MOLECULAR AND CELLULAR BIOLOGY, Aug. 1991, p. 4207–4216
0270-7306/91/084207-10$02.00/0

Vol. 11, No. 8

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

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

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

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^{46} and Met^{60}, 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.

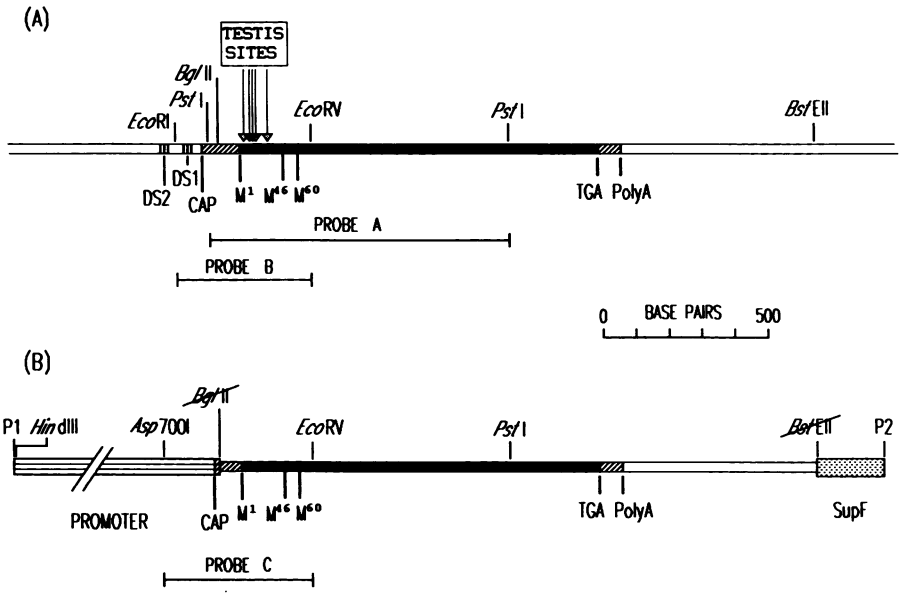

FIG. 1. (A) Diagram of the HSV-1 *tk* gene. DS1 and DS2 are the transcriptional control regions (38); M^1, M^{46}, and M^{60} are the first three ATG codons of the reading frame. The testis transcription initiation sites (TESTIS SITES) are described in the text. The fragment used as a reporter sequence 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 5×10^5 dpm of antisense probe was hybridized overnight at 45°C with 5 μg of total testis RNA. Digestion was with 40 μg of RNase A and 650 U of RNase T_1 per ml for 30 min at 37°C.

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

Immunostaining of testis sections. Deeply anesthetized mice were perfused with 2% paraformaldehyde, and tissues were postfixed with modified Bouin fixative, dehydrated in graded alcohols, and embedded in paraffin wax. Dewaxed 4-μm sections were treated with 1% H_2O_2 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[a]	n[b]	TK activity (pmol/min/mg of protein)					
			Testis		Liver		Preputial gland	
			Mean ± SE[c]	Range[d]	Mean ± SE	Range	Mean ± SE	Range
BS6-*tk-supF*	S	5	84.0 ± 20.7	31–140	4.39 ± 2.04	0.05–12.7	27.4 ± 13.3	0.04–83
BS6-*tk-supF* (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-*tk-supF*	S	3	60.7 ± 9.2	47–83	0.47 ± .005	0.46–0.48	0.01 ± 0.01	0.0–0.04
BS6*Sau2-tk-supF*	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-*tk-supF*	S	3	54.5 ± 9.0	40.4–73.3	0.01[e]		0.35[e]	

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

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_0 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

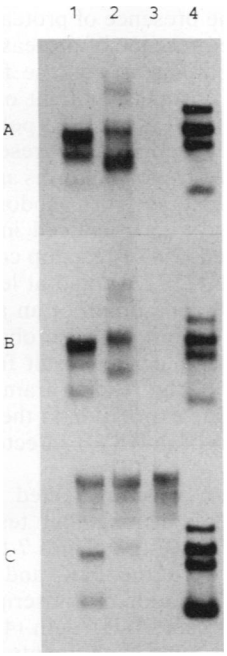

FIG. 2. Mapping of HSV-1 *tk*-specific RNA in extracts of line 78 testis. Identical RNA transfers were probed with a full-length HSV-1 *tk* double-stranded DNA probe (A), a 5′ double-stranded DNA probe (B), and a 5′ single-stranded antisense RNA probe (C). Lanes contain RNA samples as follows: 1, line 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).

