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A polyoma-based episomal vector efficiently expresses exogenous genes in mouse embryonic stem cells

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ABSTRACT

We describe the ability of novel episomally maintained vectors to efficiently promote gene expression in embryonic stem (ES) cells as well as in established mouse cell lines. Extrachromosomal maintenance of our vectors is based on the presence of polyoma virus DNA sequences, including the origin of replication harboring a mutant enhancer (PyF101), and a modified version of the polyoma early region (LT20) encoding the large T antigen only. Reporter gene expression from such extrachromosomally replicating vectors was approximately 10-fold higher than expression from replication-incompetent control plasmids. After transfection of different ES cell lines, the polyoma virus-derived plasmid variant pMGD20neo (7.2 kb) was maintained episomally in 16% of the G418-resistant clones. No chromosomal integration of pMGD20neo vector DNA was detected in ES cells that contained episomal vector DNA even after long term passage. The vector's replication ability was not altered after insertion of up to 10 kb *hprt* gene fragments. Besides undifferentiated ES cells, the polyoma-based vectors were also maintained extrachromosomally in differentiating ES cells and embryoid bodies as well as in established mouse cell lines.

INTRODUCTION

Establishment of the mouse embryonic stem (ES) cell system has opened new ways to study gene function in a living organism. ES cells are isolated from the inner cell mass of preimplantation embryos and retain their pluripotency when cultured under suitable conditions (1). Microinjection of ES cells into a recipient blastocyst and reimplantation into pseudopregnant mothers results in the formation of a chimeric embryo which can transmit the ES cell genotype to the next generation (2). Thus, specific genetic changes of the ES cell genotype generated by homologous recombination *in vitro* can be transferred into living mice (reviewed in ref. 3). On the other hand, ES cells can be induced to differentiate into embryo-like structures known as embryoid bodies (EBs) which resemble the 6–8 day egg cylinder stage observed during normal mouse embryogenesis (4). Since embryoid bodies recapitulate several aspects of early mouse development, they have been proposed as an *in vitro* model of embryogenesis (4–8).

To explore the feasibility of maintaining exogenous DNA sequences on episomal plasmids in ES cells, we have constructed the polyoma-based vector pMGD20neo that can replicate extrachromosomally in these cells (9). The polyoma virus, one of the smallest oncogenic viruses known, is a DNA virus existing in certain laboratory mouse colonies and in some wild mice as a silent infection (reviewed in refs 10,11). The viral genome consists of a double-stranded, circular DNA of ~5.3 kb. The polyoma virus early region, which is transcribed early during the viral life cycle, is one of the rare examples in eukaryotic cells in which all three potential reading frames are used to encode three different proteins. Alternative splicing of the common precursor transcript results in three mRNAs encoding for: (i) large tumor (T) antigen, a 100 kDa nuclear protein essential for initiating viral DNA replication; (ii) middle T, a 48 kDa protein involved in cellular transformation (12); and (iii) small T, a 22 kDa protein of ill-defined function(s). The vector pMGD20neo (Fig. 3A) contains a modified segment from the polyoma early region that can only express large T (LT20), thereby avoiding expression of the oncogenic middle T. This vector also contains the mutated PyF101 enhancer-ori segment (13) that permits efficient expression of large T and DNA replication in the early embryo as well as in ES cells. We recently showed that pMGD20neo is maintained episomally at about 10–30 copies per cell for at least 74 ES cell generations in the presence of G418 (9). In the present work, we analyzed the ability of polyoma-based vectors to enhance gene expression, to support homologous recombination in ES cells, and to replicate in established mouse cell lines and differentiating ES cells.

MATERIALS AND METHODS

Construction of vectors

pUC/LT20 and derivatives. The wild type polyoma enhancer was replaced by the 802 bp *Bgl*I–*Bam*HI fragment from plasmid pPyF101 (13) harboring the mutant enhancer. To restrict the expression of the early region, the *Ava*I site at bp 660 (numbering

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according to ref. 14) was cleaved, blunted and ligated to the *Hae*III (bp 782) site. This deletion removed the 5′ splice donor site at bp 748 for both middle T and small T reading frames. Subsequently, the *Kas*I–*Hin*cII fragment (2757 bp) of the polyoma early region including this deletion was subcloned together with a *Bam*HI–*Kas*I segment composed of a 130 bp DNA sequence containing the SV40 early region polyA site in addition to the polyoma origin of replication and PyF101 enhancer into pUC19 resulting in pUC/LT20.

