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Introduction and Recovery of a Selectable Bacterial Gene from the Genome of Mammalian Cells

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The simian virus 40 (SV40)-pBR322 recombinant, pSV2, carrying the origin of SV40 replication and the *gpt* gene of *Escherichia coli*, has been stably introduced into Chinese hamster ovary *hprt*$^-$ cells. All *gpt*-transformed cell lines were found to contain one or more insertions of pSV2 sequences exclusively associated with high-molecular-weight DNA. Additional analyses showed that at least one integrated copy in each cell line retained an intact *gpt* gene and flanking SV40 sequences required for expression of xanthine-guanine phosphoribosyltransferase. Most cell lines contained pSV2 sequences which had integrated with partial sequence duplication. Upon fusion with COS-1 cells, a simian cell line permissive for autonomous pSV2 replication, most *gpt*-transformed cell lines produced low-molecular-weight DNA molecules related to pSV2. The majority of these replicating DNAs were indistinguishable from the original transfecting plasmid in both size and restriction enzyme cleavage pattern. In addition, the recovered DNA molecules were able to confer ampicillin resistance to *E. coli* and to transform mouse L cells and Gpt$^-$ *E. coli* to a Gpt$^+$ phenotype. These studies indicate that all of the genetic information carried by this SV40-plasmid recombinant can be introduced into and retrieved from the genome of mammalian cells.

Over the past few years, there has been considerable interest in the use of DNA-mediated gene transfer and the exploitation of this technology for gene cloning. Despite the fact that a few eucaryotic genes have been isolated by this approach, progress in this area has been limited and it remains, in most instances, a difficult problem to recover a selectable marker after its introduction into recipient cells. One possible approach to this problem is suggested by the literature on biological rescue of integrated simian virus 40 (SV40) from host genomes. When SV40 sequences are introduced into nonpermissive cells by selection for transformation by using viral infection or DNA transfection, it is frequently possible to rescue viral DNA from the integrated state by fusion of the transformed cells to permissive simian cells (1, 3, 16, 29). Such excision of integrated viral sequences and their subsequent replication in heterokaryons as superhelical DNA require an intact origin of viral replication (10, 26) and is mediated by SV40 T antigen (3, 25) and permissive factors present in the nuclei of simian cells (28).

Recently, Hanahan et al. have shown that pBR322 DNA ligated to the early region of SV40 can be properly rescued after its nonselected introduction into mouse cells (12). However, in these studies, pBR322 sequences could not be recovered from many of the transformants since rescue was dependent upon expression of T antigen, which was highly variable among "convected" cell lines. It may be possible to circumvent this problem by providing T antigen in *trans* by fusion to COS-1 cells, a line of simian cells which produces T antigen constitutively (9).

Principally because of the cloning potentialities, we have been interested in studying the factors influencing the introduction and recovery of genetic markers of selectable phenotype. To address this problem we have used pSV2, a SV40-pBR322 recombinant which carries a selectable bacterial marker in lieu of early viral sequences. Although pSV2 does not encode T antigen, it contains the origin of viral replication as well as the SV40 early promoter which, in mammalian cells, regulates the expression of *Ecogpt*, a 1.0-kilobase pair (kbp) *Escherichia coli* sequence coding for the enzyme xanthine-guanine phosphoribosyltransferase (18). This bacterial enzyme is similar to the analogous mammalian enzyme hypoxanthine phosphoribosyltransferase (HPRT), but is able to utilize xanthine in addition to hypoxanthine as a substrate in the synthesis of the precursors of GMP. Consequently, selective culture conditions may be used to isolate wild-type or HPRT-deficient mammalian cells expressing the *E. coli gpt* gene (19).

In the work presented in this paper, we have

introduced pSV2 into an HPRT-deficient line of Chinese hamster ovary (CHO) cells and examined the excision of the plasmid from the integrated state after fusion with COS-1 cells. This experimental protocol should circumvent the irregular behavior of the transformed cells examined by Hanahan et al.

