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Maintenance of an extrachromosomal plasmid vector in mouse embryonic stem cells

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ABSTRACT **We have constructed and characterized a polyoma virus-based plasmid that is maintained as an autonomously replicating extrachromosomal element (episome) in mouse embryonic stem (ES) cells. Plasmid pMGD20neo contains the polyoma origin of replication harboring a mutated enhancer (PyF101), a modified polyoma early region that encodes the large tumor (T) antigen only, and a gene that confers resistance to G418 (*neo*). After transfection, the plasmid replicates in ES cells and is maintained as an extrachromosomal element in 15% of G418-resistant clones. Integration of the plasmid DNA is undetectable for at least 28 cell generations. In one clone, the transfected DNA persists unaltered as an episome at 10–30 copies per cell for at least 74 cell generations in the presence of G418. Cells that maintain the autonomously replicating plasmid can efficiently replicate and maintain a second plasmid that carries the polyoma origin of replication. Independent vector-containing ES cell lines showed no significant alteration of the karyotype, and two cell lines yielded several chimeric animals when introduced into blastocysts, suggesting that the presence of an episomal element and expression of polyoma large T do not eliminate the ES cells' ability to populate an embryo. This system offers an efficient means for manipulating and analyzing various aspects of gene expression in ES cells.**

Many different genes have been disrupted by homologous recombination in mouse embryonic stem (ES) cells (1, 2), providing new insights into gene expression and function *in vivo* (3). Moreover, expression of yeast artificial chromosomes in ES cells and their transfer into the germ line have recently been demonstrated, illustrating that ES cells can incorporate large pieces of DNA into their genomes without losing their totipotency (4, 5). We have explored the possibility of generating polyoma virus-based vectors that can be maintained in ES cells as autonomously replicating episomes. By analogy with bacteria, the establishment of episomal vectors in ES cells, particularly ones containing DNA segments of interest, may prove to be useful for analyzing and modifying gene expression in ES cells during embryonic development and possibly in the germ line.

Polyoma virus DNA replicates as free, unintegrated minichromosomes in infected mouse cells (6). Three related proteins, encoded by the polyoma virus early region, are expressed shortly after infection: large tumor (T) antigen, middle T, and small T. These proteins are translated from three different mRNAs generated by alternative splicing of the primary transcript (7). Large T is required for initiating viral DNA replication (8, 9), while middle T is responsible for the viral transforming activity (10), and small T is suspected of having a subsidiary role in DNA replication (11) and/or in transformation (12, 13). Polyoma virus mutants whose DNA lacks the entire large T intron (14) or where the splice sites used in the formation of the properly processed middle T and small T mRNAs are absent can replicate to a limited extent because they express large T, but they are unable to transform cells because they cannot form middle T (15). Thus, replication of polyoma DNA requires only the viral origin of replication (ori) as a cis sequence, the trans-acting protein large T, and the cellular enzymes and accessory proteins needed for DNA synthesis. Infection of embryonal carcinoma cells (16) or ES cells (17) with wild-type polyoma or transfection with viral DNA fails to yield progeny virus. However, several polyoma mutants are able to propagate in mouse embryonal carcinoma cells by virtue of one or multiple changes in the enhancer region (18).

In the present work, we describe the construction and characterization of the polyoma virus-based vector pMGD20neo, which can be maintained as an extrachromosomal element in ES cells without impairing their ability to contribute to the formation of chimeric animals.

MATERIALS AND METHODS

Vector Constructions. The fragment LT20 consists of the polyoma virus early region in which the wild-type enhancer/origin and the coding region for all three T proteins have been replaced by the PyF101 mutant enhancer/origin (18) and a modified gene for large T, respectively (Fig. 1*A*). To eliminate the expression of middle and small T, the sequence between bp 663 and bp 782 (nomenclature according to ref. 19) was deleted. The LT20 fragment was joined to a segment containing the simian virus 40 (SV40) early region poly(A) site and then inserted into the *Dra* II site of PGKneopolyA, which is a derivative of pMC1neopolyA (20) carrying the PGK promoter from PGKneo bpA (21). More detailed information and the plasmid itself are available for distribution. PGKhphΔLT20 (Fig. 1*B*) differs from pMGD20neo in having a 1249-bp *Nco* I–*Xba* I deletion in the large T coding sequence and an *hph* gene (22) in place of the *neo* gene.