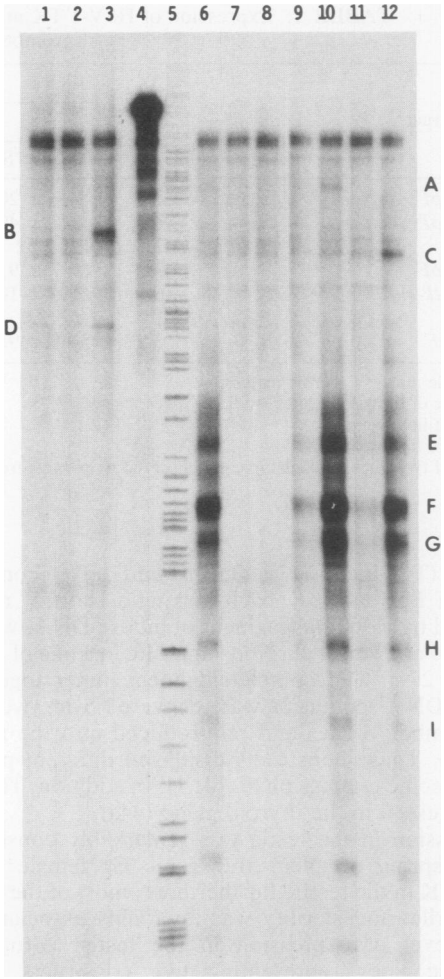

FIG. 3. Mapping of 5′ ends of HSV-1 *tk*-specific RNA in livers and testes of transgenic mice. The RNA riboprobe was transcribed from a fragment of BS6-*tk-supF* which overlaps the MUP gene cap site (see Materials and Methods). Lanes: 1 and 7, tRNA controls; 2, RNA from nontransgenic liver; 3, RNA from 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[a]

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

[a] Methionine initiation codons are as follows: M^1, +79; M^{46}, +214; M^{60}, +256.

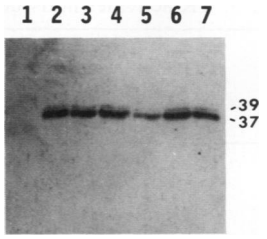

FIG. 5. HSV-1 TK-specific polypeptides in testis extracts from different lines of transgenic mice. The positions of the 39K and 37K polypeptides are marked. Lanes contain testis extracts as follows: 1, nontransgenic control; 2, line 64 (BS6-*tk-supF*); 3, line 78 (BS6-*tk-supF*); 4, line 46 (BS6-*tk-supF*); 5, line TK16 (HSV-1 *tkDS2*−); 6, line TG66 (TG-*tk-supF*); 7, line S87 (BS6*Sau2-tk-supF*).

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 promotorless 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 *Eco*RI-*Bst*EII 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_0 transgenic males carrying the HSV-1 *tkDS2*− fragment and in male transgenic offspring of a G_0 female (founder of line TK16). Three of the G_0 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_0 males carrying *tk* constructs (1). Such males are probably mosaics with nontransgenic precursor cells in their germ line (see below). The third expressing G_0 male and line TK16 males were sterile (Table 3). In nuclease protection experiments, testis RNA from line TK16 showed the same pattern of 5′ ends within the HSV *tk* coding region as did testis RNA from mice carrying the promoter constructs (Fig. 3, lane 11). Furthermore, line TK16 testis contained the same shorter HSV TK polypeptides that were observed in the testis of those mice (Fig. 5, lane 5). These results argue convincingly that the expression of HSV-1 TK in the testes of transgenic mice is not due to the various tissue-specific promoters when these are present. Instead, it appears to be due to a cryptic promoter within the HSV-1 *tk* reporter gene, located downstream of the TATA box-containing promoter which is utilized during HSV infection.

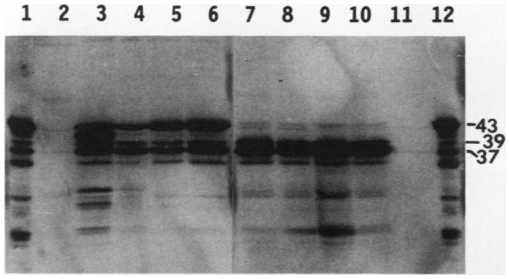

FIG. 4. Effect of protease inhibitors on HSV-1 TK polypeptides in extracts of transgenic preputial glands and testes. Each lane was loaded with 50 μg of protein. Proteins were transferred to a filter and developed with anti-HSV-1 TK antibody as described in Materials and Methods. The positions of the 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).

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[a]	TK22, fertile	TK39, sterile	TK40, fertile[a]	
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[b]	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

[a] Fertile nontransmitting.
[b] ND, not determined.