MATERIALS AND METHODS

Cell culture. Mouse L929 cells and CHO DR31 *hprt*$^-$ *aprt*$^-$ *glyB* cells have been described previously (17). Simian COS-1 cells came originally from Y. Gluzman (9) and were kindly provided by J. Hassel, McGill University, Montreal, Canada. All cell lines were maintained in α-minimal essential medium supplemented with 10% fetal calf serum.

Plasmids and bacterial strains. *E. coli* HB101 containing pSV2 (18) was kindly provided by P. Berg, Stanford University, Stanford, Calif. The C600 *r*$^-$ *m*$^+$ strain of *E. coli* came originally from R. Schimke and was obtained through the courtesy of K.-L. Lee, Oak Ridge National Laboratory, Oak Ridge, Tenn. The *gpt* strain of *E. coli*, CGSC 6186 Δ(*pro-lac*) *purE*, was obtained from B. Bachmann, Yale University, New Haven, Conn. Supercoiled plasmid DNAs were prepared from 1-liter cultures and banded in cesium chloride by centrifugation at 44,000 rpm in a Ti 70.1 rotor for 48 h at 20°C. Plasmid preparations from 50-ml amplified cultures were prepared as described elsewhere (L.-C. Tsui, M. L. Breitman, L. Siminovitch, and M. Buchwald, Cell, in press). Transformation of *E. coli* with plasmid or low-molecular-weight DNAs purified from mammalian cells was carried out by the method of Dagert and Ehrlich (8). *E. coli* CGSC 6186 was transformed with plasmid DNAs, and *gpt*$^+$ colonies were scored on minimal medium plates containing thiamine, glucose, Casamino Acids, and guanine (60 μg/ml).

DNA transformation of mammalian cells and selection of *gpt* transformants. Cultures of DR31 *hprt*$^-$ cells (5×10^5 cells per 10-cm dish) were treated with DNA-calcium phosphate coprecipitates containing 100 ng of circular pSV2 and 20 μg of carrier DR31 DNA by the method of Graham and van der Eb (11) as modified by Wigler et al. (30). At 48 h after exposure to DNA, cells were trypsinized and replated in selective HAT medium containing 10% dialyzed fetal calf serum and hypoxanthine (15 μg/ml), aminopterin (2 μg/ml), and thymidine (10 μg/ml) (HAT). After 10 to 14 days, several HAT-resistant clones were isolated and grown into mass culture. Mouse L929 cells were transformed as described above, using 1.0 μg of plasmid DNA without carrier. Selection of *gpt* transformants was carried out in HAT medium supplemented with xanthine (250 μg/ml) and mycophenolic acid (25 μg/ml) as described by Mulligan and Berg (19).

Preparation of cellular DNA. Approximately 2×10^8 cells grown in 150-mm^2 flasks were trypsinized and collected by centrifugation. Cell pellets were washed once with phosphate-buffered saline, resuspended in 1.0 ml of phosphate-buffered saline, and lysed by addition of 4.0 ml of a solution containing 1% Triton X-100, 3 mM $MgCl_2$, and 10 mM Tris-hydrochloride (pH 8.0). Nuclei were pelleted at 2,000 rpm in an International Centrifuge, suspended in 5.0 ml of TNE buffer (10 mM Tris-hydrochloride [pH 7.5]–100 mM NaCl–1.0 mM EDTA), and lysed by addition of 125 μl of 20% sodium dodecyl sulfate. The lysate was treated with 200 μg of self-digested proteinase K per ml for 3 h at 37°C and extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and once with chloroform. An equal volume of 4.0 M ammonium acetate was added to the aqueous phase, and nucleic acid was precipitated by addition of 2.5 volumes of ethanol. The precipitate was dissolved in 5.0 ml of 10 mM Tris-hydrochloride (pH 8.0)–0.1 mM EDTA and treated with 20 μg of RNase A per ml (heated for 10 min at 100°C) for 2 h at 37°C. After digestion, the DNA solution was extracted with phenol-chloroform-isoamyl alcohol as described above, adjusted to 2% potassium acetate, and precipitated in ethanol. The DNA precipitate was washed twice with ethanol, vacuum dried, dissolved in TE buffer (10 mM Tris-hydrochloride [pH 7.5]–1.0 mM EDTA), and stored at 4°C.