ES Cell Culture and Electroporation. The ES cell line CCE (23) was maintained continuously on gelatin-coated plates without feeder cells in Dulbecco's modified Eagle's medium (DMEM) containing 1000 units of murine leukemia inhibitory factor per ml (GIBCO/BRL), 20% heat-inactivated fetal calf serum (Gemini Biological Products, Calabasas, CA), 0.1 mM 2-mercaptoethanol, and 1× minimum essential medium nonessential amino acids (GIBCO/BRL) in a 37°C, 5% CO_2-humidified incubator. Routinely, 10^7 ES cells in 800 μl of HBS (20 mM Hepes/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM dextrose, pH 7.05) were electroporated with 10 μg of vector DNA at 240 V/1080 μF using a Bio-Rad Gene Pulser, and, after 10 min, the cells were transferred to a 150-mm plate. Selection for *neo* or *hph* relied on the ability to grow in either G418 (250 μg/ml) or hygromycin B (0.11 μg/ml), respectively.

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Abbreviations: ES, embryonic stem; SV40, simian virus 40; PGK, phosphoglycerate kinase.

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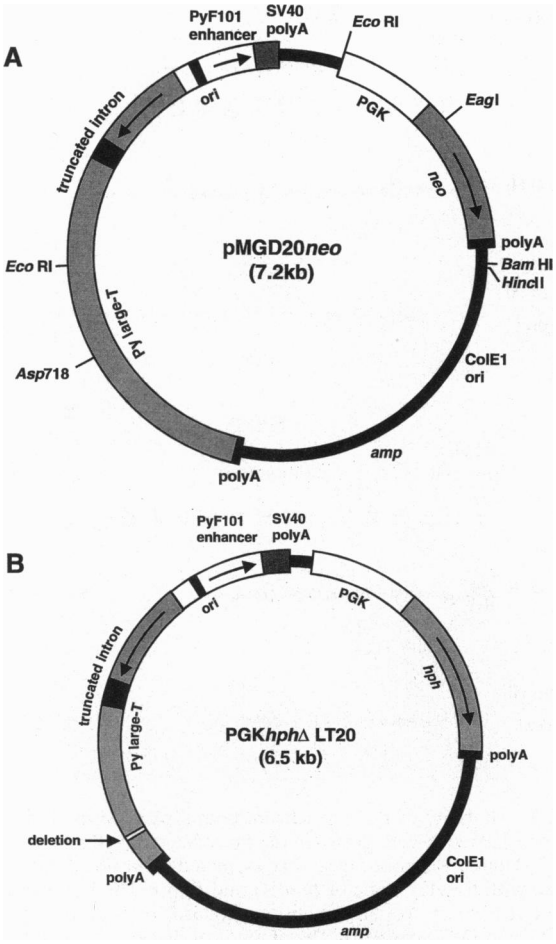

FIG. 1. Plasmids pMGD20neo and PGKhphΔLT20. (*A*) pMGD20neo contains the mutant enhancer PyF101, the polyoma wild-type ori, and LT20 fragment. The *neo* gene is expressed from the phosphoglycerate kinase (PGK) promoter. An SV40 poly(A) processing site was cloned into the polyoma late region. Restriction sites used in this study are indicated. (*B*) PGKhphΔLT20 is similar to pMGD20neo except that PGKhph replaces the PGKneo transcription unit, and the large T segment contains a large deletion in its coding sequence.

