice from the G<sub>1</sub> and G<sub>2</sub> generations (17), possibly due to a change in the properties of the locus during propagation. The levels of expression in GH-deficient males and females were not significantly different, and in this respect the expression of BS6-tk mirrors that of the endogenous *Mup* genes. However, the level of expression in GH-deficient mice of both sexes was the same as that in normal females. In contrast, the endogenous *Mup* genes are expressed at a much lower level in GH-deficient mice than in normal females (Fig. 5). We suggest that this difference is due to constitutive (GH-independent) expression of BS6-tk. High constitutive expression could obscure a relatively much smaller difference in expression between normal females and GH-deficient males (see below).

The difference between normal males and GH-deficient males in the expression of BS6-tk was significant and greater than 2-fold. Treatment of GH-deficient males with GH induced expression by a factor of about 3, to a level that was in fact significantly higher than that in the uninduced normal males. Thus about 50% of the expression of the hybrid gene in normal males appears to be GH-dependent. We attribute the remaining 50% to GH-independent constitutive expression, which at this level would explain both the low sex ratio and our inability to detect a difference between normal females and GH-deficient mice. The HSV-tk reporter is not expressed in the liver when not appropriately attached to a liver-specific promoter or when attached to a shorter region (314 bp) of the BS6 promoter (31). Thus both the GH-inducible and the proposed constitutive expression of BS6-tk seem to be due to sequences residing in the BS6 promoter.

## DISCUSSION

### Expression of *Mup* Genes is Regulated by the Pattern of Circulating GH

Data presented here confirm that the effects of the sex hormones on *Mup* gene expression are mediated by the different patterns of circulating GH in the two sexes (10). GH-deficient male and female mice express the *Mup* genes at the same very low level, which is lower than the levels of expression in normal males or fe-

**Table 2.** GH Induction of the Line 78 BS6-tk Hybrid Gene in GH-Deficient Males

Background and genotype <sup>b</sup>	Hormone treatment	Thymidine kinase activity <sup>a</sup>	
		Males (n)	Females (n)
C57BLx CBA	None	22.75 ± 1.29 <sup>c</sup> (4)	10.10 ± 1.10 <sup>c,d</sup> (4)
C57BLx CBA	Testosterone	ND	21.10 ± 1.33 <sup>d</sup> (3)
C57BL <i>lit</i> /+	None	20.13 ± 1.30 <sup>e</sup> (4)	12.77 ± 1.27 (3)
C57BL <i>lit</i> / <i>lit</i>	None	8.87 ± 0.58 <sup>e,f</sup> (2)	12.67 ± 1.30 <sup>f</sup> (3)
C57BL <i>lit</i> / <i>lit</i>	Buffer	11.15 ± 1.85 <sup>g</sup> (2)	ND
C57BL <i>lit</i> / <i>lit</i>	GH	32.70 ± 1.17 <sup>g</sup> (3)	ND

<sup>a</sup> Units of thymidine kinase activity, picomoles of TMP formed per min/mg protein.

<sup>b</sup> Maintenance of line 78 on the C57BLx CBA background and transfer of the transgene to C57BL *lit*/*lit* and *lit*/+ genotypes is described in *Materials and Methods*.

<sup>c-g</sup> The significance of the differences between samples (*t* test)<sup>c-g</sup> are: <sup>c</sup>, *P* < 0.005; <sup>d</sup>, *P* < 0.001; <sup>e</sup>, *P* < 0.001; <sup>f</sup>, *P* < 0.002; <sup>g</sup>, *P* < 0.005; ND, not determined.

males. When GH was administered in a regime that crudely mimics the endogenous GH pattern of males, expression of the *Mup* genes was induced, giving expression comparable to that of normal males. The *Mup* genes were induced by testosterone in normal but not in GH-deficient female mice, demonstrating a requirement for GH. Furthermore, normal females could be induced by episodic GH administration alone, showing that the sex steroid status of the female can be overridden in this way and also that male levels of testosterone are not required for expression when GH is administered episodically. Since the sex steroids control the secretion pattern of GRF (6, 7), it is likely that induction by testosterone is mediated by the masculinization of the endogenous GH pattern of the treated females.

