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The P4 promoter of the parvovirus minute virus of mice is developmentally regulated in transgenic P4-*LacZ* mice

Claytus Davis,[a] Niva Segev-Amzaleg,[b] Irit Rotem,[b] Michal Mincberg,[b] Nava Amir,[b] Sara Sivan,[b] Inna Gitelman,[a] and Jacov Tal[a,b,*]

[a] *Department of Molecular Genetics of Development, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel*
[b] *Department of Virology, Faculty of Health Sciences, Ben Gurion University of the Negev, Beer Sheva, Israel*

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Abstract

Activation of the minute virus of mice (MVM) P4 promoter is a key step in the life cycle of the virus and is completely dependent on host transcription factors. Since transcription-factor composition varies widely in different cell types, there is the possibility that only some cell types in the host organism have the capacity to initiate expression from the P4 promoter and therefore that the promoter may be a factor in determining the tropism of MVM. In this study, the ability of various cell types to activate P4, independent of the other virus–host interactions, was examined in transgenic mouse lines bearing a β-galactosidase reporter sequence driven by the P4 promoter. It was found that *lac*Z was expressed during embryogenesis and in the adult in a cell-type-specific and differentiation-dependent pattern. The data are consistent with cell-type and stage-specific activation of the P4 promoter having a role in determining the host cell-type range of MVM. The ability of some parvoviruses to replicate in, and kill oncogenically transformed cells, and to destroy induced tumors in laboratory animals is the basis of recent approaches to use MVM-based vectors in cancer gene therapy. Since these vectors rely on the activation of the P4 promoter by the target tissues, understanding the promoter dependence on cell-type and differentiation status is important for their design and potential use.

Keywords: Minute virus of mice; MVM; P4; Transgene; Mouse; Gene regulation

Introduction

Minute virus of mice (MVM), a natural murine pathogen, is a small icosahedral virus that contains a single-stranded, linear DNA genome, 5.1 kilobases (kb) in length. The entire coding content of MVM, clustered in its viral (minus) strand, is organized in two partially overlapping transcription units having promoters at map units 4 (P4) and 38 (P38) (Astell et al., 1986; Pintel et al., 1983; Sahli et al., 1985). Messenger RNAs generated from P4 encode the viral regulatory proteins NS1 and NS2 (Cotmore and Tattersall, 1986b). Messenger RNAs generated from the P38 promoter encode the viral capsid proteins VP1 and Vp2 (Labieniec-Pintel and Pintel, 1986). NS1, the major regulatory protein, is a multifunctional phosphoprotein (Cotmore and Tattersall, 1986a). It plays a key role in productive infection through its various biochemical functions, including site-specific endonuclease, ATPase, and helicase activities (Cotmore and Tattersall, 1995; Jindal et al., 1994; Nuesch et al., 1995), viral genome amplification, and *trans*-regulation of transcription from both P4 and P38 viral promoters (Doerig et al., 1988, 1990; Legendre and Rommelaere, 1994; Lorson et al., 1996). The P4 promoter is regulated by NS1 in a dose-dependent fashion (Hanson and Rhode, 1991). NS2, of which three isoforms are made as a result of alternative splicing, is required for infection and capsid assembly in cultured murine cells (Cater and Pintel, 1992; Cotmore et al., 1997) and for the pathogenicity of the virus in vivo (Brownstein et al., 1992). The accumu-

* Corresponding author. Fax: +972-8-627-6215.
E-mail address: ytal@bgumail.bgu.ac.il (J. Tal).

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lating data suggest that, during MVM infection, NS2 acts as a modulator of NS1 activity in a cell-type-specific manner (Brandenburger et al., 1990; Legrand et al., 1993).

Because of its limited coding capacity, the replication of MVM (similar to that of all other autonomous parvoviruses) relies heavily on cellular factors, some of which have been identified. In tissue culture, MVM replication depends on cellular functions expressed transiently during S-phase (Deleu et al., 1999; Tattersall, 1972) and production of a transcriptionally active double-stranded viral genome occurs only in mitotically active cells. Productive infection also depends on the differentiation state of the host cell. The early embryo and embryonic stem cells are resistant to viral replication, but differentiated derivatives may be sensitive (Gardiner and Tattersall, 1988; Guetta et al., 1986; Miller et al., 1977; Spalholz and Tattersall, 1983). Cell type has also been shown to be a determining factor in restriction of infectivity. The best studied example of this is the allotropism displayed by the fibrotropic (p) and lymphotropic (i) strains of MVM, which can establish productive infections in mouse fibroblast and T-lymphocyte cell lines, respectively, but not in each other's permissive host (Spalholz and Tattersall, 1983; Tattersall and Bratton, 1983). In this case, the different tropisms are due to a few amino acid variations in the viral capsid protein VP2 (Gardiner and Tattersall, 1988; Maxwell et al., 1995). The regulatory NS proteins have also been implicated in host-range determination (Rubio et al., 2001).

Activation of the regulatory P4 promoter is a critical step in the MVM viral life cycle. Although the activity of P4 is later modulated by its own gene products, initial activation is completely dependent on host cell factors. The P4 promoter contains multiple transcription factor-binding sites (Faisst et al., 1994), including E2F (Deleu et al., 1999), NF-Y (Gu et al., 1995), SP1 (Pitluk and Ward, 1991), and CRE (Perros et al., 1995, 1999). It has also been shown to interact with a heterogeneous nuclear ribonucleoprotein-like protein designated NSAP1 (Harris et al., 1999). Some cell lines exhibit increased P4 promoter activity in S-phase (Deleu et al., 1998), which is important for productive infection (Deleu et al., 1999). The P4 promoter is regulated by NS1 in a dose-dependent fashion (Hanson and Rhode, 1991). To date, no transcription factor has been shown to interact with the P4 promoter in a developmentally dependent fashion, but the central role of NS1 in the MVM life cycle and the dependence of MVM on host differentiation state suggested the possibility that P4 could be developmentally regulated. Indeed, the demonstration that the Ets factor-binding site (EBS) was required for full P4 activity in ras-transformed rat fibroblasts cell line but did not play a significant role in their parental, untransformed cells (Fuks et al., 1996; Spegelaere et al., 1991) and the observation that deletions of upstream P4 promoter elements had cell-type-specific effects on the promoter activity (Spegelaere et al., 1994) both lend support to the hypothesis.

