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Episomal Maintenance of a Bovine Papilloma Virus Vector in Transgenic Mice

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We have used a bovine papillomavirus-based vector to generate transgenic mice. Transgenic mice result from either pronuclear or cytoplasmic injections of the vector into fertilized eggs. Of 30 mice generated by microinjection, 27 (90%) contained the vector in its episomal form, at less than one copy per cell. This represents a highly efficient means of gene transfer in which the transgene is in a controlled genetic environment.

Gene transfer to mouse embryos has become an effective method to generate model systems for the study of gene expression in vivo (12). In transgenic mice many of the transgenes are expressed appropriately in a tissue-specific manner. However, there is considerable variation in the level of expression when comparing the same transgene in different animals (13). In addition, a low level of expression is often observed in inappropriate tissues. These quantitative inconsistencies are usually attributed to the site of integration for the transgene.

Integration of the transgene appears to be random, with the possibility of multiple integrations occurring at different points in the mouse's genome (12). At these sites the transgene is usually incorporated as a multicopy tandem repeat. Although there is little direct evidence, it is believed that the host flanking DNA sequences and chromatin structure, as well as the unnatural repeat organization of the transgene, influence the expression of the transgene to result in the variable expression observed. These problems may be overcome if methods that use homologous recombination could be devised. However, at this time the low efficiency of such methods precludes their use. The alternative is to generate mice in which the transgene is present in an unintegrated form. Since the gene is no longer influenced by the DNA of the host, it should then be theoretically possible to modify the expressiosn of the gene through the use of tissue-specific enhancers and promoters. Our goal is to establish such a system. Toward this end we have been studying the suitability of bovine papillomavirus (BPV)-based vectors for the generation of transgenic mice.

BPV is a double-stranded DNA virus belonging to the papovavirus family. The DNA alone is capable of transforming rodent cells in culture and causing tumors in mice (3, 6). In the transformed cells the viral genome exists as an unintegrated low-copy episome. Both transformation and episomal maintenance properties are encoded in a subgenomic fragment termed BPV_{69T} which contains eight open reading frames (E1 to E8) (6, 7). Plasmid constructions containing the intact BPV-1 genome or BPV_{69T} have been used to express a variety of genes in cultured cells (5, 10, 11). These properties make BPV an attractive choice for generating transgenic mice.

The vector used in these experiments is pBPVΔ MMT.CAT (Fig. 1). It contains the BPV_{69T} fragment from the unique *Hin*dIII to *Bam*HI (converted to *Sal*I) restriction sites, with an 880-base pair deletion from the unique *Bst*XI site to the adjacent *Acc*I site. This destroys the E5 transforming region as well as the E2, E3, and E4 open reading frames (17), although the plasmid maintenance elements are retained. The vector also contains the mouse metallothionein-1 (MMT) promoter fused to the bacterial chloramphenicol acetyltransferase (CAT) gene.

The transgenic mice were generated essentially as described by Brinster et al. (1). Single-cell mouse embryos were obtained from superovulated ICR females mated with $B6C3F_1$ males. After microinjection of approximately 200 molecules of pBPVΔMMT.CAT, the embryos were transferred to the oviducts of synchronized ICR foster mothers. After the pups were weaned, DNA was isolated from tail biopsies and analyzed by Southern blotting (9, 15).

In general, the methods used to generate transgenic mice have been optimized to favor the integration of a linear DNA fragment. Since our objective was to avoid integration, we injected undigested DNA into the male pronucleus or the cytoplasm. The injected DNA consisted predominantly of forms I and II, and the efficiencies of microinjection and embryo transfer are described in Table 1. The surprising feature of this system is the exceptionally high frequency of transgenic mice. Virtually all of the pups screened were transgenic. This is a 5- to 10-fold increase in efficiency over the injection of linear DNA fragments (1). This high efficiency was observed for both nuclear and cytoplasmic injections.