DNA Extraction and Analysis. Low molecular weight DNA was extracted using a modification of the method described by Hirt (24), which separates plasmids of the size used here from high molecular weight chromosomal DNA. After rinsing the cells twice with cold phosphate-buffered saline (PBS), 1% Nonidet P-40 in PBS was added carefully to the plates and incubated for 1 min at room temperature (25). Following aspiration of the fluid, the cells were treated according to the standard Hirt method. Total DNA extraction and Southern blot analysis were performed by standard methods.

Cytogenetic Analysis. Karyotype analysis was performed on ES cell cultures using standard cytogenetic methods. In short, cultures were suspended with trypsin following mitotic arrest with colcemid (final concentration, 0.05 μg/ml) for 30 min at 37°C, treated with hypotonic KCl solution (0.075 M) for 15 min at room temperature, and fixed twice with 3:1 methanol/acetic acid fixative. Chromosome slide preparations were made by standard procedure and analyzed using the GTW-banding method (26).

RESULTS

Construction of pMGD20neo. To construct an autonomously replicating episomal plasmid in mouse ES cells, we chose the polyoma early region segment to supply the cis-acting ori function and the trans-acting initiator function (large T). First, the polyoma segment spanning bp 664–781, which is located in the intervening sequence of large T and contains the splice sites for processing the middle and small T mRNAs, was deleted from the transcription unit (LT20), thereby preventing the formation of the middle T and small T mRNAs but permitting large T mRNA production. Second, the wild-type polyoma enhancer-ori was replaced with the mutant enhancer-ori from PyF101 (18) to enable expression and replication in ES cells. This modified polyoma early region was inserted into PGKneopolyA, yielding plasmid pMGD20neo (Fig. 1*A*), which also contains an SV40 early poly(A) site upstream of the enhancer-ori segment to minimize any inhibitory effects of transcription emanating from the polyoma late region promoter. Transfection of linearized pMGD20neo into mouse 3T3 cells led to stable transformants that are G418 resistant and express large T as judged by Western blot analysis (data not shown).

Establishment of Stable ES Cell Lines Containing Episomal pMGD20neo DNA. ES cells (CCE) were electroporated with supercoiled pMGD20neo DNA and grown in a medium containing G418 until independent colonies could be picked and subcultured. Plasmid DNA was extracted from such subcultures using a modified version of the Hirt procedure to separate the lower molecular weight plasmid DNA from the cell's chromosomal DNA (24). Because the transfected pMGD20neo DNA had been prepared in *Escherichia coli*, it carries a characteristic methylation pattern that renders it digestible by *Dpn* I endonuclease (27). However, that methylation pattern is lost after replication in mammalian cells, and replicated DNA becomes resistant to *Dpn* I digestion. Approximately 15% of the 87 G418 resistant clones examined contained *Dpn* I-resistant extrachromosomal plasmid DNA. Three independent clones (clones 1.15, 1.19, and 1.24) were characterized further; their plasmid DNA was extracted and digested with either *Bam*HI to linearize the plasmid or *Bam*HI plus *Dpn* I or *Dpn* I alone. Southern blot analysis of the digested products revealed that virtually all of the nonchromosomal DNA is identical in size to the transfecting DNA and resistant to *Dpn* I digestion (Fig. 2*A*). Thus, the transfected DNA had probably undergone multiple rounds of replication. Judging from the intensity of the plasmid bands relative to the standards, these clones contain at least 5–10 copies of the plasmid DNA, but this is probably an underestimate because only about 50% of plasmid DNA is recovered in the Hirt extracts. Fig. 2*B* shows that the recovered extrachromosomal DNA is predominantly supercoiled with a trace of nicked circular DNA and is indistinguishable in size from the transfected plasmid. To examine the extrachromosomal DNA from these clones in more detail, it was transfected into *E. coli*. The plasmid DNA recovered from multiple independent *E. coli* transformants could not be distinguished from the original pMGD20neo DNA with respect to its size or restriction patterns after digestion with enzymes that make frequent cuts in pMGD20neo. By this criterion, we judge that the extrachromosomal DNA in clones 1.15, 1.19, and 1.24 is either identical to or very closely resembles the transfected plasmid. Furthermore, the plasmid DNA recovered from the various *E. coli* clones is as proficient as pMGD20neo, but not more so, in establishing ES clones containing episomal DNA. We surmise, therefore, that the episomal DNA maintained in the transformed ES cells is not substantially altered in ways that enhance its ability to become established as an episome.