Potential cell-type and differentiation state-specific activation of the P4 promoter is particularly relevant in view of the ability of MVMp and other autonomous parvoviruses to destroy induced tumors in laboratory animals (Faisst et al., 1998; Guetta et al., 1986) and to preferentially replicate in, and kill, oncogenically transformed cells in vitro but not their untransformed parental cells (Cornelis et al., 1988; Guetta et al., 1990; Mousset et al., 1986; Salome et al., 1990; reviewed in Rommelaere and Cornelis, 1991). Interactions between the transcription products from the P4 promoter, the NS1 proteins, and the host cell are believed to account for the antineoplastic activity of some parvoviruses (Rommelaere and Cornelis, 1991; Vanacker and Rommelaere, 1995). Experimental systems using this antineoplastic activity for the design of therapeutic vectors have relied upon the activity of the native P4 promoter (reviewed in Palmer and Tattersall, 2000).

In the current study, transgenic mouse lines were generated bearing a *lac*Z reporter gene under the regulation of the MVM P4 promoter. This permitted the characterization of the activation of the P4 promoter in the context of its normal range of potential host cells and independent of other viral components. The tissue and cell-type and differentiation-state dependence of activation of the transgenic P4 promoter reported here are consistent with the hypothesis that activation of the P4 promoter by the host cell is partially responsible for determination of the MVM host cell-type range.

Results

Establishing P4-LacZ transgenic mouse lines

Three of the 28 offspring from injected eggs were identified as transgenic by dot blot and Southern blot analyses. These progenitors were used to generate the independent transgenic lines p4:LacZ-10, p4:LacZ-11, and p4:LacZ-17; each of those showed Mendelian inheritance of the transgene. Southern blot analysis indicated that the transgenic lines carried one to three copies of MVMP4-lacZ, each at a different chromosomal location. The activity of the P4 promoter was initially assessed by X-gal staining in embryos at 9.5, 11.5, 12.5, 13.5, and 14.5 days postcoitum (dpc) collected from $T_g^+ \times$ wt crosses. The staining was observed from 11.5 dpc on, and the pattern was the same in all three lines: strong in the ventral CNS and eyes and weaker in the heart and limb mesenchyme. Constancy of expression pattern in independent transgenic lines is the accepted indication that the expression reflects the intrinsic activity of the transgene rather than chromosomal position effect. The line exhibiting the most intense staining, p4:LacZ-17, was used for further analyses. A heterozygous line was maintained by

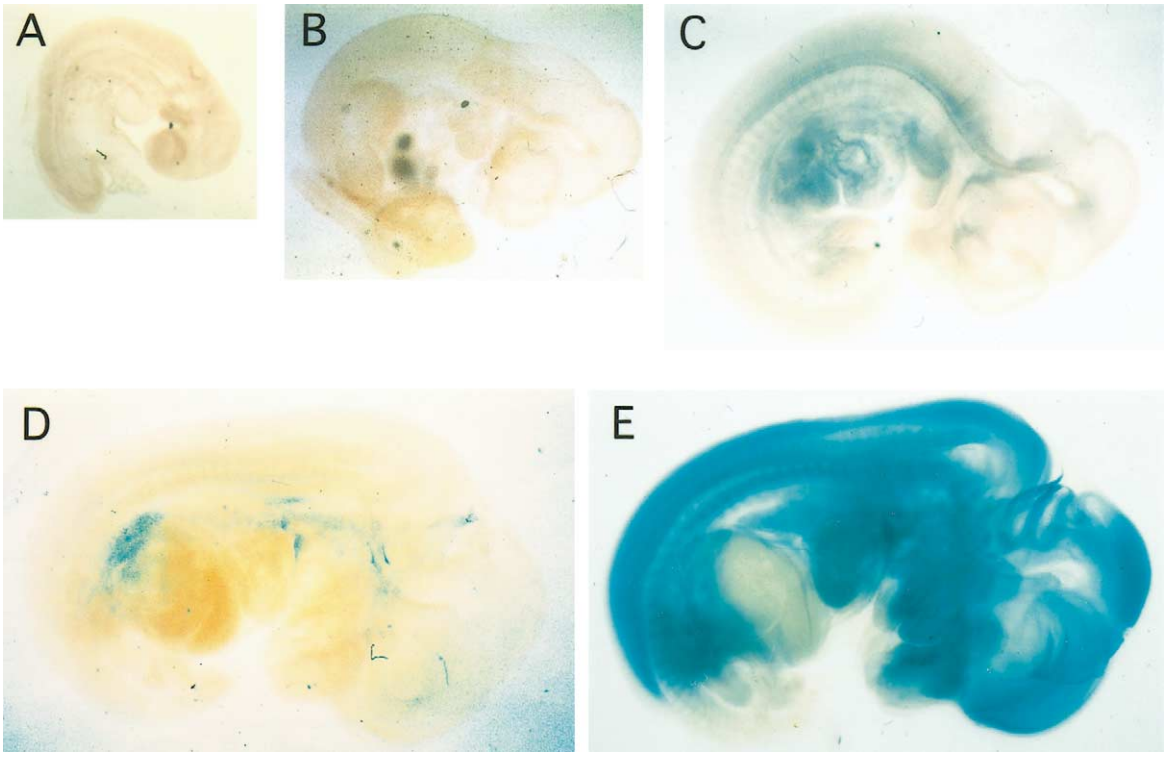


Fig. 1. Transgene expression in middevelopment mouse embryos. Heterozygous transgenic BALB/c animals were mated to BALB/c mice and embryos were collected on the embryonic days indicated. Embryos were stained in a whole-mount format with the β-galactosidase substrate X-gal as described under Materials and methods and cleared in BABB for photography. (A) 8.5 dpc embryo; (B) 9.5 dpc embryo; (C) 11.5 dpc transgenic embryo; (D) 13.5 dpc wild-type negative control embryo; (E) 13.5 dpc transgenic embryo.