pMGD20neo. A *Bgl*II linker was introduced into the *Dra*II site of pMC1neopolyA (15) and followed by the substitution of the *Eco*RI–*Bss*HII (820 bp) fragment of the resulting pMC1neopolyA^{BglII+} with the 1047 bp segment (*Eco*RI–*Bss*HII) from PGKneobpA (16) generating PGKneopolyA. Subsequently, this vector was cleaved with *Bgl*II and *Ssp*I and the *Bam*HI–*Hin*cII fragment from pUC/LT20 was inserted, giving rise to pMGD20neo (see Fig. 3A). Note that the *neo* gene used in our preparations does not contain the point mutation in nucleotide 2096 which has been shown to reduce the resistance of transformants to G418 selection (17). Following the same cloning steps, we generated a variant of pMGD20neo in which the LT20 fragment was replaced by the cDNA (pPyLT1) version of large T (18).

pMC1neo-hGH and derivatives. The SVtk hybrid promoter (*Pvu*II–*Bgl*II fragment) used to drive *neo* expression in pSVtkneoβ (19) was linked to the hGH reporter gene fragment *Bam*HI–*Ssp*I (20), and the resulting segment was subcloned into the *Nde*I–*Bam*HI site of pMC1neopolyA^{BglII+} (see above) thereby replacing the *neo* cassette. Insertion of the MC1neopolyA cassette into the *Bgl*II site resulted in pMC1neo-hGH while coinsertion with a *Bam*HI–*Hin*cII fragment containing either the PyF101/LT20 polyoma sequence (from pUC/LT20) or the corresponding wild type (PyF101/wt) or cDNA (PyF101/LT1) viral sequences resulted in plasmid pMC1neo-hGH-PyF101/LT20 and its corresponding derivatives (wt and LT1).

pRVi6.8-LT20. The RVi6.8 *hprt* fragment—which contains the *neo* expression cassette in the inverted orientation compared with RV 6.8 (21)—was subcloned into an *Xho*I-deficient variant of pBluescript SKII (Stratagene) previously digested with *Eco*RI and *Sma*I. Subsequently, insertion of the *Hin*cII–*Sma*I fragment of pUC/LT20 (containing the polyoma replication unit) into the *Sma*I site yielded pRVi6.8-LT20.

pHPT$^{+1\ kb}$ LT20. The 10 kb *Bgl*II–*Bgl*II fragment encompassing the *hprt* exons 6–9 and a *neo* cassette was isolated from vector pHPT$^{+1\ kb}$ (22) and subcloned into the *Bam*HI site of pUC/LT20 yielding pHPT$^{+1\ kb}$ LT20.

Cell culture

The ES cell line CCE (23) was grown on gelatine-coated dishes without feeder cells in freshly prepared Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 20% heat-inactivated (56°C, 30 min) foetal calf serum (FCS; Boehringer–Mannheim), 1000 U/ml leukemia inhibitory factor (LIF; Gibco), 150 µM monothioglycerol (Sigma), 1× minimal essential medium (MEM) non-essential amino acids (Gibco), 100 U/ml penicillin (Gibco) and 0.1 mg/ml streptomycin (Gibco) in a humidified environment containing 5% CO_2 at 37°C and passaged every 2–3 days as described (7,8). Routinely, 10^7 ES cells in 800 µl PBS were electroporated with 10–20 µg DNA using a BioRad Gene Pulser at 240 V/960 µF. Selection for *neo* was started 24–40 h later using G418 (500 µg/ml active substance). Selection against a functional *hprt* gene was started exactly 6 days after transfection by adding 6-thioguanine to the medium to a final concentration of 1 µg/ml. Differentiation of ES cells to EBs was performed in a semisolid medium exactly as described (7,8).

Mouse embryonic carcinoma cells (F9), mouse renal adenocarcinoma cells (RAG) and mouse L-fibroblasts (L-929) were cultured in freshly prepared DMEM supplemented with 10% FCS, 150 µM monothioglycerol, 1× MEM non-essential amino acids, 1 mM sodiumpyruvate (Gibco), 2 mM L-glutamin (Gibco) and antibiotics. Mouse erythroleukemia (MEL) cells were grown in the same medium but containing 15% FCS.

Cell analysis

Quantitation of hGH protein in the supernatant of transfected ES cells was performed using the immunoradiometric assay TANDEM-R HGH (Hybritech, San Diego, CA) following the manufacturer's instructions exactly. Low-molecular-weight DNA was extracted according to a modified Hirt protocol as described (9). Total DNA extraction and Southern blotting was performed by standard methodology. For Western blot analysis, cells were lysed with RIPA buffer [150 mM NaCl, 50 mM Tris–HCl (pH 7.2), 0.5% Nonidet P-40, 1% Triton X-100, 0.1% SDS, and 1% sodium deoxycholic acid] and spun 20 min at 50 000 r.p.m. in a Beckman TL100 ultracentrifuge. The extracts were loaded onto a 7.5% polyacrylamide gel and subsequently electroblotted onto a Nytran filter (Schleicher & Schuell). Polyclonal rat antibodies against polyoma T antigens were obtained from W. Eckhart (San Diego, CA). Chemiluminescent detection was performed using an anti-rat IgG antibody conjugated to horseradish peroxidase.