Stability of the Extrachromosomal Plasmid DNA. The stability of the extrachromosomal DNA was examined during propagation of the transformed ES cell clones for 74–95 cell generations in the presence or absence of G418. Episomal pMGD20neo DNA is maintained without significant change during 74 cell generations in G418-containing medium (Fig.

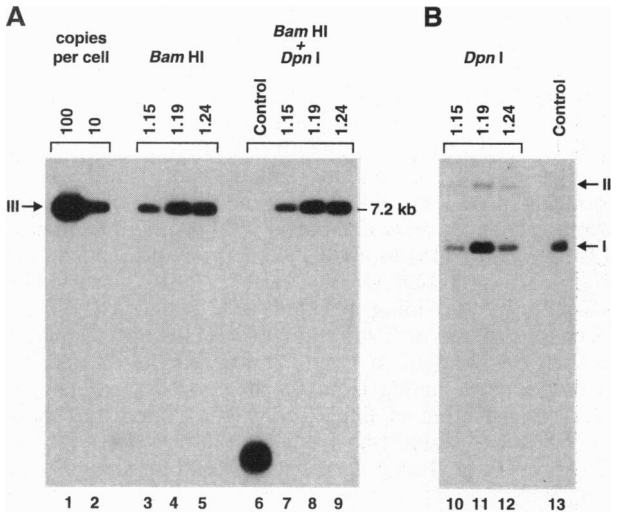

FIG. 2. Detection of stable ES cell lines harboring free copies of pMGD20neo DNA. (*A*) Southern blot analysis of plasmid-containing ES cell clones 1.15, 1.19, and 1.24. G418 was added to the medium 3 days after transfection of supercoiled pMGD20neo DNA (10 μg) into 10^7 cells, and, 10 days later, single clones were isolated and expanded. Hirt extracts of these individual clones grown for 24 days after transfection were hybridized to a polyoma large T probe. Lanes 1 and 2, size and quantity standards (100 or 10 copies of pMGD20neo DNA per cell). Lanes 3–5, product of digestion of the Hirt extract with *Bam*HI; lanes 7–9, product of double digestion with *Bam*HI and *Dpn* I. Control lane 6, pMGD20neo DNA used to transfect the ES cells is fully *Dpn* I sensitive, indicating complete digestion by this enzyme. The arrows marked I, II, and III indicate the position of supercoiled, nicked, and linear pMGD20neo, respectively. (*B*) Southern blot analysis of the plasmid DNA in the Hirt extract from the same clones after digestion with *Dpn* I only. The arrows marked I and II indicate the position of supercoiled and nicked pMGD20neo DNA, respectively.

3*A*). The plasmid was also maintained without significant loss for about 50 cell generations in the absence of selection for G418 resistance, but then the amount began to decline at about 76 generations and could be detected only after prolonged exposure of the autoradiograms (Fig. 3*B*). Nevertheless, after about 87 generations, the addition of G418 to the medium and continued propagation for another 8 cell generations resulted in the reappearance of substantial amounts of the original plasmid. Evidently, a sufficient amount of the plasmid was retained by a fraction of the cell population to ensure their survival in G418 either by allowing the plasmid copy number to increase or by selecting for cells that still maintained the plasmid at near-original levels.