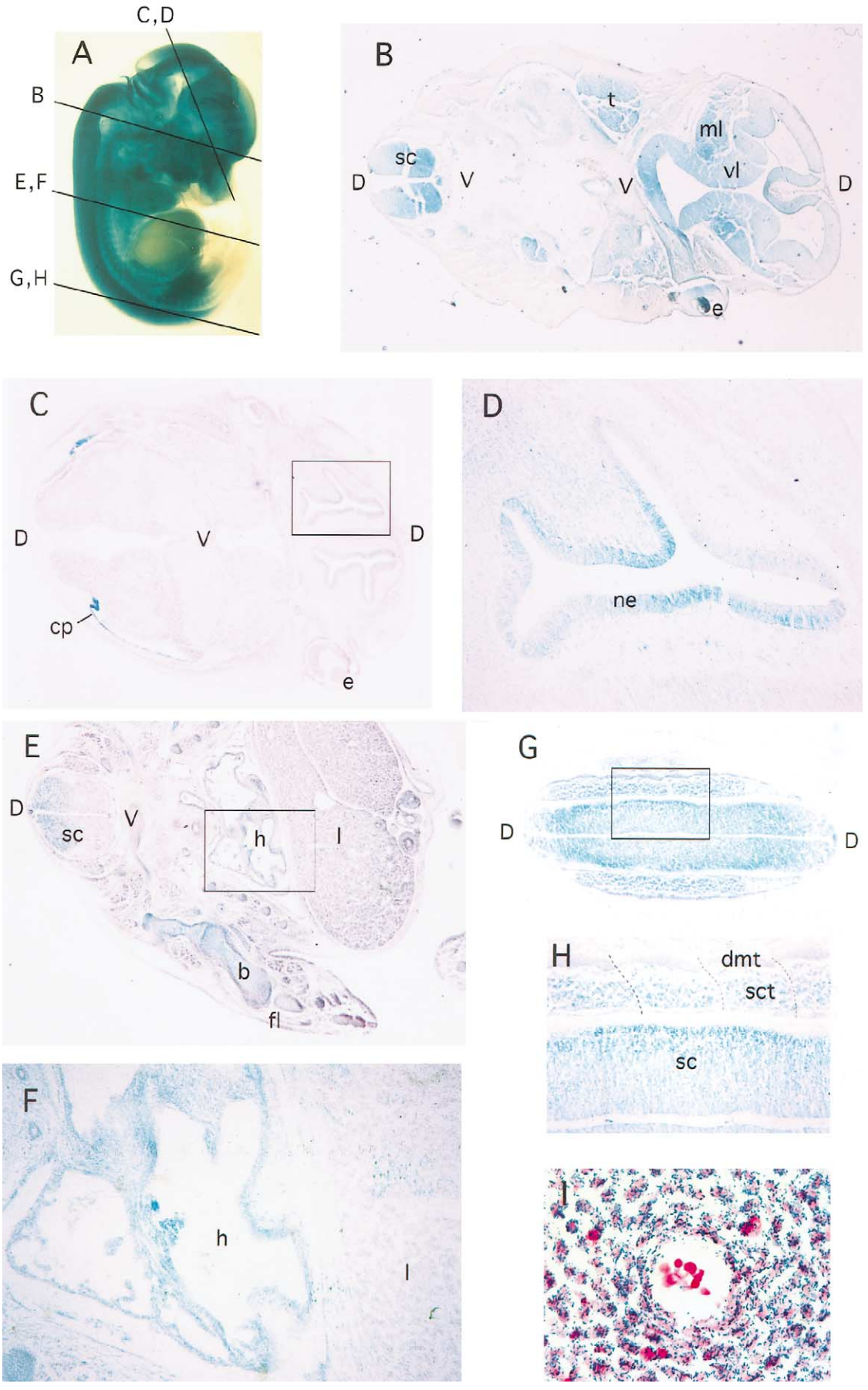
crossing to BALB/c wild-type mice and identifying heterozygous transgenic offspring by PCR analysis of tail DNA.

The transgenic P4 promoter exhibits tissue and stage-specific activity during embryonic development

To determine whether the P4 promoter was activated in a stage-specific manner, we analyzed transgenic embryos at eight different developmental stages, including: 3.5 dpc (preimplantation), 7.5 dpc (late gastrulation–neurulation), 8.5 (neural tube closure, cardiogenesis), 9.5, 10.5, 11.5, 12.5, and 13.5 dpc. Transgenic embryos were dissected out and stained with X-gal in a whole-mount format. No staining was observed in 3.5, 7.5, 8.5, 9.5, or 10.5 dpc embryos (Fig. 1A, B data not shown), indicating that the promoter was inactive during early development. Weak staining was first observed at 11.5 dpc in the developing CNS, the heart, and visceral embryonic mesoderm (Fig. 1C), indicating tissue-specific activation of the P4 promoter. By 13.5 dpc, the staining intensified and also appeared in the limb buds (Fig. 1E). In comparison, nontransgenic 13.5-dpc embryos showed only small localized patches of staining (Fig. 1D) due to endogenous β-galactosidase activity. There was no variation in staining intensity between stained embryos within a litter, indicating no variation in penetrance.

By 13.5 dpc, the rudiments of all organs have formed. This stage was chosen, therefore, for a more detailed histological analysis of sections of the embryos which had been stained as whole-mounts. The staining pattern observed in the sections (Fig. 2) indicated cell-type-specific and regional variations in activation of the transgenic P4 promoter.