On the basis of a study of dwarf rats it was proposed recently that the GH pattern may not regulate the sexual dimorphism of liver cytochrome P450, and that a contaminant of GH preparations may be the active agent (32). Although the basis of this claim has been challenged (11), the alternative explanation warrants examination here. Two considerations argue against it in the present case. First, GH treatment reverses the *lit*/*lit* phenotype, which is believed to be due to a specific defect in GH release (20). Second, we have successfully induced *Mup* synthesis with human GH, bovine GH, ovine GH, and rat GH (our unpublished results). Thus if the inducing agent is other than GH, it presumably must nevertheless be linked to GRF stimulation, and it must be present in GH preparations from four species. This is not impossible but seems the less likely explanation.

#### Hybrid *Mup* Transgenes Are Induced by Pulsatile GH

In order to establish whether *cis*-acting DNA sequences are involved in the response of the *Mup* genes to GH, we generated lines of transgenic mice carrying two *Mup*- $\alpha_{2u}$ -globulin hybrid transgenes with different *Mup* gene promoters but otherwise with the same structure. No consistent difference in the expression of the two transgenes was observed. Their expression was highly

dimorphic and dependent upon a male pattern of circulating GH: GH-deficient mice of either sex failed to express the transgenes and were fully induced by episodic GH administration. Like the expression of endogenous *Mup* genes, transgene expression was not inducible by testosterone in GH-deficient females. In at least one transgenic line the expression of the transgene in normal females could be induced by episodic GH treatment, showing that, as in the case of the endogenous *Mup* genes, the sex hormone status of the animal can be overridden by GH. Also in line with the response of the endogenous *Mup* genes, this indicates that induction by episodic GH does not involve any direct effect of sex steroids on the liver.

#### The BS6 Promoter Contains a GH-Responsive Element

The expression of the BS6-tk hybrid gene was previously shown to be sexually dimorphic (17). Run-on transcription experiments demonstrated that sexually dimorphic expression of both the resident *Mup* genes and the BS6-tk transgene was due to different rates of transcription in the two sexes (16, 17). If sexually dimorphic expression is dictated by the GH pattern, the BS6 promoter region should contain a response element which responds to GH stimulation. Here we verified this by introducing the BS6-tk transgene into a line of GH-deficient mice. Hepatic expression of BS6-tk in GH-deficient males was significantly lower than in normal males, and higher levels of expression were induced when GH-deficient mice were treated with GH, confirming the presence of a GH-responsive element in the promoter.

The response of the BS6-tk transgene to GH, and also the degree of sexual dimorphism it displayed, were small relative to the responses of the BS6- $\alpha_{207}$  transgene. One possible explanation is that mouse or rat sequences downstream of the promoter, which are present in BS6- $\alpha_{207}$  but not in BS6-tk, are responsible for the greater responses of BS6- $\alpha_{207}$ . This explanation is reinforced by the fact that a rat  $\alpha_{207}$  transgene shows extreme sexual dimorphism in the mouse (33).

If so, the implication would be that there is a second response element in the downstream region. An alternative explanation, advanced above, is that BS6-tk has constitutive transcriptional activity, effectively a high GH-independent background level of transcription, which obscures the true extent of GH induction. If the putative downstream response element suppressed transcription in the absence of GH, the two explanations could relate to a single response element (or cluster). At this time the basis of the low response of BS6-tk remains unresolved. It is interesting to note that a fragment (-639 to +1395) of the rat  $\alpha$ 207 gene was actively transcribed in male but not in female rat liver nuclear extracts (34).