Is Plasmid DNA Integrated into ES Cell Chromosomal DNA? We have considered the issue of whether the ES cell clones containing extrachromosomal plasmid DNA also contain integrated copies in their chromosomes. To this end, we examined the fragment patterns generated by digestion of the total DNA (which contains chromosomal and episomal DNA) from clones harboring extrachromosomal DNA (1.19 and 1.24) or integrated plasmid DNA only (1.17) with restriction enzymes that either fail to cleave pMGD20neo DNA (*Bgl* II and *Ssp* I) or cleave once (*Asp*718 and *Hin*cII) or twice (*Eco*RI) (Fig. 4). Enzymes that do not cleave pMGD20neo but cleave ES cell DNA should yield, besides the supercoiled and occasionally nicked circular and linear forms, additional fragments characteristic of the structure, number, and location of integrated plasmid DNA. Enzymes that cleave pMGD20neo once will produce, besides full-length linear DNA indicative of episomal DNA and any free or integrated catameric DNA, other fragments whose size depends on the flanking sequence and structure of integrated DNA. The fragment pattern with *Eco*RI endonuclease, which cuts pMGD20neo DNA twice,

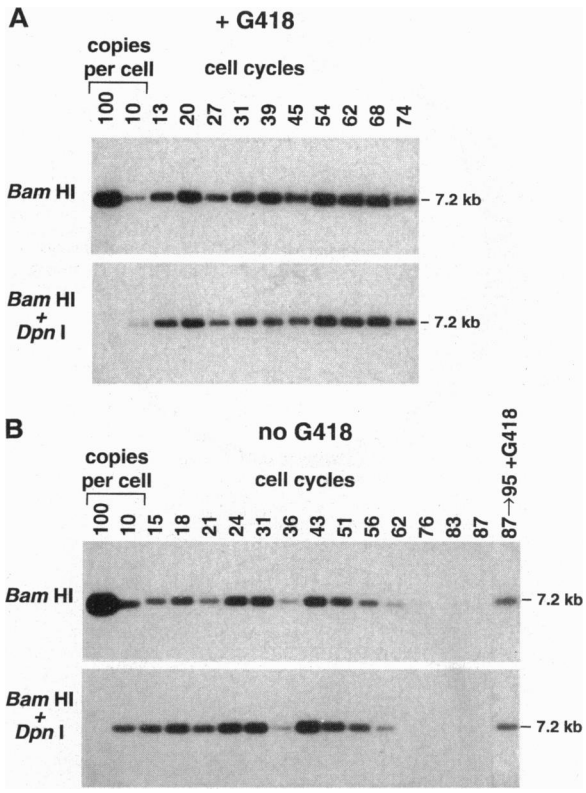

FIG. 3. Stability of the extrachromosomal plasmid in clone 1.19. (*A*) Clone 1.19 cells were grown in the presence of G418 and passaged every 3–4 days. Plasmid DNA was extracted at various stages and digested with *Bam*HI alone or *Bam*HI and *Dpn* I. The first two lanes on the left provide size and quantity standards (100 or 10 copies of pMGD20neo DNA per cell). The amount of plasmid per cell (10–30 copies) remained stable for at least 74 cell cycles when the experiment was stopped. (*B*) Clone 1.19 was grown in the absence of G418 for 87 cell cycles. After this time G418 selection was reconstituted and allowed to proceed for 8 cell generations.