The CNS exhibited region-specific variation in staining intensity. In the rostral CNS, staining was stronger in the ventral areas, including the floorplate of the hindbrain and anterior spinal cord. In the caudal CNS, on the other hand, the staining was stronger in the dorsal part of the neural tube (Fig. 2B, E). Undifferentiated cells of the germinal layer and differentiated postmitotic neurons of the mantle layer both stained with approximately equal intensity (Fig. 2B). Staining was observed in the neural crest-derived trigeminal and spinal ganglia (Fig. 2B and data not shown). Weaker staining was also seen in the retina and lens of the developing eye (Fig. 2B). The nasal epithelia and the endothelia of some, but not all, blood vessels stained (Fig. 2C, D, I). Gut epithelium and surrounding smooth muscle stained. Staining was strong in cardiomyocytes and heart fibroblasts but absent or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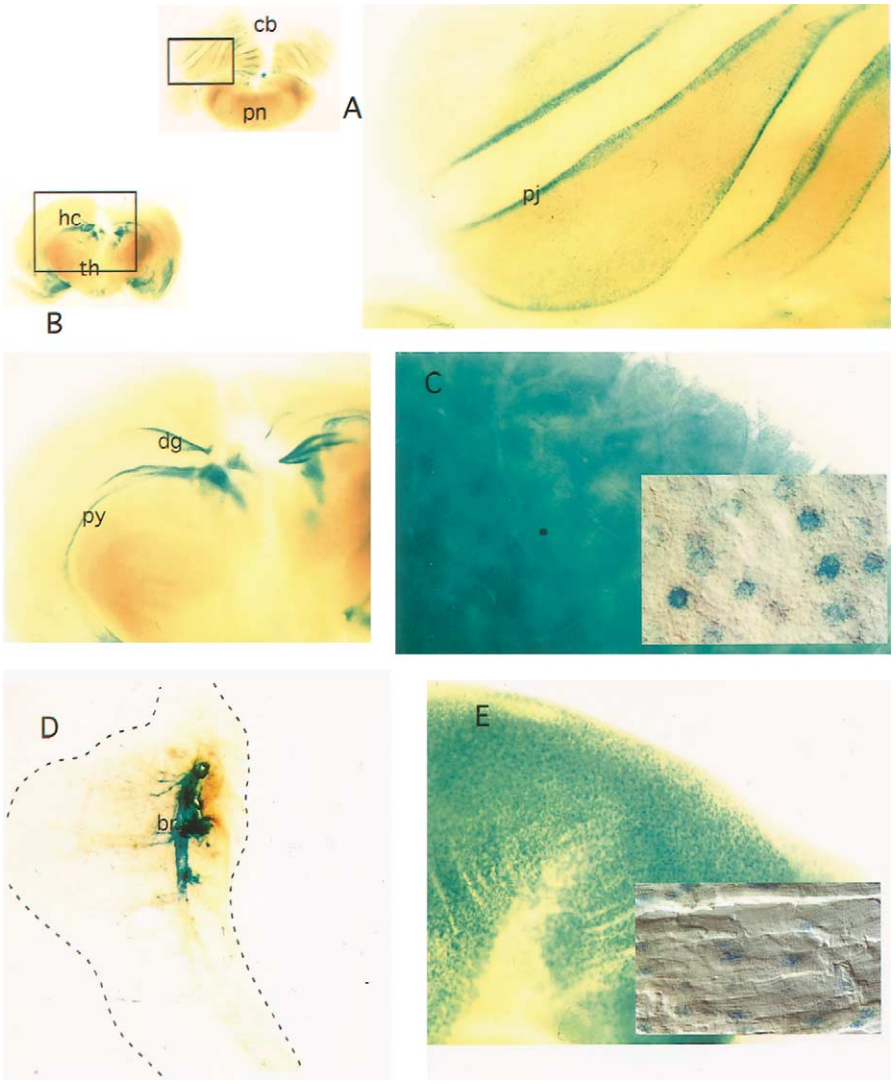

Fig. 3. Transgene expression in adult organs. Organs dissected from adult transgenic mice were stained in a whole-mount format with the β-galactosidase substrate X-gal as described under Materials and methods and cleared in BABB for photography. (A) Cut face of pontine portion of the brain and posterior aspect of the cerebellum and enlargement of boxed region showing punctate staining on folia surfaces. (B) Face of coronal slice through the hippocampus and thalamus of brain and enlargement of boxed region. (C) External aspect of tip of one lobe of the liver and section showing widespread staining in hepatocytes (largely nuclear localization). (D) Lobe of the adult lung. Dashed line demarks the border of the organ. (E) Cut face of the adult heart and section showing widespread staining in cardiomyocytes (largely nuclear localization). cb, cerebellum; pn, pons; hc, hippocampus; th, thalamus; pj, Purkinje neurons; py, pyramidal cell layer; dg, dentate gyrus; br, bronchi.

extremely weak in hepatocytes (Fig. 2E, F). In the caudal embryo, where differentiated somites were still present, staining was observed in the sclerotome (which will give rise to much of the axial skeleton) and in chondroblasts comprising the limb bud cartilage (future site of bones of the limbs) (Fig. 2E, G, H). The dermatome (future part of dermis) and the myotome (the most primitive myoblasts) did not not show any P4 activity (Fig.

Fig. 2. Distribution of transgene expression in 13.5 dpc embryonic tissues. 13.5 dpc transgenic embryos were stained in a whole-mount format with the β-galactosidase substrate X-gal, embedded in wax, and sectioned as described under Materials and methods. (A) Transgenic 13.5 dpc embryo stained in whole-mount format. Labeled lines indicate the level of the sections in the corresponding figure panels. (B) Section through embryonic head at level indicated in (A). (C) Section through embryonic head at level indicated in (A). (D) Detail of boxed region in (C). (E) Section through thorax and abdomen at level indicated in (A). (F) Detail of boxed region in (E). (G) Section through tail just posterior to the hind limb bud at the level indicated in (A). (H) Detail of the boxed region in G. Dashed lines delineate individual somites. The sclerotome and dermomyotome portions of one somite are indicated. (I) Blood vessel in developing bone primordium counterstained with eosin showing expression in endothelial cells and surrounding chondroblasts. sc, spinal cord; t, trigeminal ganglia; ml, marginal layer of developing brain; vl, ventricular layer of developing brain; e, eye; D, dorsal aspect of brain and spinal cord; V, ventral aspect of brain and spinal cord; cp, choroid plexus; ne, nasal epithelium; h, heart; l, liver; b, developing bone; fl, developing forelimb; dmt, dermomyotome portion of somite; sct, scleratome portion of somite.