#### Expression of the Resident *Mup* Genes and the MUP- $\alpha$ 207 Hybrid Genes in Female Mice

Two observations suggest that in liver subjected to the female GH regime MUP expression is feminized rather than simply not induced: 1) The level of expression of the resident *Mup* genes is higher in females than in GH-deficient mice of either sex. Furthermore, when individual urinary MUPs were resolved by IEF it was apparent that some proteins are more abundantly expressed in males and others more abundantly expressed in females, relative to the basal patterns observed in urine from GH-deficient mice [(15) and Fig. 6A]. Similarly, mRNA species derived from different *Mup* genes are expressed at different relative levels in males and females (14). 2) In one transgenic line (BL1-39) a significant level of BL1- $\alpha$ 207 hybrid gene mRNA was detected in normal females, but no or very low expression was detected in GH-deficient mice of the same line; thus, in this line the female and GH-deficient transgene phenotypes resemble the endogenous MUP phenotypes. The most likely explanation of these observations is that female liver exposed to a relatively constant level of GH is feminized, while male liver exposed to pulses of GH is masculinized, both relative to the GH-deficient state. Feminization may generate a different stable level of the same *trans*-acting protein(s) involved in masculinization, or may lead to the appearance of qualitatively different hepatic *trans*-acting proteins. Masculinization and feminization may involve different signal transduction pathways. Different pathways of signal transduction are involved in the GH induction of *c-fos* and insulin-like growth factor-I in preadipocytes (35).

BL1 is the most highly expressed *Mup* gene in BALB/c female mice, where it represents 75% of the total *Mup* mRNA (14). The expression level of the endogenous BS6 *Mup* gene in female mice is not known, although translation of BS6 mRNA generates a protein which comigrates with a MUP present in female urine (Johnson, D., unpublished observations). The BS6- $\alpha$ 207 hybrid gene was not expressed in female mice (six out of six lines), and only one out of three lines expressed the BL1- $\alpha$ 207 hybrid gene in females. Thus, the general pattern of expression of the two hybrid

genes seems to be the same, given that the expression observed in females of one line (BL1-39) is a special property of the foreign DNA or its site of integration in that line, that is, to mutation, to the arrangement of the foreign genes, or to a chromosomal position effect (25, 36). However, because we established only three lines which expressed BL1- $\alpha$ 207, we cannot completely exclude the possibility that BL1- $\alpha$ 207 is less sexually dimorphic and that failure of expression in females carrying the BL1-5 and BL1-35 integrations is due to one or more of the causes of aberrant expression listed above.

#### Activity of the *Mup* Gene Promoter in the Preputial Gland Is Modulated by Sequences 3' of the Cap Site

BS6-tk is expressed in the liver and also at high levels in the preputial glands and testis (17, 25), although the resident *Mup* genes are not expressed in the two latter tissues. The HSV-tk reporter directs its own expression to the testis, using a cryptic housekeeping-type, TATA-independent, promoter (31). However, expression in the preputial gland appears to be a property of the *Mup* promoter because a *Mup* promoter-T antigen reporter gene is also expressed in the preputial gland (37) and because other hybrid genes containing HSV-tk as the reporter are not expressed in this tissue (31). With the exception of one line (see below), the BS6- $\alpha$ 207 gene was not expressed or was expressed only at an extremely low level, in the preputial glands of transgenic mice. It therefore appears that a sequence which prevents expression in the preputial gland is located in the BS6- $\alpha$ 207 gene downstream of the promoter. In contrast to the *Mup* genes, the  $\alpha$ <sub>2u</sub>-globulin genes are highly expressed in rat preputial glands, and in contrast to BS6- $\alpha$ 207, the  $\alpha$ 207 gene was expressed at high levels in the preputial glands of transgenic mice (33). This suggests that the sequence in question is located within one of the two *Mup* regions of BS6- $\alpha$ 207 which are not shared with either BS6-tk or  $\alpha$ -207. The first maps from the junction between the BS6 promoter and HSV-tk in the 5'-noncoding region to the BS6- $\alpha$ 207 junction at the *Eco*RI site in exon 2; the second is the region 3' of the *Aval* site in exon 6.