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

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

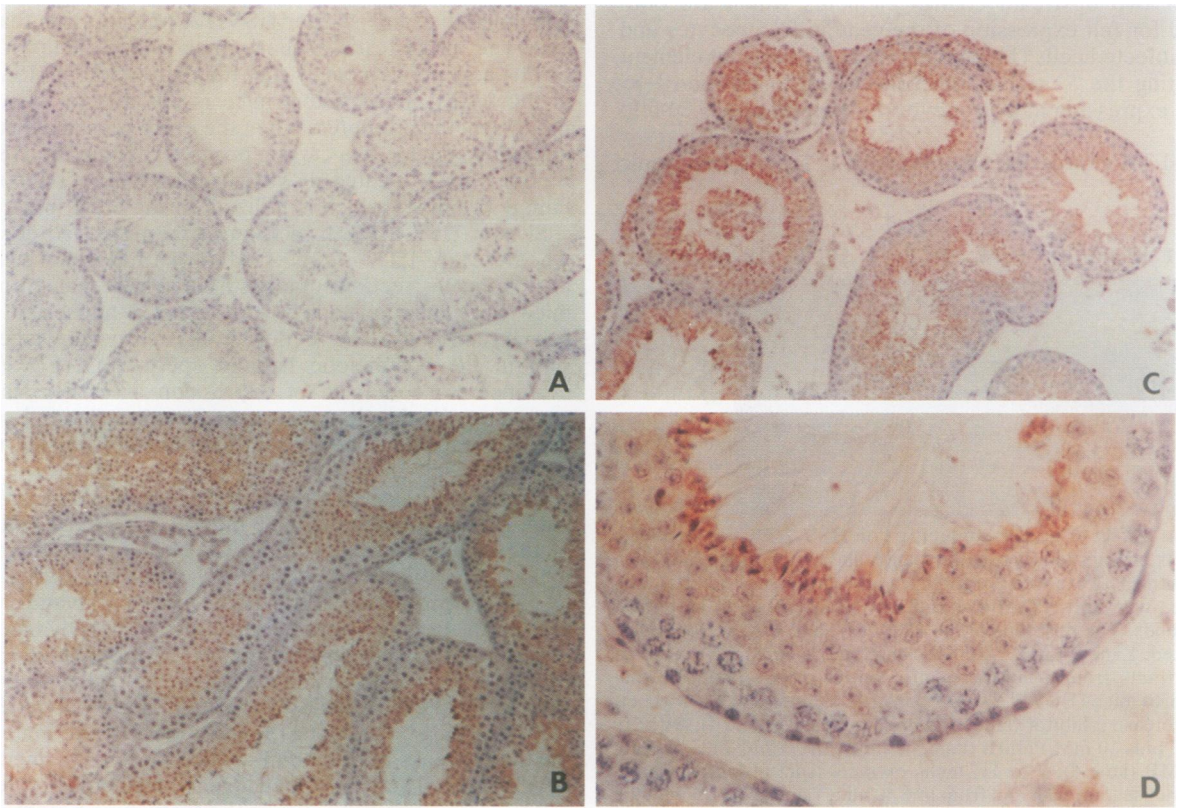

FIG. 6. Testes of BS6-*tk-supF* transgenic mice immunostained with anti-HSV-1 TK serum. (A) Nontransgenic testis; (B) transgenic testis from line TG66 (TG-*tk-supF*); (C and D) transgenic testis from line 78 (BS6-*tk-supF*). Magnifications: A, B, and C, ×70; D, ×280.