should also reveal fragments diagnostic of integrated plasmid DNA. Fig. 4*A*, which shows the patterns produced by two noncutters, reveals predominately supercoiled and nicked circular episomal DNA and trace amounts of several other plasmid DNA species that are present in the transfecting DNA. However, there are no unique bands suggestive of integrated copies in clones 1.19 and 1.24. By contrast, digestion of cellular DNA from clone 1.17 with the same two restriction enzymes—*Bgl* II and *Ssp* I—yields a new band indicative of an integrated form. The results after digestion of the total DNA from clones 1.19 and 1.24 with the enzymes that cleave once (*Asp*718 and *Hin*cII) or twice (*Eco*RI) also fail to reveal bands indicative of integrated DNA (Fig. 4 *B* and *C*). Indeed, the two expected fragments from the cleavage of the episomal DNA by *Eco*RI endonuclease are evident in the DNA from clones 1.19 and 1.24 but these fragments are absent from clone 1.17 DNA. A similar analysis of five additional G418 resistant clones that lacked episomal DNA revealed varying size fragments indicative of integrated sequences; however, the fragment patterns indicated that in each case only a portion of the transfecting plasmid was stably integrated. We suspect that such stable transformants were formed by integration of varying size fragments derived from the transfecting DNA, at least one of which must contain an intact *neo* gene. On the basis of these data, we surmise that the extrachromosomal plasmid DNA in clones 1.19 and 1.24, and the others with similar properties, is being maintained as autonomously replicating episomes by virtue of their cis-acting ori sequence and their ability to express the trans-acting replication initiator, polyoma large T.

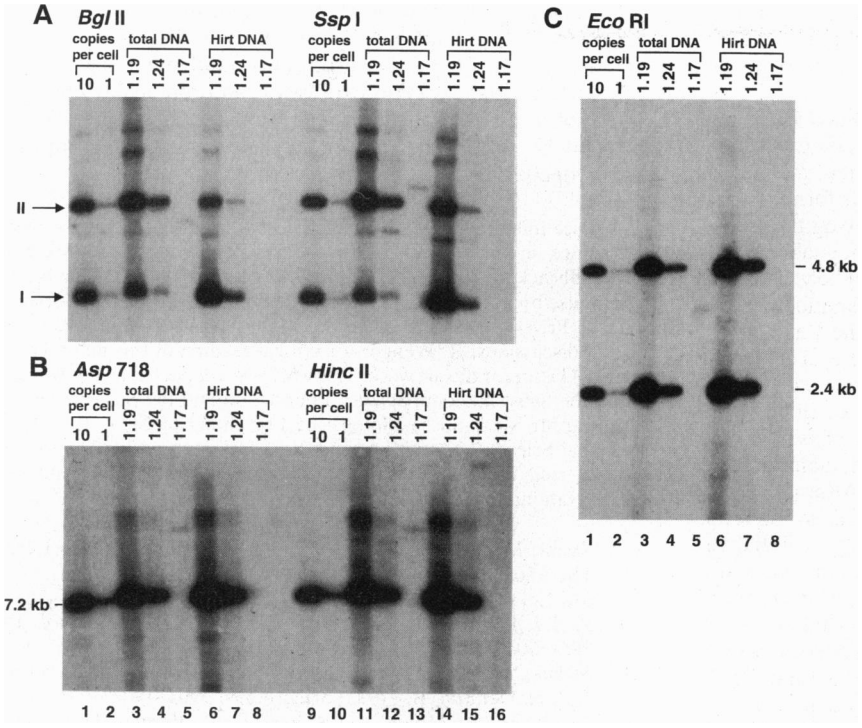

FIG. 4. Southern blot analysis of total and Hirt DNA extracts from G418-resistant ES cell clones. Total and Hirt DNA were extracted from 10^6 cells of G418-resistant clones 1.19 and 1.24, which contained extrachromosomal copies of pMGD20neo DNA, and from clone 1.17, which lacks any episomal DNA but which contains an integrated *neo* gene. These clones had undergone about 28 cell generations following selection for G418 resistance. Genomic DNA (10 μg) isolated from ES cells was added to the Hirt-extracted DNA prior to preparing the restriction digest. Total and Hirt DNA extracts were incubated with *Bgl* II or *Ssp* I, neither of which cleaves pMGD20neo DNA (*A*), or with *Asp*718 or *Hinc*II, each of which linearizes the plasmid (*B*), or with *Eco*RI (*C*), which cleaves the plasmid twice. DNA from pMGD20neo mixed with genomic DNA from ES cells (10 μg) was used as size and quantity standard: lanes 1 or 9 and 2 or 10 correspond to 10 or 1 plasmid copies per cell, respectively. An aliquot of each restriction enzyme mix was tested for complete digestion. 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. 1*A*).