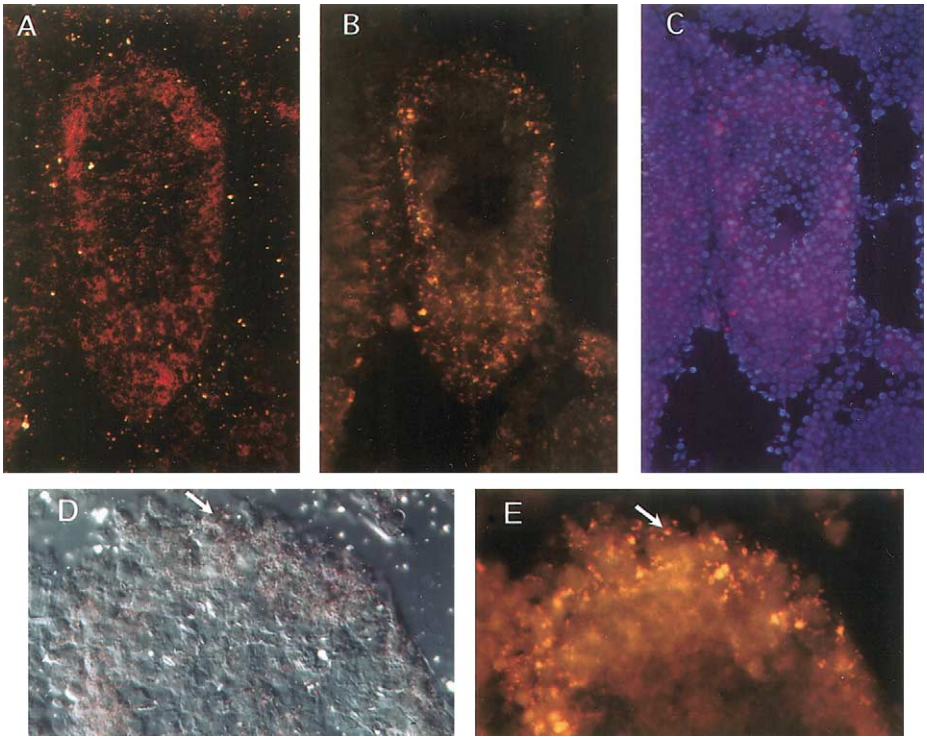

Fig. 4. Coincidence of transgene expression and productive MVM infection. Section through a testicle of 5-week-old BALB/c transgenic male which had been injected with infectious MVMp and killed 10 days later. Testicles were stained in whole-mount format for P4 driven β-galactosidase activity, sectioned, and stained for the presence of MVM coat proteins by fluorescent immunohistochemistry using an anti-MVM coat protein primary antibody and a Cy3-coupled secondary antibody. (A) Dark field image. The birefringent insoluble X-gal cleavage product indicating β-galactosidase activity appears bright pink. (B) Same field as in (A) under fluorescent illumination showing the distribution of Cy3-labeled MVM coat proteins. (C) DAPI counterstaining of same field showing the distribution of all cell nuclei. (D–E) Higher magnification showing single-cell resolution of top of tubule in (A–C). (D) DIC/dark field double exposure showing X-gal reaction product (pink). (E) Same field as in (D) under fluorescent illumination showing the distribution of Cy3-labeled MVM coat proteins. Arrows point to an individual cell showing both β-galactosidase activity and MVM coat proteins.

2G, H). Widespread, low-level staining was observed in mesenchyme.

Tissue-specific activity of the transgenic P4 promoter continues into adulthood

A tissue-specific pattern of MVM-P4-lacZ expression was retained in the adult. In the brain, it was expressed in the Purkinje cells of the cerebellum (Fig. 3A), in the dentate gyrus and the pyramidal cells of the hippocampus (Fig. 3B), and in neuronal nuclei in both the dorsal and the ventral thalamus and the pons (Fig. 3B, data not shown). Staining was widely distributed in hepatocytes (Fig. 3C). In the lungs, it was restricted to the epithelia of the larger bronchi only (Fig. 3D). P4 activitity was retained in adult cardiomyocytes (Fig. 3E). In the testes, staining was observed in the cortical portion of the seminiferous tubules coinciding with the position of the spermatagonia (Fig. 4). More differentiated germinal cells, closer to the lumen, did not stain. Specific staining was observed consistently in the heart, testicles, and ovaries of transgenic animals. The staining pattern in the liver, lungs, and brain was consistent, but was not observed in all transgenic animals examined, suggesting that the transgene may be subject to an age-dependent variable silencing in these tissues in the adult, a phenomenon well described in some transgenic lines (e.g., Cohen-Tannoudji et al., 2000; Robertson et al., 1996). Staining in negative control, nontransgenic animals was consistent and limited (in the tissues examined) to the spleen, the kidney, and sporadically in the interstitial cells in the testes. Therefore, we could not determine whether the transgene was also expressed in these tissues.

Transgenic P4 activity in the testes is restricted to cell types susceptible to MVM infection

The tissue-specific expression of the P4 transgene, described here, suggests that P4 activation may contribute to MVM tropism. One organ in the adult in which the comparison of distribution of infection versus transgene expression is feasible is the testes, which are experimentally accessible and express the transgene in mitotic cells. The testes of sexually mature, 6-week-old transgenic male BALB/c mice were injected with 3×10^4 PFU of MVMp and analyzed 10 days later. The virus was detected with anti-MVM viral coat protein antibody (Fig. 4B,E). To en-

sure that signal was due to de novo virus production, not the inoculum, control animals were injected with an equal dose of UV-irradiated MVMp. The testes were examined in cryosections for cell-specific expression of the transgene and for the presence of the viral coat antigens.

Transgenic P4 expression (Fig. 4A, D) and viral particles (Fig. 4B, E) colocalized to the cortical portion of the seminiferous tubules, where the spermatagonia are located. Neither signal was observed in the more differentiated germinal cells closer to the lumen. We did not observe any detectable viral coat protein signal in the more than 100 sections of testes collected from the animals injected with UV-irradiated MVM, indicating that the immunohistochemical signal observed was due to de novo virus production, not the injected virus.