Restriction analyses and DNA-transfer hybridization. Restriction enzymes were purchased from Boehringer-Mannheim Corp. or New England Biolabs and used as recommended by the supplier. Restricted DNAs were electrophoresed through 5% polyacrylamide gels (acrylamide–bis-acrylamide, 29:1) or through 0.6 or 0.7% agarose gels in gel buffer containing 90 mM Tris-borate and 3 mM EDTA (pH 8.3). DNA bands were visualized by using a long-wavelength UV transilluminator after staining with ethidium bromide (1.0 μg/ml).

For DNA-transfer hybridization, agarose gels were treated as described by Wahl et al. (27) and transferred to nitrocellulose filters (Schleicher and Schuell BA85) by the procedure of Southern (24). Filters were prehybridized and then hybridized with ^{32}P-labeled DNA (10^5 cpm/cm^2) as described (27). High-specific-activity [^{32}P]pSV2 probes (5×10^8 cpm/μg of DNA) were prepared by using a nick-translation kit (Amersham). After hybridization overnight at 42°C, filters were washed five times at room temperature with 50 ml of 2× SSC–0.1% sodium dodecyl sulfate (1× SSC = 150 mM NaCl–15 mM sodium citrate) followed by three cycles of alternating washes in 2× SSC–0.1% sodium dodecyl sulfate at 50°C and 0.2× SSC–0.1% sodium dodecyl sulfate at 42°C. Filters were air-dried and exposed to Kodak XAR-5 film with Cronex Lightning-Plus intensifying screens for 1 to 4 days at −70°C.

Cell fusion. Approximately 10^6 DR31 cells and 2×10^6 COS-1 cells were mixed and plated together in 10-cm dishes. After 24 h, the medium was removed, and cells were exposed for 1 min to 4 ml of α-minimal essential medium (prewarmed to 37°C) containing 40% polyethylene glycol (PEG 1000, Koch Light) and 20% dimethyl sulfoxide (20). Cells were then washed three times with prewarmed α-minimal essential medium containing 10% dimethyl sulfoxide and once with α-minimal essential medium containing 10% fetal calf serum and incubated at 37°C. At 2 to 4 days after PEG treatment, low-molecular-weight DNAs were prepared as described elsewhere (Tsui et al., in press) and analyzed by transfer hybridization.

RESULTS

Selection of *gpt* transformants. The SV40-plasmid recombinant pSV2 described by Mulligan and Berg (18) is shown in Fig. 1. To study the