ES Cells Containing pMGD20neo as an Episome Are Also Able To Maintain a Second Plasmid Containing a Polyoma ori. We sought to determine if ES cell clones that contain episomal pMGD20neo DNA can support the replication and maintenance of a second plasmid that contains the polyoma ori. Accordingly, we compared the outcome of transfection of clone 1.19 cells with PGKhphΔLT20, a plasmid that contains the same ori segment as in pMGD20neo, a large T gene with a 1249-bp deletion in its coding sequence (Fig. 1*B*), and a gene that confers resistance to hygromycin B, with PGKhph, a similar plasmid that lacks the polyoma segment. Each of these plasmids transforms wild-type ES cells to hygromycin resistance equally efficiently, most likely by becoming integrated into ES chromosomal DNA. When these plasmids were transfected into clone 1.19 cells, the yield of hygromycin B-resistant clones was about 100 times greater with PGKhphΔLT20 than with PGKhph. This difference is attributable to the ability of PGKhphΔLT20 to replicate and be maintained as an episome in clone 1.19 cells. This is supported by the finding that 85% of the hygromycin-resistant clones contained extrachromosomal PGKhphΔLT20 DNA. By contrast, the few clone 1.19 cells transformed with PGKhph were devoid of episomal forms of PGKhph DNA. We conclude that the efficient establishment of PGKhphΔLT20 DNA as an episome in clone 1.19 cells stems from its ability to replicate from its own ori in the presence of large T provided by the already established episomal pMGD20neo DNA.

Karyotypes of Transfected ES Cell Lines. To determine if maintenance of the plasmid DNA and/or expression of polyoma large T alters the genotype of the ES cells in untoward ways, the karyotype of ES cell clones 1.15, 1.19, and 1.24 was analyzed. Two clones, 5.1 and 5.3, in which the plasmid PGKneopolyA had integrated, were also examined. The analysis was performed by the GTW-banding method (26). Of the 21 metaphases from ES clone 1.19 that were counted and sexed, 19 had 40 chromosomes, the normal mouse diploid number. A duplication of the long arm of chromosome 14 was found in 4 cells. With clone 1.24, 17 of 20 cells contained 40 chromosomes. About 90% of the cells from clone 1.15 had 41 chromosomes due to a trisomy of chromosome 1, but there was no overt gross aneuploidy detected in any of the cells examined. Cells from clones 5.1 and 5.3 were abnormal, having either a clonal karyotype of 41 chromosomes (5.1) or an abnormal chromosome count of 39, missing the Y chromosome (5.3). Thus, we conclude that neither the presence of an episomal element nor expression of polyoma large T alters the ES cells' karyotype in any significant manner.

DISCUSSION

One of the aims of this project was to determine if the efficiency of targeting mutations to chromosomal genes in ES cells could be improved by recombination with corresponding homologous DNA segments maintained on episomal vectors. We considered the polyoma virus replication system for developing an autonomously replicating plasmid because there have been reports of the maintenance of polyoma-based plasmids in mouse cells. Polyoma-related DNAs that persisted as episomes have been found in mouse L (tk^-) cells (28) and in the embryonal carcinoma line F9 (29). However, this DNA had numerous sequence changes compared to the input DNA (30). Moreover, 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 about 1000 copies per cell, but this number decreases to <1 copy per cell after 60 days (31).

In our plan to use the polyoma DNA replication system for maintenance of episomes in mouse ES cells we were mindful that the polyoma early region encodes three proteins, only one of which, large T, is involved in replication; the other two, middle T and small T, have been implicated in oncogenic transformation (10), properties that could alter the ES cell's totipotency. We first used a large T cDNA fragment (14) in our constructs, but these plasmids did not express detectable amounts of large T protein after transfection into ES cells nor were they replicated, suggesting that an associated splicing event is needed for efficient gene expression (32). Therefore, a 118-bp segment located within the large T gene's intron was deleted, thereby removing the 5′ splice site for splicing the middle T and small T mRNAs and part of the coding sequences for these two proteins. Plasmid pMGD20neo contains the promoter-enhancer-ori derived from PyF101 (18), the modi-

fied large T coding sequence (LT20), and a transcription unit in which the PGK promoter expresses the *neo* sequence. This plasmid expresses large T protein after transfection into mouse cells and transforms cells to G418 resistance.