RESULTS

Efficient expression of large T antigen from the modified polyoma early region (LT20) in ES cells

Since the transforming activity of polyoma middle T might alter the pluripotency of the ES cells, we sought to express large T only (required for viral DNA replication) from constructs harboring either the large T cDNA derived from pPyLT1 (18) or our polyoma LT20 version (9) which contains a mutated intervening sequence lacking the 5′ splice donor site for middle T and small T reading frames (see Materials and Methods). Both large T-encoding DNAs were fused to a mutant version of the polyoma enhancer-ori segment termed PyF101 (13). Subsequently, these polyoma early region variants were subcloned into a modified version of pMC1neopolyA (15) containing the human growth hormone (hGH) reporter gene (pMC1neo-hGH). After electroporation and G418 selection for 17 days, the cellular extracts of pooled ES cell colonies were tested for expression of large T by Western blot analysis using a polyclonal antibody recognizing all three T antigens (Fig. 1). As positive control we used the T antigens-producing cell line MOP6 (24), and for negative control, untransfected NIH3T3 and ES cells as well as ES cells transfected with plasmids which do not contain sequences encoding polyoma large T (–/– and PyF101/–). ES cells transfected with vector pMC1neo-hGH-PyF101/LT1 containing the large T cDNA produced an unspecific banding pattern which was indistinguishable from the negative controls (Fig. 1). In contrast, transfection with an analogous plasmid harboring the LT20 version (pMC1neo-hGH-

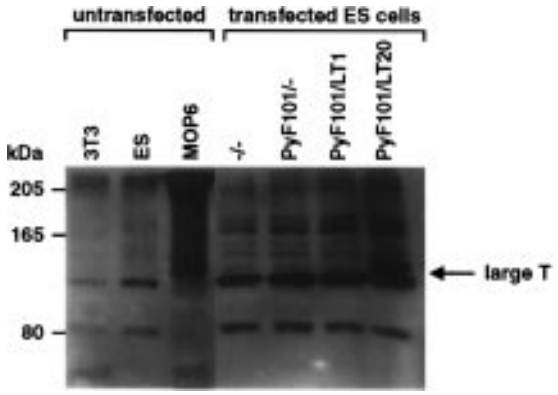

Figure 1. Western blot analysis of large T-expressing ES cells. ES cells were transfected with variants of pMC1neo-hGH harboring the PyF101 promoter linked to the large T gene from LT1 or LT20. Transfections of ES cells with the empty pMC1neo-hGH vector is indicated as (–/–). After transfection with 10µg of circular DNA, the ES cells were selected with G418 for 17 days and cellular extracts from pooled colonies were tested for large T expression. MOP6 cells were used as a positive control and untransfected ES cells and NIH3T3 as negative controls. Only construct pMC1neo-hGH-PyF101/LT20 led to large T protein production

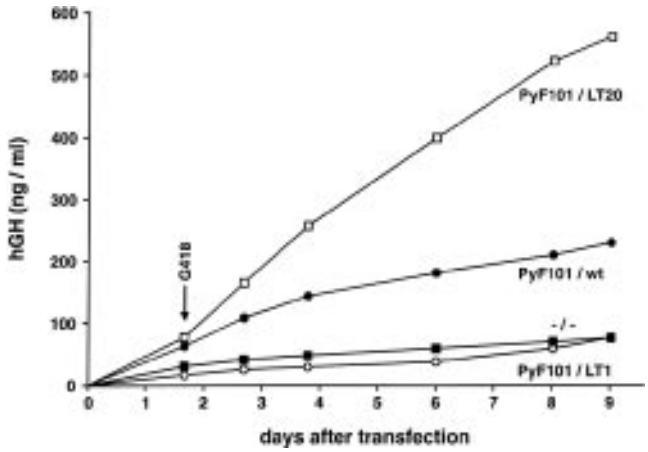

Figure 2. The presence of large T allows efficient expression of a hGH reporter gene. ES cells were transfected with equimolar amounts of the empty pMC1neo-hGH vector (–/–) or with the variants pMC1neo-hGH-PyF101/LT20, pMC1neo-hGH-PyF101/LT1 and pMC1neo-hGH-PyF101/wt. G418 selection was started 40 h later and hGH concentrations in the media were determined every 1–2 days.

PyF101/LT20) resulted in efficient production of the viral replication protein, indicating that an intervening sequence in the polyoma early region is necessary for efficient expression of large T.