One of the lines (BL1-5) expressed the hybrid gene at high levels in the preputial gland. Since this is an isolated case (one out of nine lines), high level expression in the preputial gland may be assumed to be due to mutation, gene arrangement, or the chromosomal environment of the foreign DNA in line BL1-5. In most transgenic lines the BS6-tk hybrid gene is expressed in the preputial gland, but in line 64 expression was not detected (17). We have shown that this was due to a chromosomal position effect; the transgene was reisolated from line 64 and when reintroduced into the genome of mice, preputial gland expression was restored (25). The expression of *Mup* transgenes in the preputial gland appears to be particularly volatile.



## MATERIALS AND METHODS

### Hybrid Genes

Hybrid genes were assembled by making use of restriction sites (*EcoRI* in exon 2 and *Aval* in exon 6) which are conserved between *Mup* and  $\alpha_{2u}$ -globulin genes. The BS6- $\alpha_{207}$  hybrid gene was constructed from three DNA segments: a 2.75-kb *HindIII-EcoRI* 5'-fragment of MUP gene BS6 (27), which extends from 2.2 kb upstream of the cap site to exon 2, was linked to a 1.9-kb *EcoRI-Aval* fragment (exons 2-6) of the  $\alpha_{2u}$ -globulin gene 207 (38), and a 3.4-kb *Aval-BamHI* 3'-fragment of *Mup* gene BS6, which includes 1.8 kb of 3'-flanking sequences. A 408-bp *BamHI-XbaI* fragment containing the *Escherichia coli* SupF gene is located immediately 3' of the MUP- $\alpha_{2u}$ -globulin gene. The BL1- $\alpha_{2u}$ -globulin gene was constructed by replacing the 2.2-kb BS6 promoter region with the homologous region of BL1. For microinjection, each hybrid gene together with SupF was released from the vector (17) by digestion with *XbaI* and purified by gel electrophoresis.

### Transgenic Mice

*Lit/lit* mice were maintained on the C57BL/6 background. To produce transgenic mice carrying the *lit* allele, *lit/lit* male studs were mated with normal 3-week-old C57BL/6 superovulated female mice. The fertilized eggs (50-70% of the those recovered) were injected in the male or female pronucleus. From 310 eggs injected with BS6- $\alpha_{207}$ , 46 pups were born, 11 transgenic mice were obtained, and lines were established from 7 of these. Two hundred fifty-six eggs were injected with BL1- $\alpha_{207}$ , 47 mice were born, 4 transgenic mice identified, and all were bred to establish transgenic lines. Founders were back-crossed with *lit/lit* mice or mice heterozygous for the *lit* mutation; lines were also maintained in this manner. Two lines of mice carrying the BS6- $\alpha_{207}$  hybrid gene (lines BS6-6 and BS6-10) were produced by injecting eggs recovered from C57BL/6  $\times$  CBA/Ca F<sub>1</sub> females mated with F<sub>1</sub> males; these lines were maintained by back-crossing transgenic with non-transgenic F<sub>1</sub> mice. Transgenic mice were identified by Southern blot analysis or polymerase chain reaction of tail DNA, using a SupF probe or SupF-specific primers, respectively.

### Hormone Treatments

Mice were treated with rat GH (a gift from the U.S. National Hormone and Pituitary Program, NIDDK, Baltimore, MD) by injecting 100  $\mu$ g/20 g body wt ip every 12 h for a period of 5 days (10, 21, 24, 39). Testosterone was administered as a 30-mg sc implant, and the mice were examined after 10 days.