About 15% of the G418-resistant clones obtained following transfection of ES cells with pMGD20neo contained extrachromosomal plasmid DNA; the remainder harbored integrated forms of the plasmid but no detectable episomal forms. What governs which of the two outcomes occurs following the transfection is unclear. Stable modification of the DNA enabling it to be maintained as an episome appears to be unlikely. Plasmid DNA recovered from each of three clones that contained extrachromosomal DNA was indistinguishable from the transfecting DNA as judged by their restriction patterns with several enzymes; moreover, transfection of the recovered DNAs from each of the clones into ES cells yielded the same percentage of clones with episomal DNA. Perhaps only cells at a particular phase of their cell cycle (S phase?) are able to establish and maintain the plasmid as an autonomously replicating episome. Alternatively, it is possible that there is a wide variation among cells in the level of large T and that this influences the probability of initiating replication soon enough to result in stabilization of the plasmid's extrachromosomal state. Once achieved, however, the structure and number of extrachromosomal copies of pMGD20neo (10–30 per cell) are quite stable, particularly if the continued presence and expression of *neo* is selected for with G418. Once stabilized, there do not appear to be any structural rearrangements or significant change in the plasmid's copy number after 74 cell generations, and, surprisingly, even after about 50 cell generations in the absence of selection. Considering the absence of integration into the ES cell DNA up to at least 28 generations, we surmise that the episomal state of pMGD20neo reflects its ability to replicate autonomously, rather than as part of the cell's chromosomal DNA.

Because the maintenance of pMGD20neo as an episome is associated with the continued expression of polyoma large T, we expected that another plasmid containing a polyoma ori would be maintained in a similar episomal state. Indeed, PGKhphΔLT20, which lacks a functional large T gene but contains an intact ori and PyF101 enhancer segment, could be established as an episome at high frequency (85%) following transfection into clone 1.19 cells. We presume that the increased frequency at which the second transfecting ori-containing plasmid is established as an episome is a consequence of the preexistence and continued presence of large T in all the transfected cells. Consequently, as the transfected cells cycle through S phase there is a high likelihood that a DNA containing an ori will be replicated and be maintained thereafter. We presume that plasmids containing a polyoma ori, a selectable marker, and any other gene(s) of interest could be established and maintained in such cells similarly. The capability to establish plasmids as episomes in ES cells should find utility for a variety of studies of gene regulation in these cells.

One of our concerns in undertaking this investigation was that maintenance of a plasmid expressing polyoma large T would affect the ES cell's totipotency. At present, this is still an open issue. However, ES cells in which pMGD20neo has been maintained as an episome have few chromosomal anomalies; the karyotypes of most cells in such a population have the normal number and morphologies of mouse chromosomes. Moreover, we have found that the modified ES cells are able to populate the somatic cells of developing embryo and to yield viable, apparently normal chimeric mice following microinjection into host blastocysts (M.G. and G.D., unpublished observation), indicating that neither the expression of polyoma large T nor the presence of pMGD20neo DNA as an extrachromosomal element prevents the ES cells from populating an embryo. From an experimental point of view, plasmids carrying genes that can serve as reporters of various differentiated states or that can modulate the responses to induction may allow one to study the molecular mechanisms in differentiating ES cells (33) and in developing embryos. In summary, this vector system should be useful for studying a variety of phenomena such as gene expression and molecular complementation, DNA replication and recombination, and genetic control of differentiation in ES cells and during murine embryogenesis.

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