The polyoma PyF101/LT20 sequence increases expression of an hGH reporter gene in ES cells

We were interested in determining the ability of the polyoma sequence present in our constructs to increase expression of other genes located on the same plasmid. Therefore, we measured the expression levels of the reporter gene hGH after transfection of ES cells. Since hGH protein is secreted by the cells (20), kinetic analysis of hGH expression was performed from periodically collected samples of the culture medium. Forty hours after transfection with circular plasmid DNA, the media was replaced and G418 selection was started. Every 1–2 days, the hGH concentrations were determined and the G418-containing medium was replaced. Figure 2 illustrates a typical transfection experiment: ES cells electroporated with the basic vector pMC1neo-hGH (–/–) only, or with the same vector containing the polyoma large T cDNA sequence (PyF101/LT1) showed about the same hGH concentrations. This observation was consistent with the immunoblot presented in Figure 1 where no polyoma large T was detected when expressed from a cDNA construct. In contrast, ES cells transfected with pMC1neo-hGH harboring either the polyoma wild type early region segment (PyF101/wt) or our modified large T version (PyF101/LT20) increased their hGH expression by a factor of 3–5 and 6–10, respectively.

Elevated hGH production in transfected cells expressing large T is probably accounted for by an increased number of vector template: after transfection, the vector DNA containing the polyoma origin of replication should undergo efficient large T-mediated replication. To confirm this, we transfected ES cells with vector pMGD20neo (9) which is a modified version of pMC1neo-hGH-PyF101/LT20 and contains a SV40 poly A site in the polyoma late region, as well as the phosphoglycerate kinase (PGK) promoter (25) to express the *neo* gene but lacks the hGH gene (Fig. 3A). Approximately 10^7 ES cells were electroporated with circular pMGD20neo (10 µg), plated into 6 cm dishes and grown in the absence of G418 for 1–4 days. Low-molecular-weight DNA was extracted every day according to a modified Hirt protocol (9) and digested with *Bam*HI, which cuts the vector only once, or with both *Bam*HI and *Dpn*I. The transfected vector DNA is bacterially methylated and can be cleaved by *Dpn*I. In contrast, eukaryotically replicated DNA which does not maintain the bacterial methylation is resistant to *Dpn*I digestion (26). Following electrophoresis, blotting and hybridization with a ^{32}P-labeled *neo* probe, *Dpn*I-resistant plasmid-size DNA was detected as early as one day after transfection (Fig. 3B). Phosphorimager quantitation revealed that about 80% of the linear DNA produced by cleavage with *Bam*HI was resistant to *Dpn*I digestion after the second day, indicating that the replicated pMGD20neo DNA persisted. In contrast, the Hirt-extracted DNA isolated two days after transfection with the plasmid pPGKneo, that cannot replicate because it lacks the LT20 segment, was sensitive to *Dpn*I digestion (Fig. 3B). Comparable transfections were carried out with a modified form of pMGD20neo in which the LT20 segment was replaced by the cDNA version of the large T transcription unit (LT1), but in this case *Dpn*I-resistant DNA was not detected (data not shown). Apparently, the cDNA version of large T produces too little viral protein (Fig. 1) to sustain measurable amounts of plasmid DNA replication. Taken together, these results indicate that pMGD20neo is efficiently replicated in ES cells after transient transfection suggesting that the elevated hGH levels are a consequence of increased numbers of DNA template.

Long-term maintenance of episomal pMGD20neo DNA shows no chromosomal integration

We recently reported that 15% of the 87 G418-resistant CCE ES cell clones have been found to maintain pMGD20neo episomally (9). Further transfection experiments (Table 1) using this CCE ES cell line (derived from the mouse strain 129/Sv) as well as the 129/OLA-derived ES cell line E14 and its *hprt*-deficient subclone