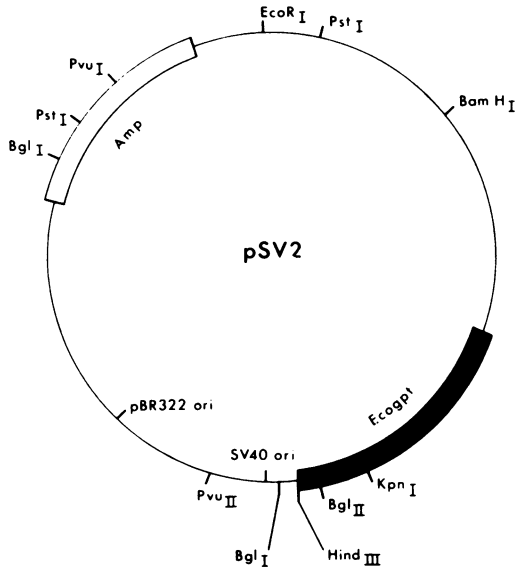

FIG. 1. Restriction map of pSV2. The pBR322 sequences in pSV2 are located on the left side of the molecule beteen the *Eco*RI and *Pvu*II restriction sites. The sequences extending from the *Pvu*II site to the beginning of *Ecogpt* (shaded box) are derived from SV40 and contain the origin of SV40 DNA replication and the SV40 early promotor. The sequences of pSV2 extending from the end of *Ecogpt* to the *Eco*RI site are also derived from SV40. Contained within this segment, between the *Bam*HI site and the end of *Ecogpt*, are the intervening sequences of small t antigen and the early polyadenylic acid addition signal of SV40 transcription.

recovery of the plasmid from mammalian cells, pSV2 was introduced into CHO DR31 *hprt*$^-$ cells as a circular molecule by the calcium phosphate coprecipitation method of Graham and van der Eb (11). Cells stably expressing the *E. coli gpt* gene were selected in HAT medium. After 10 to 14 days, several colonies were isolated, grown into mass culture, and analyzed for the presence of plasmid sequences. Figure 2 shows a blot hybridization analysis of five representative transformants after digestion of cellular DNAs with *Xba*I, a restriction enzyme that does not cut pSV2. Hybridization with a nick-translated [^{32}P]pSV2 probe revealed that all cell lines contained plasmid sequences associated with high-molecular-weight DNA. Three of these cell lines, DR31/3, DR31/7, and DR31/8, contained multiple insertions; one, DR31/6, contained two insertions; and one, DR31/1, carried a single insertion of pSV2 DNA.

Although equal amounts of cellular DNAs were analyzed, marked differences were observed in the intensity of the hybridizing bands. Since mapping and reconstruction experiments suggested that the band in DR31/1 corresponded to a single insertion of plasmid DNA (see Fig. 3 and 10), the weaker bands detected in many of the transformants probably represent insertions that were only present in some of the cells. However, it is also possible that the weaker bands represent small fragments of pSV2 which had integrated into high-molecular-weight DNA. Such fragmentation is apparent for two low-intensity bands in DR31/8 which are smaller than linear (form III) pSV2 DNA.

As shown in Fig. 2, lane 1, both supercoiled and relaxed circles (forms I and II) of pSV2 DNA were readily detected by our blotting procedure. The absence of such bands in the *Xba*I-cut material shows that no free pSV2 persisting as circular molecules was detectable in any of the cell lines. This conclusion was supported by blot analysis of Hirt-fractionated (13) low-molecular-weight cellular DNAs. No evidence of free plasmid was found under condi-

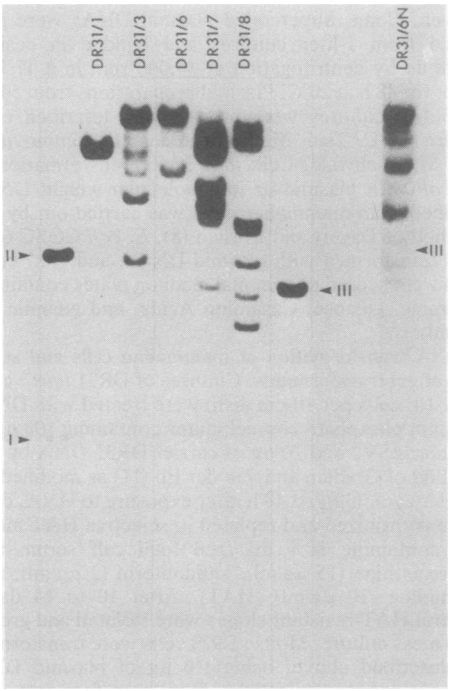

FIG. 2. Discrete insertions of pSV2 sequences in *gpt*-transformed cell lines. Transformant DNAs (5 μg) were digested to completion with restriction endonuclease *Xba*I, electrophoresed through a 0.6% agarose gel, transferred to a nitrocellulose filter, and hybridized with a ^{32}P-labeled pSV2 probe. In reconstruction experiments, 3 pg of forms I and II or 3 pg of form III of pSV2 was mixed with 5 μg of DR31 DNA and digested with *Xba*I. The lane marked DR31/6N corresponds to a subsequent analysis of DR31/6 after passage of the cell line in nonselective medium for approximately 100 generations.

tions that would have detected as little as 1 free copy of pSV2 in 50 cells (data not shown).