### RNA Analysis

RNA preparation and Northern blot analysis were carried out as described previously (17). Blots were hybridized with an  $\alpha_{2u}$ -globulin-specific 50-mer oligonucleotide (5'-CAAATATAG-TTCCCTGCACTCTCCATTTTCCTTAATACGGAAGCTTGA-AGC-3'), end labeled with [ $\gamma$ -<sup>32</sup>P]ATP. The oligonucleotide corresponds to the antisense strand of the last 19 bp of exon 2 and the first 31 bp of exon 3 of the 207  $\alpha_{2u}$ -globulin gene and differs from the *Mup* group 1 genes by 40% of its nucleotide sequence. Northern blots were reprobed with an apolipoprotein-A1 cDNA to check the integrity of the RNA samples. The apolipoprotein-A1 genes show little sensitivity to GH. Primer extension analysis of transcriptional start sites was performed by a modification of a method described (40) using the  $\alpha_{2u}$ -globulin-specific 50-mer oligonucleotide as a primer. Ten micrograms of total liver RNA and 0.2 pmol end-labeled primer were used in each extension reaction. Extension products were fractionated on a 6% acrylamide-7 M urea gel and visualized by autoradiography.

### Protein Analysis

Mouse and rat urine was collected by bladder massage. The urine was dialyzed at 4 C against 0.1 M NaCl, 10 mM Tris-HCl, pH 7.6, for 24 h and subsequently against distilled water for a further 24 h. Samples were stored at -20 C until required. Agarose IEF gels were prepared and stained as recommended by the ampholine manufacturer (Pharmacia, Piscataway, NJ). Gels consisted of 1% agarose, 12% sorbitol, and 6.3% Pharmalyte (86% Pharmalyte 4.2-4.9 and 14% Pharmalyte 3-5.5). The anode and cathode electrodes were soaked in 0.05 M H<sub>2</sub>SO<sub>4</sub> and 1 M NaOH, respectively. Gels were stained with Coomassie Blue. Samples prepared for Western Blot analysis were run on a 15% sodium dodecyl sulfate polyacrylamide gel (41). Proteins were transferred onto Immobilon (Millipore, Watford, Hertfordshire, UK) by the semidry blotting method (42) and challenged with rabbit anti- $\alpha_{2u}$ -globulin serum raised against crystallized  $\alpha_{2u}$ -globulin (a gift of Lindsay Sawyer, Department of Biochemistry, University of Edinburgh).

### Acknowledgments

We are grateful to Philip Feigelson for supplying us with rat  $\alpha_{2u}$ -globulin gene  $\alpha_{207}$  and for an initial supply of anti- $\alpha_{2u}$ -globulin antiserum, to Lindsay Sawyer for providing crystallized  $\alpha_{2u}$ -globulin, to Graham Bulfield for a gift of *lit/lit* mice and to the U.S. National Pituitary Program for a gift of rat GH. Assistance was provided by Melville Richardson, Mark Lawson, Gary Brown, Helen McIlroy, and Ann Walker.

Received September 16, 1991. Accepted December 3, 1991.

Address requests for reprints to: Dr. John Bishop, Department of Biological Sciences, University of Maryland, Baltimore County, Baltimore, Maryland 21228.

This work was supported by the Agricultural and Food Research Council, the Medical Research Council, and the Agricultural and Food Research Council Centre for Genome Research.

### REFERENCES

1. Saunders A, Terry LC, Audet J, Brazeau P, Martin JB 1976 Dynamic studies of growth hormone and prolactin secretion in the female rat. *Neuroendocrinology* 21:193-203
2. Tannenbaum GS, Martin JB 1976 Evidence for an endogenous ultradian rhythm governing growth hormone secretion in the rat. *Endocrinology* 98:562-570
3. Bilezikjian LM, Vale WW 1983 Stimulation of adenosine 3',5'-monophosphate production by growth hormone-releasing factor and its inhibition by somatostatin in anterior pituitary cells *in vitro*. *Endocrinology* 113:1726-1731
4. Evans RM, Birnberg NC, Rosenfeld MG 1982 Glucocorticoid and thyroid hormones transcriptionally regulate growth hormone gene expression. *Proc Natl Acad Sci USA* 79:7659-7663
5. Plotsky PM, Vale W 1985 Patterns of growth hormone-releasing factor and somatostatin secretion into the hypophysial-portal circulation of the rat. *Science* 230:461-463
6. Jansson JO, Ekberg S, Isaksson OG, Eden S 1984 Influence of gonadal steroids on age- and sex-related secretory patterns of growth hormone in the rat. *Endocrinology* 114:1287-1294
7. Jansson JO, Frohman LA 1987 Differential effects of neonatal and adult androgen exposure on the growth