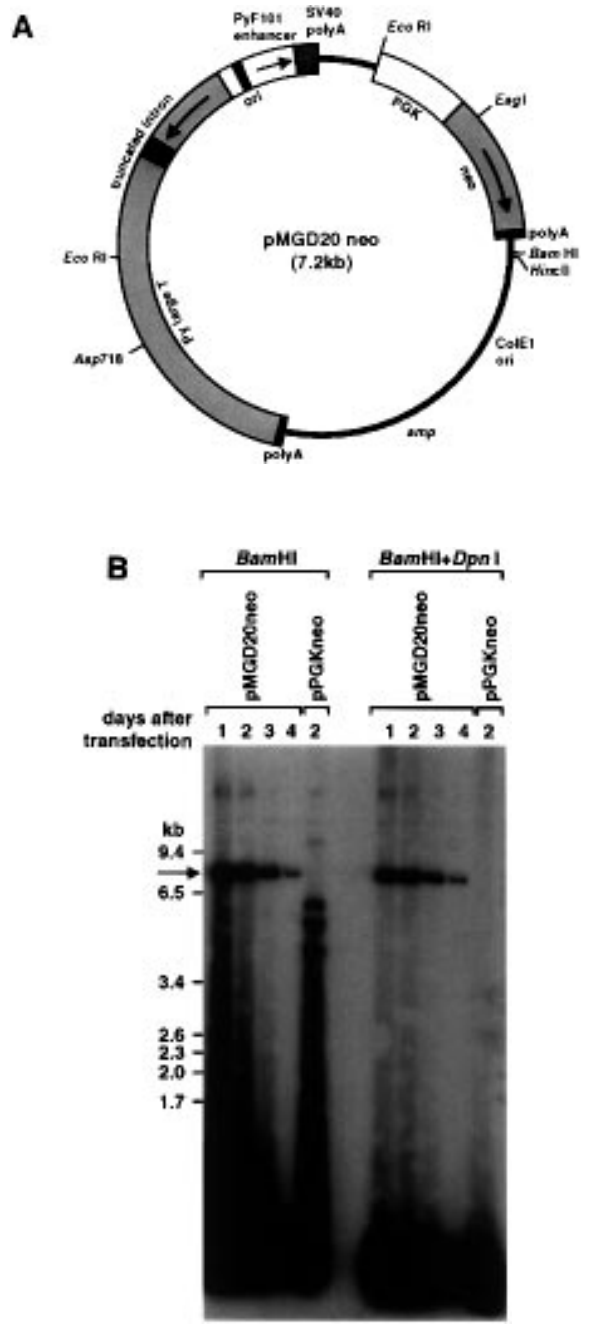

Figure 3. Transient replication of pMGD20neo DNA in ES cells. (**A**) Plasmid pMGD20neo (9) contains the mutant polyoma enhancer-ori segment PyF101, a modified polyoma early region (LT20) expressing large T only and the *neo* gene expressed from the phosphoglycerate kinase (PGK) promoter in order to confer resistance to G418. An SV40 polyA processing site was cloned into the polyoma late region. Restriction sites used in this study are indicated. (**B**) After electroporation of 10^7 ES cells with 10 µg of supercoiled pMGD20neo DNA, the cells were plated onto 6 cm dishes and grown in the absence of G418 for 1–4 days. Comparable transfections were made with equimolar amounts of supercoiled pPGKneo DNA which is replication-incompetent. After the stated periods of time, low-molecular-weight DNA was isolated by a modified Hirt procedure (9) and digested with *Bam*HI which cleaves pMGD20neo once (left side) or with both *Bam*HI and *Dpn*I (right side). The digested samples were electrophoresed in a 0.8% agarose gel, blotted and hybridized with a ^{32}P-labeled *neo* DNA probe. Unreplicated plasmid and its degradation products (smear) are fully sensitive to the combined digestion with *Bam*HI and *Dpn*I, whereas replicated pMGD20neo DNA is resistant to *Dpn*I digestion.

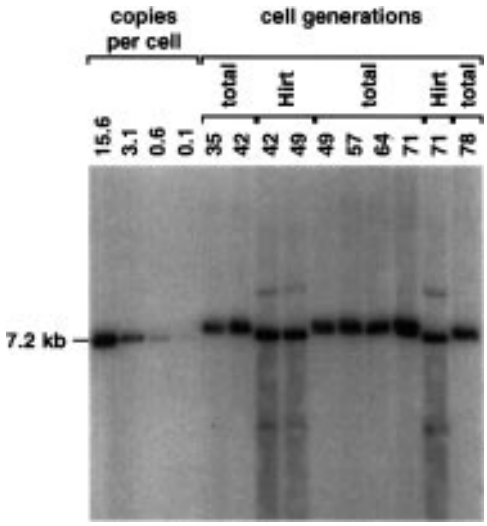

Figure 4. Extrachromosomal pMGD20neo DNA does not integrate into the chromosome for at least 78 cell generations. ES cell clone 1.19 (9) harboring pMGD20neo as an episome at 10–30 copies per cell, was cultured for up to 78 cell generations in the presence of G418. At the indicated time points, total DNA as well as low-molecular-weight (Hirt) DNA was extracted and digested with *Asp*718 which cleaves pMGD20neo once. After electrophoresis and Southern blotting, the filters were hybridized with a ^{32}P-labeled probe containing most of the pMGD20neo DNA but lacking the PGK promoter segment *Eco*RI–*Eag*I (see Fig. 3A).