Figure 2 is a Southern blot using DNA isolated from 10 of the 24 mice resulting from pronuclear injections. It is apparent that in all 10 of the samples there are three bands corresponding to the expected fragments for digestion of pBPVΔMMT.CAT with the restriction enzyme *Eco*RI. Of the 30 mice screened, one was negative and two (Fig. 2, no. 54, for example) contained extra bands suggestive of integration or rearrangement of the plasmid. Thus, 27 of 30 mice contained only the expected bands consistent with intact episomal copies of the vector. However, the intensity of hybridization is considerably less than the standard of one copy per diploid mouse genome, indicating that all of the mice are mosaic. The intensity of hybridization corresponds to approximately 10% of the cells containing one episome (Fig. 3 and 4), although other distributions with more copies in fewer cells are certainly possible. In fact, transmission data (below) suggest that germinal cells contain approximately one copy or more per cell. In general, the degree of hybridization was similar among the 29 positive mice, with

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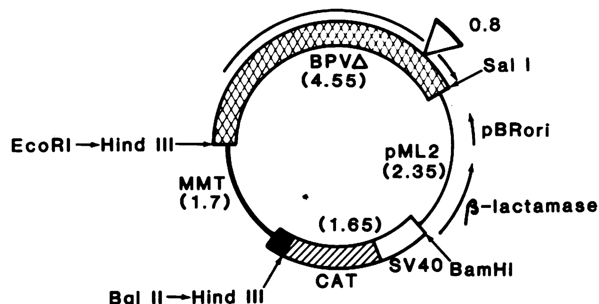

FIG. 1. Construction of pBPVΔMMT.CAT. The deleted BPV_{69T} fragment with the adjoining pML2 sequences was obtained from the vector BPVΔneo (M. Reff, unpublished data). After deletion of the neo cassette from BPVΔneo, the CAT/simian virus 40 (SV40) sequences from the vector $pA_{10}CAT_2$ (4) were inserted to generate pBPVΔCAT. This intermediate was used as a probe for Southern blotting and also to titrate control mouse DNA for copy number standards. Finally, the *Eco*RI/*Bgl*II MMT promoter fragment (with *Hin*dIII linkers) from the vector pdBPV-MMT neo (342-12) (5) was inserted to yield pBPVΔMMT.CAT.

the variation between samples probably not being greater than two- or threefold.

To examine the uniformity of the mosaicism within the animal, DNA from tissue samples of mouse 61 was analyzed by Southern blotting. All of the tissues examined were mosaic, with the spleen, testes, skin, and muscle containing more copies than the liver, kidney, intestine, and brain (results not shown). No distinguishing phenotype is associated with mice carrying pBPVΔMMT.CAT.

Two offspring from the mating of a transgenic BPV male to an ICR female were screened by Southern blotting of DNA

TABLE 1. Efficiencies of microinjection and embryo transfer for pBPVΔMMT.CAT

Parameter	No. (%)	
	Cytoplasmic	Nuclear
Injected	366	1,156
Survived	138 (38)	670 (58)
Transferred	138 (38)	634 (55)
Pups born	13 (3.6)	34 (2.9)
Pups weaned	6 (1.6)	24 (2.1)
Transgenic	6 (1.6)	23 (2.0)

from tail biopsies (Fig. 4). Both are positive and mosaic, carrying an average of fewer than one episome per cell. Four additional offspring from the same litter and five from the mating of a transgenic BPV female also were screened. All appeared positive but the bands were just visible, suggesting a decrease in copy number from the F_0 generation. This high frequency of transmission for episomal elements was observed also by Rassoulzadegan et al. (14). It implies that the copy number of the episome in germinal cells must be close to one per cell.

To examine the stability of the episome with maturation of the animal, DNA was isolated from tissues of 6-month-old transgenic mice. The weak signals in the original blots make quantitation difficult; however, it appeared that there could be some loss with age. We are currently doing studies to see if there is a continuous or significant loss accompanying ontogeny of the mice.