Discussion

The construction of three independent mouse transgenic lines bearing *Lac*Z reporter sequences under the regulation of the MVM nonstructural P4 promoter is described here. Promoter-*Lac*Z transgenes have been shown to faithfully reproduce the expression patterns of numerous genes (Cui et al., 1994). However, site-specific variations in the pattern of expression may result if transgenes come under the influence of local tissue-specific enhancer/repressor sequences. The standard approach to preclude studying such an artefact has been to generate independent transgenic lines and compare their patterns of expression. Despite different chromosomal locations, all three of our independent transgenic lines exhibited the same pattern of P4 promoter activity, indicating that the transgene expression in the embryo was due to transgenic sequences, not their chromosomal environment.

The tissue distribution of staining clearly showed that the transgene exhibited stage, tissue, and differentiation-specific patterns of activity during embryogenesis and in the adult; the transgenic P4 promoter activity depended upon both the differentiation state and the cell type. It was silent during early embryogenesis, at stages containing many committed and some differentiated cell types. In older embryos, expression in particular cell lineages clearly depended upon the differentiation state. For instance, the early somites, which will give rise to many mesodermally derived tissues, did not express the transgene, but by the time the somite had differentiated into dermatome, myotome, and sclerotome, the transgenic promoter was active in the sclerotome portion only and continued to be active in the bone primordia of the axial skeleton, which are sclerotome-derived. In the CNS, expression in the undifferentiated neuroepithelium did not begin until day 11.5 dpc, 3–4 days after the formation of this tissue. Expression remained at the same level in the young postmitotic neurons of the mantle layer, which are derived from the still mitotic, underlying neuroepithelium. However, by adulthood, expression was observed in a very small subset of the differentiated neuronal cell types. In the adult testes, where differentiation status of the germ line is organized in a gradient from exterior to interior of the seminiferous tubules, the still mitotic germinal cells—the spermatagonia—activated the P4 promoter, whereas the more mature, postmeiotic spermatids did not.

Any relevance of the transgene expression pattern to understanding normal virus–host interactions depends upon how closely the expression pattern of the integrated P4 promoter reflects its activity in the normal context of the episomal viral genome. Certainly some viruses having a distinct tissue or cell-type tropism contain promoter elements which direct expression to the same cell types in transgenic animals (Baskar et al., 1996b; Cid et al., 1993; Honigwachs et al., 1989). The sites of activity of the major immediate-early promoter (MIEP) of human cytomegalovirus (HCMV) during murine embryogenesis in transgenic setting correlate with known target tissues of congenital viral infection in human fetuses (Fritschy et al., 1996; Koedood et al., 1995) and may partially determine the tissue tropism of the virus in utero (Baskar et al., 1996a). It is worthwhile noting that the regulatory promoter (P6) of human parvovirus B19 was sufficient to confer upon a helper-dependent parvovirus, adenoassociated virus (AAV), the ability to replicate independently in the B19 normal host erythroid cells (Wang et al., 1995). In the case of the MVM P4-reporter transgene described here, the viral promoter sequence included the entire region upstream of the NS genes comprising all known promoter elements (Fig. 5) (Deleu et al., 1998). P4 promoter activity has been studied in episomal contexts, both viral and plasmid (Ahn et al., 1989; Christensen et al., 1993; Cornelis et al., 1988; Deleu et al., 1999; Fuks et al., 1996; Gu et al., 1995; Hanson and Rhode, 1991; Harris et al., 1999; Perros et al., 1995; Spegelaere et al., 1991), and in chromosomally integrated stable transfectants (Deleu et al., 1998). The only observed context-dependent modulation of P4 activity was shown to be mediated by NS1 (Hanson and Rhode, 1991). There is, therefore, reason to think that tissue distribution of the transgenic promoter activity may be similar to that of the promoter in the wild-type context.

Further support for this possibility comes from comparing both the temporal and the spatial patterns of activation of the transgenic promoter with the cell-type host range of MVM (Table 1). The general timing of embryonic susceptibility to MVM infection matches the timing of transgene expression. Pregnant mice infected during early gestation were reported to develop placental infection, while the embryos were unaffected. Infection during mid and late gestation resulted in intermittent transplacental fetal infection (Kilham and Margolis, 1970, 1971, 1975; Margolis and Kilham, 1975). Embryonic stem cells (equivalent to the inner cell mass cells of the blastocyst, in which we do not observe transgenic P4 promoter activity) are resistant to infection. Similarly, mouse embryonal carcinoma cells (ECC) are resistant to MVM replication, whereas more