E14TG2a (27) confirmed this observation: 32 out of 204 (16%) of the G418-resistant ES cell clones contained the plasmid as an extrachromosomal element. Previously, analysis of individual clones (e.g. clone 1.19) derived from experiment 2 (Table 1) revealed that the transfected DNA persisted as an episome without detectable chromosomal integration of plasmid DNA for 28 cell generations (9). To investigate whether plasmid copies integrate into the chromosome as the cells are further passaged in culture, we analyzed chromosomal DNA from ES cells grown for 78 cell generations. Total DNA (containing chromosomal and episomal DNA) as well as low-molecular-weight DNA was extracted from the vector-containing clone 1.19 grown in the presence of G418. The isolated DNA was digested with *Asp*718 which linearizes pMGD20neo and thus gives rise to a 7.2 kb fragment indicative of episomal DNA as well as of any integrated concatemeric DNA. However, integrated DNA will also produce additional fragments of varying sizes. As shown in Figure 4, Southern blot analysis failed to detect any additional bands indicative of integrated copies for up to 78 cell generations after transfection, implying that pMGD20neo DNA is maintained solely as an extrachromosomally replicating plasmid in these ES cells during long term passage. Moreover, the episomal DNA was stable during this long-term passage as the plasmids retained a functional bacterial gene that confered resistance to ampicillin in *Escherichia coli* (data not shown).

Gene targeting in ES cells using an episomal vector

As our polyoma-based vector is maintained episomally during a prolonged period of time, we sought to exploit this property to increase the efficiency of homologous recombination events in ES cells. To this end, we compared the targeting frequency of a ‘classical’ linearized targeting vector with a circular replication-

competent vector containing the identical target fragment. We decided to disrupt the endogenous X-linked *hprt* gene in male ES cells since the ability to select for and against its expression made *hprt* a convenient gene for targeting experiments (28,29). The *hprt* gene was targeted by using either pRVi6.8 (30) harboring *hprt* exons 2 and 3, the latter exon being disrupted by insertion of a *neo* expression cassette derived from pMC1neopA, or pHPT$^{+1\ kb}$ (22) containing *hprt* exons 6–9, exon 8 being disrupted again by the same insertion (Fig. 5). Both *neo*-containing *hprt* sequences were subcloned into pUC/LT20 which contains the identical viral replication unit as pMGD20neo (i.e. the polyoma origin of replication, PyF101 and LT20) giving rise to pRVi6.8-LT20 (13.8 kb) and two variants of pHPT$^{+1\ kb}$ LT20 (16 kb) which differ solely by the orientation of the *hprt* sequence (Fig. 5). Southern blot analysis of ES cells transfected with these circular targeting vectors revealed that all *hprt*-containing plasmids replicated as efficiently as the original pMGD20neo vector for at least 17 days in the presence of G418 as judged by resistance to *Dpn*I cleavage. This indicates that the polyoma replication unit is able to support replication of plasmid DNA with a total length of at least 16 kb (data not shown).

Table 1. G418 resistant ES cell clones containing extrachromosomal pMGD20neo DNA

Exp. #	ES cell line	Number of G418^R clones tested	Clones replicating pMGD20neo DNA
1	CCE	21	3
2	CCE	21	3
3	CCE	10	2
4	CCE	19	2
5	CCE	16	2
6	E14	37	6
7	CCE	7	1
8	E14TG2a	12	1
9	CCE	15	2
10	CCE	23	6
11	CCE	23	4
	Total	204	32 (16%)

Targeting frequency to the *hprt* locus in ES cells transfected with replication-competent circular vectors were compared with cells transfected with 'classical' linearized targeting vectors (lacking any polyoma sequences). After transfection, the cells were double-selected with G418 for the presence of the *neo* gene and with the purine analog 6-thioguanine (6-TG) against the presence of a functional *hprt* gene. Figure 5 summarizes the outcome of these targeting experiments: while double resistant (G418^r, 6-TG^r) colonies derived from ES cells transfected with linear, non-replicating vectors were found at 6–9 clones per 10^6 transfected cells, the ratio dropped to 0.3 clones per 10^6 cells transfected with the circular replication-competent variants. Evaluation of the double-resistant clones by Southern blot analysis (data not shown) confirmed that five out of seven clones targeted with the linearized control vectors, as well as the clones targeted with the circular replication-competent constructs, had undergone homologous recombination. Thus, the gene targeting efficiency in ES cells could not be enhanced by inserting the targeting sequence into an episomal vector.

Polyoma-based vectors are maintained episomally in differentiating ES cells and in several mouse cell lines

Spontaneous differentiation of ES cells occurs by culturing the cells in the absence of feeder cells and/or of the leukemia inhibitory factor (LIF). To analyze whether the episomal maintenance of pMGD20neo in ES cells is affected as the cells differentiate, we cultured the vector-containing clone 1.19 (9) in the absence of LIF for up to 27 cell generations. As shown in Figure 6, ES cell differentiation did not alter the episomal state of pMGD20neo as judged from Southern blot analysis of Hirt-extracted DNA digested with *Asp*718. ES cells can also be differentiated into EBs if cultured in a semisolid medium lacking LIF (7). Under these conditions, ES cell clone 1.19, like its ES cell progenitor, differentiated into typical embryoid bodies. DNA extracted by the Hirt procedure at day 4 and 9 of differentiation contained episomal plasmid DNA that was indistinguishable in size from that found in the parental clone 1.19 cells (data not shown).