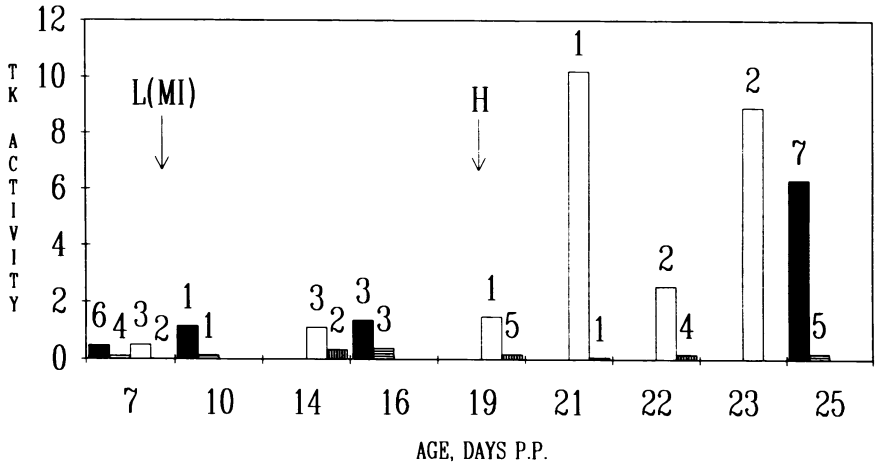

FIG. 7. Time course of HSV-1 TK expression in testis during sexual maturation. Testes of pups from crosses of line 40 and line 46 females with nontransgenic males were assayed as described prior to determining their transgenic status. Ordinate, HSV-1 TK activity, expressed as picomoles of TMP synthesized 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	5×10^3	10^5	5×10^4
Contents of vas deferens	Numerous sperm 80% normal Motility normal	Very few sperm None with normal morphology Nonmotile			
HSV-1 TK activity[a] (line mean ± SE)		140 ± 6.7		139 ± 17.6	

[a] From Al-Shawi et al. (1).

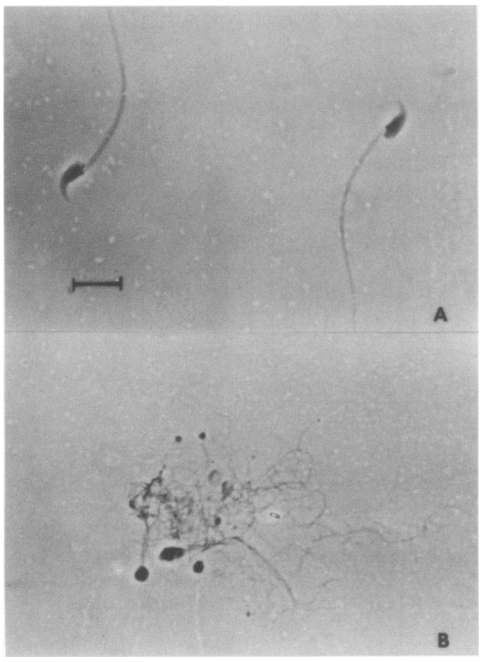

FIG. 8. Normal and defective spermatozoa. The contents of the vas deferens of nontransgenic (A) and line 40 (B) mice were stained with eosin. The bar represents 10 μm.

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–β-interferon–*tk* construct, Iwakura et al. (24) observed minority transcripts (about 20%) which hybridized with both β-interferon and *tk* probes, while about 80% of the transcripts reacted only with the *tk* probe and closely resembled the testis *tk* transcripts that we observed (Fig. 2). The cryptic *tk* promoter evidently did not abrogate (or abrogate 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^{60}, the third methionine of the reading frame, which lies within the strongest translational initiation consensus of the first three ATG codons. M^{60} lies within a region of the enzyme shown by site-directed mutagenesis to be the ATP-binding pocket (29). Consequently, the enzymatic activity of the 37K protein is likely to be less than that of full-length TK, and the level of TK expression in the testis may be considerably higher than indicated by enzyme 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_α^d gene was at

first attributed to the expression of E_{α}^{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–β-interferon–*tk* foreign gene caused male sterility, which was attributed to the expression of β-interferon (24). HSV-1 TK activity at levels similar to those we have observed was detected in the testes of the transgenic mice, and it was not convincingly demonstrated that β-interferon rather than HSV-1 TK was the cause of sterility.

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

Causes of male sterility. Our data indicate that sterility in mice with an intermediate level of TK expression may be due to expression in the postmeiotic spermatids. Since mammalian sperm do not undergo postmeiotic mitosis, this would exclude effects involving the synthesis of DNA, such as hypermutation. The nonmotile sperm produced by these sterile mice are present in normal numbers and are mainly normal in appearance, suggesting that their lack of motility may be due to an impoverished energy source. HSV-1 TK is an aggressively promiscuous nucleoside kinase, is only poorly susceptible to end-product inhibition, and vigorously phosphorylates mononucleotides as well as nucleosides (10, 41). Extracts of transgenic testis show elevated levels of both nucleoside kinase and nucleotide kinase activity (34a). Normal mouse sperm remain motile for several hours in the absence of an exogenous energy source, suggesting that they possess a sizable energy store (9). Thus, the lack of sperm motility may derive from a failure of energy storage due to ATP depletion during maturation.

The gross morphological defects and almost complete failure of sperm maturation that are observed in mice with higher levels of testis HSV-1 TK activity are likely to have a more radical cause. Possibly the low-level TK activity present prior to the first meioses, and tentatively attributed to expression in premeiotic germ cells, interferes with DNA synthesis or some other vital cellular function in lines with 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. Irmiere, 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. **Irmiere, 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 Eαd MHC class II gene in transgenic mice. EMBO J. **4**:2225–2230.
38. **Richardson, W. D., B. L. Roberts, and A. E. Smith.** 1986. 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. **Shackleford, 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.