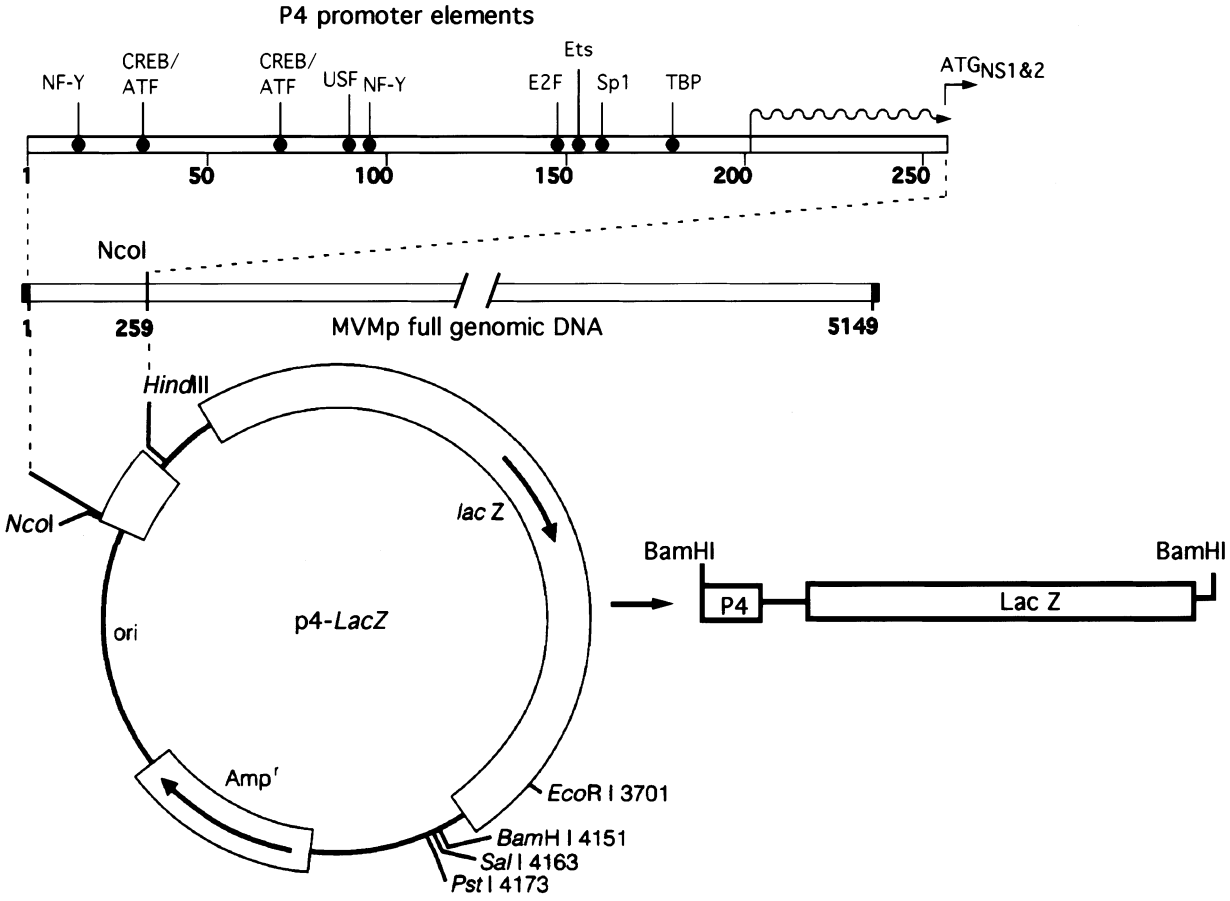

Fig. 5. The P4-LacZ transgene construct. The SV40 promoter/enhancer of the plasmid pSV-β-galactosidase (Stratagene) was excised by *Hin*dIII and *Nco*I cleavage and replaced by a 259 *Bam*HI-*Nco*I left-end fragment of the MVM genome, containing the P4 promoter and its upstream elements (top). The promoter elements (Deleu et al., 1998) are indicated: NF-Y, CREB/ATF, USF, E2F, Ets, Sp1, and TBP. Wavy arrow indicates start of transcription. $ATG_{NS1\&2}$ marks start of coding sequence of NS1 and NS2 proteins. The transgene was excised for injection by cleavage with *Bam*HI (right).

differentiated ECC-derived cell lines are suceptible (Miller et al., 1977). In our initial studies on embryonic succeptibility to MVM infection, we observed infection in embryonic spinal ganglia, chondroblasts, and notochord (Table 1) (Itah et al., unpublished results), tissues which express the transgene but have not been previously identified as MVM permissive. In the transgenic mice, the P4 promoter was inactive until midgestation and was then widely expressed in the late-gestation embryo. Also, we have not yet identified embryonic tissues in which the transgene was silent yet were succeptible to MVM infection. Almost all cell types known to be susceptible to MVM infection also activated the transgenic P4 promoter (Table 1). The only exception was lung capillary endothelium, where infection has been described, but where we did not observe transgenic P4 activity. We observed viral infection in the spermatagonia-a mitotically active, easily accessible tissue which also expressed the transgene. There were cell types which activated the transgenic P4, but nonetheless are refractive to infection, for instance, the Purkinje neurons in the brain. This is to be expected, since host activation of the viral P4 promoter is necessary, but not sufficient for productive MVM infection. The comparison of transgenic P4 promoter activity with MVM infectivity suggests that the integrated genomic context of the transgenic promoter is not drastically altering its tissue specificity.

Nevertheless, there remains the possibility that promoter activity is differentially modulated in the viral episomal context as compared to the chromosomally integrated context. For instance, episomal viral DNA is involved in replication as well as viral gene expression, and there could be proteins interacting with the viral terminal hairpin structures that could alter the tissue specificity of the promoter. For this reason, the use of a transgenic P4 promoter in this study must be considered an experimental caveat with respect to the activity of the same promoter in its viral context. The levels of expression of transgenic and wild-type promoters are also almost certainly different. Transgene expression levels are often affected by transgene copy number, typically in inverse correlation with the number of tandem transgenes at the insertion locus (Henikoff, 1998). Most likely the promoter activity from the one to three copies that are present in the transgene is less than that observed during infection. Also, P4 promoter activity during infection is

Table 1
Correlation between transgenic P4 promoter activity and MVM tropism

Cell type	Transgene expression	Viral tropism	Reference
Endothelium	+	+	a, b
Smooth muscle	+	+	b
Mesenchyme/fibroblasts	+	+	b
Chondroblast/osteoblast (bone)	+	+	e
Undifferentiated germ cells	+	+	d
Differentiated germ cells	−	−	d
Skeletal muscle	−	−	a, b
Hepatocytes	+	−	a, b
Cardiomyocytes	+	−	a
Lens	+	−	e
Epithelium	+	nd*	a
Hematopoietic lineages	nd	+	a, b
Nervous system			
Pontine nuclei	+	+	c
Dentate gyrus	+	+	c
Proliferating neuroepithelium	+	+	c
Laterodorsal thalamic nuclei	+	+	c
Spinal ganglia	+	+	e
Pyramidal cells	+	−	c
Purkinje cells	+	−	c
External granular layer cells	nd	+†	b

Note. Tropism of MVM for murine host cells and the tissue distribution of the P4-LacZ transgene activity are listed by tissue type. Data from different age host animals, different viral strains, and different murine host strains are pooled. Viral strains and references are listed on the right. (+) Positive for either infection or expression. (−) Negative for infection or expression. (nd) Not determined. References are (a) Brownstein et al. (1991); (b) Brownstein et al. (1992); (c) Ramirez et al. (1996); (d) results of this study; (e) Itah et al. (unpublished data).