To test whether our polyoma-based vectors were able to replicate in cell lines other than ES cells, we transfected either pMGD20neo

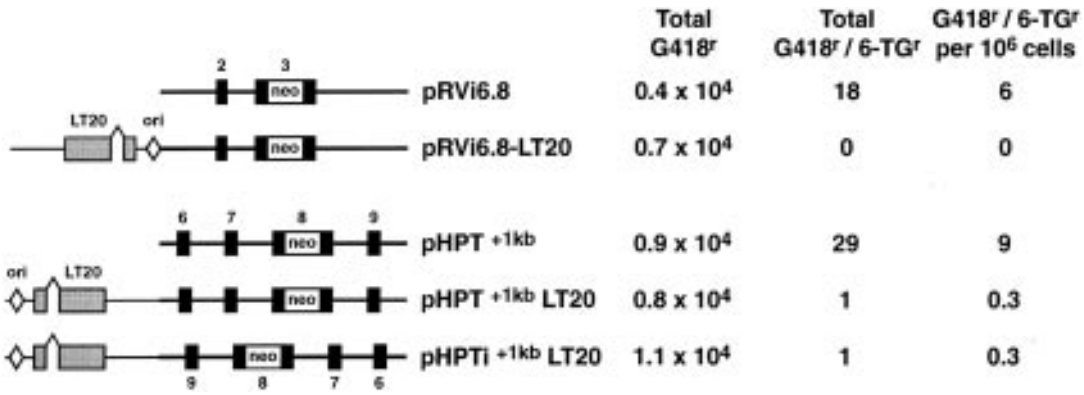

Figure 5. Gene targeting experiments in ES cell with polyoma–based targeting constructs. Using either pRVi6.8–LT20 harboring *hprt* exons 2 and 3 or pHPT^{+1kb} LT20 containing the *hprt* exons 6–9, the targeting frequencies to the *hprt* locus in ES cells transfected with these replication-competent circular vectors are compared with the ones with the corresponding 'classical' linear targeting vectors. Equimolar amounts of plasmid DNA were used for transfection. Note that the *hprt* segments are not drawn to scale and that all LT20-containing targeting vectors are transfected as circular plasmids.

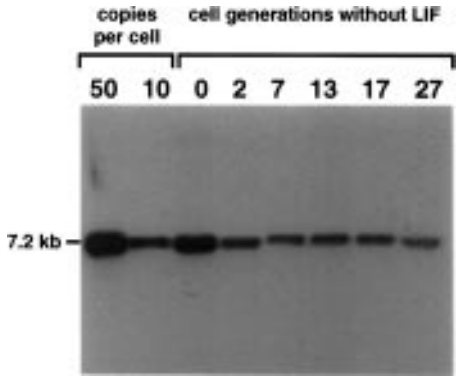

Figure 6. Vector pMGD20neo is maintained episomally in differentiating ES cells. ES cells from clone 1.19 were cultured for up to 27 cell generations without LIF, low-molecular-weight DNA was extracted at various stages, digested with *Hin*cII which cleaves the vector once and analyzed by Southern blotting as described in Figure 5. The first two lanes represent size and quantity standard (50 or 10 copies of pMGD20neo DNA per cell).

or pRVi6.8-LT20 into the following cell lines: (i) mouse embryonic carcinoma F9, initiated from a testicular teratocarcinoma (31); (ii) mouse erythroleukemia (MEL), a Friend virus-transformed erythroid precursor cell (32); (iii) mouse renal adenocarcinoma (RAG) (33); and (iv) mouse L-fibroblasts (L-929) (34). As with differentiating ES cells, Southern blot analysis using Hirt-extracted DNA from pools of G418-resistant clones selected for at least 10 days showed that in all four transfected cell lines both vectors were *Dpn*I-resistant and indistinguishable in structure from the corresponding transfected DNA (data not shown). This observation indicates that the episomal state of both vectors can be maintained in all established mouse cell lines tested.

DISCUSSION

There have been a few reports in the past on the maintenance of polyoma-based plasmids in mouse cells. Polyoma-pBR322 recombinants containing the wild-type early region and origin of replication have been shown to replicate efficiently in mouse fibroblasts and to be maintained as episomes for at least 6 days at ~1000 copies per cell (35). Plasmids recovered from individual clones were structurally identical to the parental plasmid. However, after 60 days, there was less than 1 copy of free plasmid per 10 cells. Another recombinant vector, pSV5gpt (36), which includes the polyoma early region containing two copies of ori and the coding sequence for all three T antigens, persisted episomally in mouse hepatoma cells for at least 50 generations without significant rearrangement of the vector DNA (37). On average, the cells contained 50–100 copies of plasmid DNA and only one integrated copy. The morphology of the transfected cells was significantly altered and their albumin synthesis decreased drastically, but the cause of these effects was not established. More recently, polyoma-related plasmids that persisted as episomes have been found in mouse L (tk^-) cells (38) and in the embryonal carcinoma cell line F9 (39). The polyoma-related DNA in the L (tk^-) cells had numerous sequence changes compared with the input DNA, and when transfected into F9 cells it was maintained as a plasmid in the cells although most of it was rearranged (40). The morphology of the embryonal carcinoma cells containing the episomal DNA was not altered nor was their ability to differentiate in the presence of retinoic acid affected.