Although all of the pSV2 cell lines maintained stable expression of *gpt* in the absence of selection, several transformants showed a change in the pattern of pSV2 insertions after passage in nonselective medium. Figure 2 shows a DNA blot analysis of DR31/6 cells (DR31/6N) after growth of the cell line in nonselective medium for approximately 100 generations. Several new

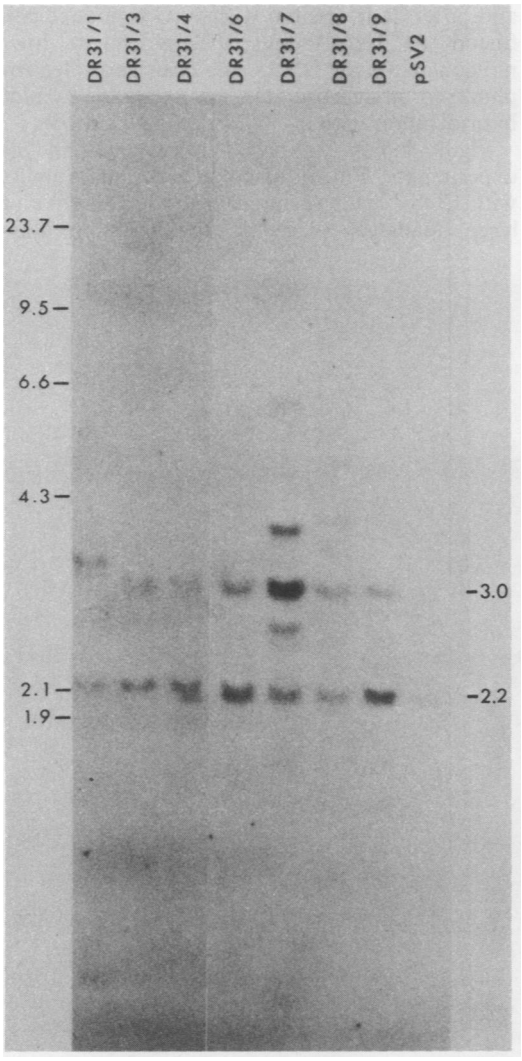

FIG. 3. Determination of intact *gpt* genes in pSV2 cell lines. Equal amounts of cellular DNAs (5 μg) were sequentially digested with *Pvu*II and *Bam*HI, electrophoresed through a 0.7% agarose gel, transferred to a nitrocellulose filter, and hybridized with ^{32}P-labeled pSV2 DNA. A single-copy reconstruction experiment was approximated by parallel digestion of 5 pg of pSV2 mixed with 5 μg of DR31 DNA. Markers (in kilobases) correspond to the *Hin*dIII digestion fragments of bacteriophage λ.