* Although not described as a target tissue in the literature, the fact that oronasal application of MVM is a typical experimental infection route suggests that epithelium may become infected.

† Infection has been described in the external granular layer and in the granular neurons of the cerebellum, which migrate from the still mitotic external granular layer. Since the granular neurons are postmitotic, it was proposed that the signal observed in these was due to infection prior to migration (Ramirez et al., 1996).

modulated by its own NS transcription products, which are absent in the transgenic system.

A tissue-specific regulation of the viral P4 promoter could be relevant to MVM host range determination. According to current understanding, a cell which is not able to initiate transcription of the P4 promoter after initial production of double-stranded viral DNA should not be able to sustain a productive MVM infection. Thus, activation of P4 by the host cell is a critical step in the viral life cycle. One of the most stringent requirements for productive MVM infection is a mitotic cell population, yet it cannot be argued that P4 activation is a universal feature of mitotic cells since the transgenic P4 promoter is silent in the early embryo. Indeed, the complex pattern of activity of the transgenic promoter in the context of the almost fully mitotic embryo raises the possibility that some cells may be refractory to infection solely because they are incapable of initiating transcription of the P4 promoter. If so, activation of P4 should be considered one of the host cell-type determinants of the MVM virus. Although the pattern of transgene activity and its correlation with the pattern of MVM infectivity suggests that this may be so, it is not proven. Further testing will come from extending the initial correlation presented here. This research program is currently being executed in the developing embryo. More definitive data would also come from observing a change in host cell-type specificity following infection with MVM bearing a modified P4 promoter.

Finally, the data suggest that the P4 promoter is worth considering in attempts to harness the ability of the autonomous parvoviruses to preferentially destroy tumors and transformed cells for therapeutic purposes. The initial experimental systems to engineer the parvoviruses as potential cancer therapy vectors have relied upon the activity of the native P4 promoter (Dupont et al., 2000; Haag et al., 2000; Kestler et al., 1999; Moehler et al., 2001; Russell et al., 1992; Telerman et al., 1993). The tissue-type and differentiation state specific activity of the transgenic P4 promoter described here may provide some indication of the types of cancers which could be potential targets for this approach.

Materials and methods

Construction of pMVMpP4-LacZ vectors

The MVMp P4-driven *Lac*Z expression vector is shown in Fig. 5. The SV40 promoter/enhancer sequence in the vector pSV-β-galactosidase (Promega) was excised by *Hin*dIII and *Nco*I cleavage and replaced by blunt-end ligation with the 259-bp *Bam*HI-*Nco*I fragment of plasmid pMM984, which contains the MVMp P4 promoter and its upstream elements (Merchlinsky et al., 1983). The junction was verified by sequencing, and β-galactosidase activity was confirmed in transfected NBE (SV40-transformed human embryo kidney) cells, a cell line in which the P4 promoter is active.

Production of transgenic mice

The P4-LacZ fragment was excised from the plasmid by *Bam*HI cleavage, DNA purified, and injected into the male pronuclei of FVB/N fertilized eggs, which were implanted into pseudopregnant female BALB/c mice. Twenty-eight offspring were obtained from injected eggs, and three of these, identified as transgenic by dot blot and Southern blot analyses of tail DNAs, were chosen for further study. For dot blot analysis the DNA was left uncut; for Southern analysis the DNA was cut with *Bam*HI, *Eco*RV, or *Hin*dIII, electrophoresed on 1% agarose gel, and transferred to nitrocellulose. Presence of the transgene was detected by hybridization with ^{32}P-labeled fragment containing MVM promoter and β-galactosidase sequences. Animals which tested positive were mated with BALB/c wild-type mice and the F1 generation mice were similarly analyzed. In

further generations, transgenic animals were identified by PCR of tail DNA using the primers 5′-GCGAAAAG-GAAGTGGGCGTGGT-3′ (nt 147–168 within the MVM sequence) and 5′-GGGATCGATCTCGCCATACAGCG-3′ (nt 210–477 within the β-galactosidase open reading frame), which amplifies a ca. 500-bp transgene-specific fragment.

Whole-mount analysis of β-galactosidase expression

The morning when a plug was observed was considered to be 0.5 dpc. Embryos were collected at 3.5 (blastocysts), 7.5 (late gastrulation), 8.5, 9.5, 10.5, 11.5, 12.5, and 13.5 dpc and then were fixed intact in 4% paraformaldehyde in PBS for no more than 40 min at room temperature. Older embryos and adult organs were bisected after 10 min and then fixed for an additional 30 min. Staining with the β-galactosidase substrate X-gal was done according to a standard procedure (Bonnerot and Nicolas, 1993). Embryos were stained for 20 to 36 h at 30°C. Samples were cleared by dehydration through an ethanol series followed by incubation in a mixture of benzyl alcohol:benzyl benzoate (1:2).

Histology

Stained 13.5-dpc heterozygous transgenic embryos were dehydrated through an ethanol series into xylene and embedded in paraffin wax. Serial sections cut at 25 μm were collected, dewaxed, and mounted in permount.

Viral infections

Sexually mature, 6-week-old transgenic males were anesthetized with Metofane to effect. CsCl-purified MVMp stock virus, titered by plaque assay on NB324K cells (Tattersall and Bratton, 1983), was diluted to 1000 PFU/μl. Thirty microliters was injected into each testicle. Negative control mice were injected with the same dose of UV-inactivated MVM. Mice were sacrificed 10 days after injection and their testes analyzed.

Combined immunohistochemistry and β-galactosidase staining

The injected testes were first stained for β-galactosidase activity in the whole-mount format as above. They were then frozen in OCT and cryosections collected for immunohistochemistry. Viral capsid proteins were identified by immunohistochemistry using a rabbit polyclonal antiserum against the MVM-coat proteins and a Cy3-labeled antiserum against rabbit IgG. Localization of *lac*Z and viral coat proteins in the same sample was accomplished by photography first under dark field illumination to detect the X-gal staining product. The same field of view was then photographed under epifluorescence to identify the viral coat proteins. Sections were counterstained with DAPI for visualization of all cell nuclei.

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