To express genes from an episomal vector rather than from integrated copies, we constructed polyoma-based vectors that were maintained as episomal elements in ES cells (9). Apart from introducing a mutant polyoma enhancer fragment (PyF101) enabeling viral replication in embryonic cells, our vectors contained two different versions of the early region aiming to express large T only (LT1 and LT20). ES cells transfected with circular plasmids containing the polyoma early region mutant LT20 fragment (which requires splicing of the primary transcript) produced sufficient amounts of large T to support replication of the introduced plasmid. This in turn led to an increased production of hGH. In contrast, ES cells transfected with plasmids harboring the polyoma large T cDNA segment (LT1) failed to produce detectable amounts of viral protein and did not replicate after transient transfection. These observations are consistent with those reported by Nilsson and Magnusson (41). Based on the stable and long-term episomal maintenance of the vector which was not impaired after insertion of an up to 6.8 kb DNA fragment (i.e. pRVi6.8-LT20) and on the efficient expression of several genes (i.e. large T, *neo*, hGH) from the replicating vector, we believe that this system will facilitate many genetic manipulations of ES cells such as efficient overexpression of a gene of interest or genetic complementation by expressing a desired cDNA library from an extrachromosomally replicating vector.

Experiments comparing the targeting efficiency of molecules with no breaks (supercoiled DNA), single-strand breaks (nicked circle DNA), and double-strand breaks (linear DNA) within the region of homology showed that linear DNA targeted at a 10-fold higher frequency than nicked circular DNA and at a 34-fold higher frequency than supercoiled DNA (42). A reason for the low targeting efficiency of the circular vectors might be the requirement for DNA strand break events to promote homologous recombination. It has been reported that double strand breaks within the region of homology increased the number of targeted events by 5- to 10-fold (21). Since these experiments were all performed with a non-replicating vector, we tested whether the use of a replication-competent vector that is present episomally in the cells during several cell cycles will enhance the frequency of homologous recombination. To this end, we targeted the mouse *hprt* locus with linear or replicating (circular) vectors. Only two homologous recombination events, however, were found when using circular replication-competent constructs. In contrast, a total of 47 $G418^r$, 6-TG^r clones was found when linear vectors were used to transfect ES cells. Thus, our data underline the requirement of DNA strand breaks to promote homologous recombination.

Transfection of replication-competent vectors into several different mouse cell lines revealed that extrachromosomal maintenance of these plasmids was not restricted to ES cells. Polyoma virus is known to infect mice mainly through the respiratory tract and is replicated predominantly in lung, liver, kidney and colon. It can be propagated in mouse fibroblasts as well as in primary cell cultures derived from mouse kidney or embryo (43). Consistent with the properties of the infectious virus, vector pMGD20neo was found to replicate efficiently in mouse L-fibroblasts, in mouse renal adenocarcinoma (RAG) cells and in the teratocarcinoma cell line F9 which closely resembles ES cells. Furthermore, the mouse erythroleukemia cell line MEL was also capable to support replication of pMGD20neo. Thus, we

presume that this polyoma-based vector carrying a gene of interest can be useful to study gene expression in a variety of cell lines.

Continued replication and persistence of a polyoma based expression vector as an episome in differentiating ES cells could provide a useful tool to analyze different states of embryonic development *in vitro*. Moreover, vector-containing ES cells yielded several viable chimeras (9) indicating that our plasmid expressing polyoma large T does not affect either the ES cells' pluripotency or normal embryogenesis. The direct microinjection of extrachromosomally maintained vectors containing a polyoma replication unit into fertilized mouse eggs might allow studies in a variety of molecular events such as DNA methylation, replication, recombination and repair during the earliest stages of development, since these events might be analyzed directly from the isolated extrachromosomal vector. Compared with linear, non-replicating constructs, polyoma-derived vectors might enable a more efficient expression of given genes in developing embryos. Studies of such genetically altered mice should not be disturbed by the polyoma large T, as the latency of tumorigenesis in transgenic mice expressing this viral protein from the polyoma early region promoter has been reported to be very long: development of pituitary tumors in these transgenic mice began at about 9–13 months of age (44,45).

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