CAT enzyme activity (2) was not detectable in any of the tissues examined, although the vector was expressed and

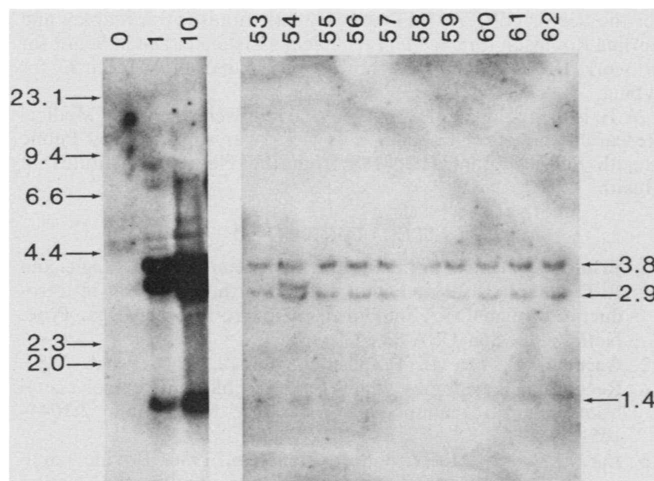

FIG. 2. Transfer of pBPVΔMMT.CAT to mice. Each lane in the left panel contains 15 μg of control mouse DNA titrated with plasmid pBPVΔCAT to the equivalent of 1 and 10 copies per diploid genome per 15 μg of DNA and digested with *Eco*RI. The right panel contains 15-μg DNA samples isolated from tail biopsies of 10 mice after injections with pBPVΔMMT.CAT. The samples were electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pBPVΔCAT. The slight difference in the mobility of the 2.9-kb fragment is due to the different *Eco*RI digestion patterns of pBPVΔCAT and pBPVΔMMT.CAT. Also, digestion with *Eco*RI releases a 2-kb fragment from pBPVΔMMT.CAT (samples 53 to 62) which does not hybridize to the probe pBPVΔCAT.

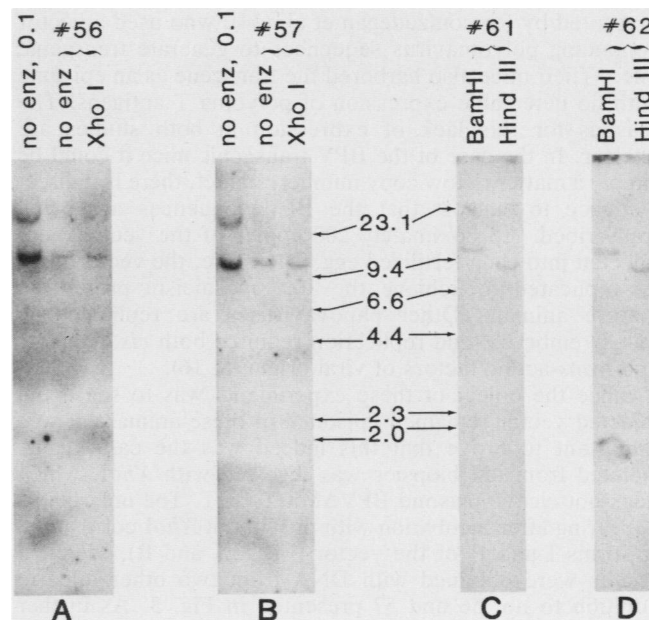

FIG. 3. Episomal maintenance of pBPVΔMMT.CAT. DNA from tail biopsies of mice 56, 57, 61, and 62 was electrophoresed through 0.8% agarose gels, transferred to nitrocellulose, and hybridized with nick-translated pBPVΔCAT. Mice 56 and 62 are females and mice 57 and 61 are males. Each lane in (A) and (B) contains 20 μg of DNA and each lane in (C) and (D) contains 15 μg. DNA in the lanes marked "no enz, 0.1" was titrated with plasmid pBPVΔMMT.CAT to the equivalent of 0.1 copy per diploid genome per 20 μg. DNA was undigested (no enz) or digested with the enzymes indicated.