- hormone secretory pattern in male rats. *Endocrinology* 120:1551-1557
8. Rumbaugh RC, Colby HD 1980 Is growth hormone the pituitary feminizing factor mediating the actions of estradiol on hepatic drug and steroid metabolism? *Endocrinology* 107:719-724
  9. Gustafsson JA, Mode A, Norstedt G, Hokfelt T, Skett P, Sonnenschein C 1980 The hypothalamo-pituitary-liver axis, a new hormonal system in control of hepatic steroid and drug metabolism. In: Litwack G (ed) *Biochemical Actions of Hormones*, 7. Academic Press, New York, pp 47-89
  10. Norstedt G, Palmiter R 1984 Secretory rhythm of growth hormone regulates sexual differentiation of mouse liver. *Cell* 36:805-812
  11. Waxman DJ, Pampori NA, Ram PA, Agrawal AK, Shapiro BH 1991 Interpulse interval in circulating growth hormone patterns regulates sexually dimorphic expression of hepatic cytochrome P450. *Proc Natl Acad Sci USA* 88:6868-6872
  12. Bishop JO, Clark AJ, Clissold PM, Hailey S, Francke U 1982 Two main groups of mouse major urinary protein genes, both largely located on chromosome 4. *EMBO J* 1:615-620
  13. Al-Shawi R, Ghazal P, Clark AJ, Bishop JO 1989 Intraspecific evolution of a gene family coding for urinary proteins. *J Mol Evol* 29:302-313
  14. McIntosh I, Bishop JO 1989 Differential expression in male and female mouse liver of very similar mRNAs specified by two group 1 major urinary protein genes. *Mol Cell Biol* 9:2202-2207
  15. Clissold PM, Hailey S, Bishop JO 1984 Messenger RNAs coding for mouse major urinary proteins are differentially induced by testosterone. *Biochem Genet* 22:379-387
  16. Derman E 1981 Isolation of a cDNA clone for mouse major urinary proteins: age- and sex-related expression of mouse urinary protein genes is transcriptionally controlled. *Proc Natl Acad Sci USA* 78:5425-5429
  17. Al-Shawi R, Burke J, Jones CT, Simons JP, Bishop JO 1988 A Mup promoter-thymidine kinase reporter gene shows relaxed tissue-specific expression and confers male sterility upon transgenic mice. *Mol Cell Biol* 8:4821-4828
  18. Eicher EV, Beamer W 1976 Inherited ateliotic dwarfism in mice. *J Heredity* 67:87-91
  19. Phillips III J, Beamer WG, Bartke A 1982 Analysis of growth hormone genes in mice with genetic defects of growth hormone expression. *J Endocrinol* 92:405-407
  20. Jansson JO, Downs TR, Beamer WG, Frohman LA 1986 Receptor-associated resistance to growth hormone-releasing factor in dwarf "little" mice. *Science* 232:511-512
  21. Beamer WH, Eicher EM 1976 Stimulation of growth in the little mouse. *J Endocrinol* 71:37-45
  22. Hammer RE, Palmiter RD, Brinster RL 1984 Partial correction of murine hereditary growth disorder by germ-line incorporation of a new gene. *Nature* 311:65-67
  23. Chubb C, Nolan C 1985 Animal models of male infertility: mice bearing single-gene mutations that induce infertility. *Endocrinology* 117:338-346
  24. Knopf JL, Gallagher JF, Held WA 1983 Differential multi-hormone regulation of the mouse urinary protein gene family in the liver. *Mol Cell Biol* 3:2232-2240
  25. Al-Shawi R, Kinnaird J, Burke J, Bishop JO 1990 Expression of a foreign gene in a line of transgenic mice is modulated by a chromosomal position effect. *Mol Cell Biol* 10:1192-1198
  26. Clark AJ, Ghazal P, Bingham RW, Barrett D, Bishop JO 1985 Sequence structure of a mouse major urinary protein gene and pseudogene compared. *EMBO J* 4:3159-3165
  27. Clark AJ, Clissold PM, Al Shawi R, Beattie P, Bishop J 1984 Structure of mouse major urinary protein genes: different splicing configurations in the 3'-non-coding region. *EMBO J* 3:1045-1052
  28. Clark AJ, Chave-Cox A, Ma X, Bishop JO 1985 Analysis of mouse major urinary protein genes: variation between the exonic sequences of group 1 genes and a comparison with an active gene outwith group 1 both suggest that gene conversion has occurred between MUP genes. *EMBO J* 3:1045-1052
  29. Shahan K, Gilmartin M, Derman E 1987 Nucleotide sequences of liver, lachrymal, and submaxillary gland mouse major urinary protein mRNAs: mosaic structure and construction of panels of gene-specific synthetic oligonucleotide probes. *Mol Cell Biol* 7:1938-1946
  30. Kulkarni AB, Gubits RM, Feigelson P 1985 Developmental and hormonal regulation of alpha 2u-globulin gene transcription. *Proc Natl Acad Sci USA* 82:2579-2582
  31. Al-Shawi R, Burke J, Wallace H, Jones C, Harrison S, Buxton D, Maley S, Chandley A, Bishop JO 1991 The herpes simplex type 1 virus thymidine kinase is expressed in the testis of transgenic mice under the control of an internal promoter. *Mol Cell Biol* 11:4207-4216
  32. Bullock P, Gemzik B, Johnson D, Thomas P, Parkinson A 1991 Evidence from dwarf rats that growth hormone may not regulate the sexual differentiation of liver cytochrome P450 enzymes and steroid 5 alpha-reductase. *Proc Natl Acad Sci USA* 88:5227-5231
  33. da Costa Soares V, Gubits RM, Feigelson P, Costantini F 1987 Tissue-specific and hormonally regulated expression of a rat alpha 2u-globulin gene in transgenic mice. *Mol Cell Biol* 7:3749-3758
  34. Sarkar P, Feigelson P 1989 Developmentally regulated male-specific transfactor(s) enable *in vitro* transcription of a cloned  $\alpha_{2u}$ -globulin gene. *Mol Endocrinol* 3:342-348
  35. Doglio A, Dani C, Grimaldi P, Ailhaud G 1989 Growth hormone stimulates *c-fos* gene expression by means of protein kinase C without increasing inositol lipid turnover. *Proc Natl Acad Sci USA* 86:1148-1152
  36. Held WA, Mullins JJ, Kuhn NJ, Gallagher JF, Gu GD, Gross KW 1989 T antigen expression and tumorigenesis in transgenic mice containing a mouse major urinary protein/SV40 T antigen hybrid gene. *EMBO J* 8:183-191
  37. Jaenisch R, Jahner D, Nobis P, Simon I, Lohler J, Harbers K, Grotkopp D 1981 Chromosomal position and activation of retroviral genomes inserted into the germ line of mice. *Cell* 24:519-529
  38. Dolan KP, Unterman R, McLaughlin M, Nakhasi HL, Lynch KR, Feigelson P 1982 The structure and expression of very closely related members of the alpha 2u globulin gene family. *J Biol Chem* 257:13527-13534
  39. Swank RT 1978 Roles of growth hormone and testosterone in the synthesis of mouse kidney glucuronidase. *Mol Cell Endocrinol* 12:139-149
  40. Ghosh PK, Reddy VB, Piatak M, Lebowitz P, Weissman SM 1980 Determination of RNA sequences by primer directed synthesis and sequencing of their cDNA transcripts. *Methods Enzymol* 65:580-595
  41. Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685
  42. Khyse-Anderson J 1984 Electrophoretic transfer of a simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to agarose. *J Biochem Biophys Methods* 10:203-209