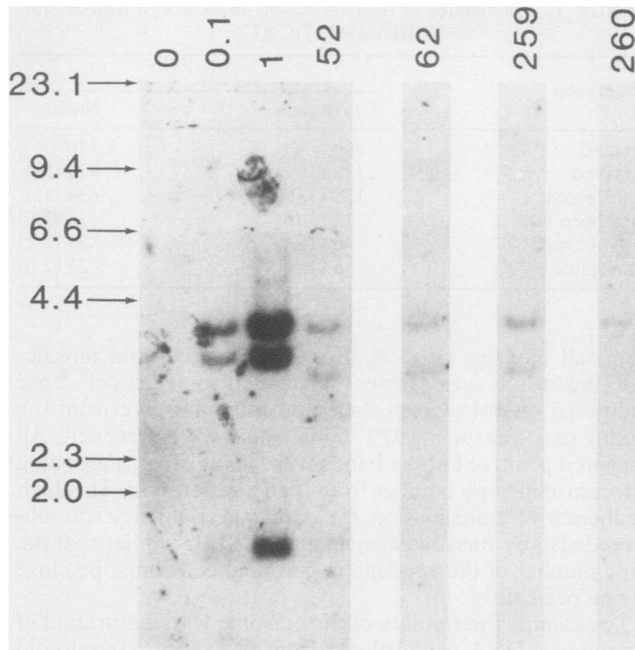

FIG. 4. Transmission of pBPVΔMMT.CAT. A 15-μg portion of DNA from tail biopsies was electrophoresed through a 0.8% agarose gel, transferred to nitrocellulose, and hybridized with nick-translated pBPVΔCAT. Mice 52 and 62 are F_0s and mice 259 (female) and 260 (male) are F_1s. Lanes marked 0, 0.1, and 1 are copy number controls as described in the legends to Fig. 2 and 3.

induced by $CdCl_2$ when transfected into mouse C127 cells (results not shown). These results are similar to those presented by Rassoulzadegan et al. (14), who used a vector containing polyomavirus sequences to generate transgenic mice. Their mice also harbored the transgene as an episome with no detectable expression of polyoma T antigens. The reasons for this lack of expression in both studies are unclear. In the case of the BPV transgenic mice it could be simply a matter of low copy number. In fact, there is indirect evidence to suggest that the BPV sequences are being transcribed. Approximately 200 copies of the vector were injected into each fertilized egg. Therefore, the vector must be replicated to achieve the 10% mosaicism present in mature animals. Other papovaviruses are replicated in mouse embryos, and replication requires both *cis* elements and *trans*-acting factors of viral origin (8, 16).

Since the object of these experiments was to see if the injected vector remained episomal in these animals, it was important to prove that this indeed was the case. DNA isolated from tail biopsies was digested with *Xho*I, which does not cleave plasmid BPVΔMMT.CAT. The only bands appearing after incubation with or without *Xho*I correspond to forms I and II of the vector (Fig. 3A and B). Identical results were obtained with DNA from two other mice in addition to no. 56 and 57 presented in Fig. 3. As further proof, DNA from mice 61 and 62 was digested with *Bam*HI, which linearizes the plasmid, or *Hin*dIII, which removes the 1.7-kilobase (kb) MMT promoter fragment (Fig. 1). Again, only the expected bands at 10.25 and 8.5 kb, respectively, were detected. The 1.7-kb MMT promoter fragment was not detected after *Hin*dIII digestion because the probe used lacks that element. This was done to avoid confusion that would result from hybridization to the endogenous metallothionein genes.

Recently, Lacey et al. (3) published a report describing a transgenic mouse carrying BPV sequences. They used a linear fragment with a repeat of the BPV_{69T} fragment. Offspring from this mouse contained integrated forms of the transgene in normal tissues and extrachromosomal forms in tumors. From these studies and ours, it appears that, although the BPV virus has the capacity to exist as an episome, integration is possible by injecting a modified linear form of the viral DNA. This idea is supported by the fact that circular polyomavirus-based vectors remain episomal in transgenic mice (14).

These experiments show that by using a BPV-based vector it is possible to generate transgenic mice in which the transgene is present in an unintegrated form. In contrast to the results of Rassoulzadegan et al. (14), who used a polyomavirus-based vector, most of the BPV transgenic mice appear to have unrearranged episomes. The authors proposed that the recombinational activity could be the result of T antigen interacting with viral sequences, although they were unable to detect T-antigen expression in the mice. All of the BPV mice are mosaic for the vector. The resulting low copy number could explain the lack of detectable CAT activity in the tissues. One possible explanation for the mosaicism is that, during development, replication of the vector does not keep pace with replication of the host DNA. Recently, it has been shown that an enhancerlike element located outside of the BPV_{69T} fragment is necessary for replication (8). Our future efforts will involve construction and injection of other BPV vectors to attempt to improve the copy number and expression. Finally, the ability to generate transgenic mice at high efficiency and with relative ease, through both cytoplasmic and pronuclear injections, adds a new dimension to the study of genes in vivo.

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