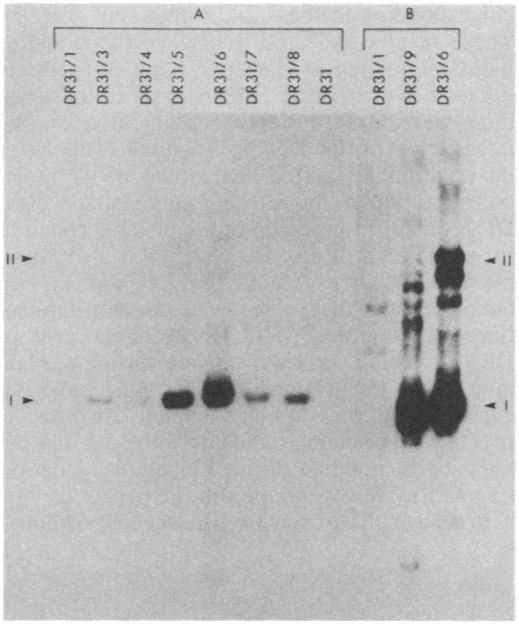

FIG. 4. Rescue of pSV2 DNA from the integrated state after fusion of *gpt*-transformed cell lines to COS-1 cells. (A) Dishes (10 cm) containing 10^6 *gpt*-transformed DR31 cells and 10^6 COS-1 cells were treated with PEG as described in the text. Two days after cell fusion, cell cultures were lysed, and low-molecular-weight DNAs were purified (Tsui et al., in press). One-half of the DNA recovered from each 10-cm plate was electrophoresed through a 0.7% agarose gel, transferred to a nitrocellulose filter, and hybridized with a nick-translated [^{32}P]pSV2 probe. (B) PEG-treated cultures contained 10^6 *gpt*-transformed DR31 cells and 2 × 10^6 COS-1 cells. Three days after cell fusion, the total low-molecular-weight DNA recovered from each plate was analyzed as described for (A).

pSV2-hybridizing bands were observed as well as a decrease in the intensity of one of the original insertions detected by transfer hybridization. Furthermore, some of the new bands were smaller than 5.2-kbp form III pSV2 DNA.

Integrity of the *gpt* gene in pSV2 cell lines. To analyze pSV2 cell lines for the presence of potentially active *gpt* genes, equal amounts of transformant DNAs were sequentially digested with *Pvu*II and *Bam*HI, electrophoresed through a 0.7% agarose gel, and analyzed by transfer hybridization with a ^{32}P-labeled pSV2 probe. Double digestion of parental pSV2 with these two single-cut restriction enzymes yielded two fragments of approximately 3.0 and 2.2 kbp. The 3.0-kbp fragment contained the pBR322-derived portion of pSV2, whereas the 2.2-kbp fragment contained the bacterial segment and flanking SV40 sequences required for expression of the *E. coli gpt* gene in mammalian cells (see Fig. 1).

All cell lines contained a hybridizing band

corresponding to the 2.2-kbp fragment (Fig. 3). These results indicated that all of the transformants contained at least one copy of an intact *gpt* gene. Furthermore, the majority of these cell lines were also found to contain at least one intact copy of the 3.0-kbp fragment. This latter observation suggested that some pSV2 molecules had integrated into high-molecular-weight DNA through the region of the plasmid required for expression of *gpt*. However, it is also possible that integrity of both the 2.2- and 3.0-kbp bands reflects the presence of tandem duplications of integrated pSV2 DNA. In the case of DR31/1, which carried a single insertion of plasmid DNA, integration of pSV2 must have occurred within the 3.0-kbp fragment. As a result, two new hybridizing bands of about 3.2 and 0.4 kbp, corresponding to pSV2-cellular junction fragments, were generated in the process.

Rescue of pSV2 from *gpt*-transformed cell lines. Fusion of SV40-transformed cell lines with permissive simian cells frequently leads to excision of integrated SV40 sequences and replication of free viral DNA (1, 3, 16, 29). Since these events are mediated by SV40 T antigen (3, 25), we investigated whether pSV2 could be rescued from the high-molecular-weight DNA of *gpt* transformants by using COS-1 cells, a line of SV40-transformed CV-1 cells which produces T antigen constitutively (9). Therefore, COS-1 and *gpt*-transformed cell lines were plated together and, after 24 h, treated with PEG to induce cell fusion. At 2 to 4 days after treatment, low-molecular-weight DNAs were purified, electrophoresed on agarose gels, and analyzed by blot hybridization with a ^{32}P-labeled pSV2 probe.

Figure 4 shows the results of two independent experiments. Fusion of most *gpt* transformants with COS-1 cells resulted in the appearance of large quantities of replicating low-molecular-

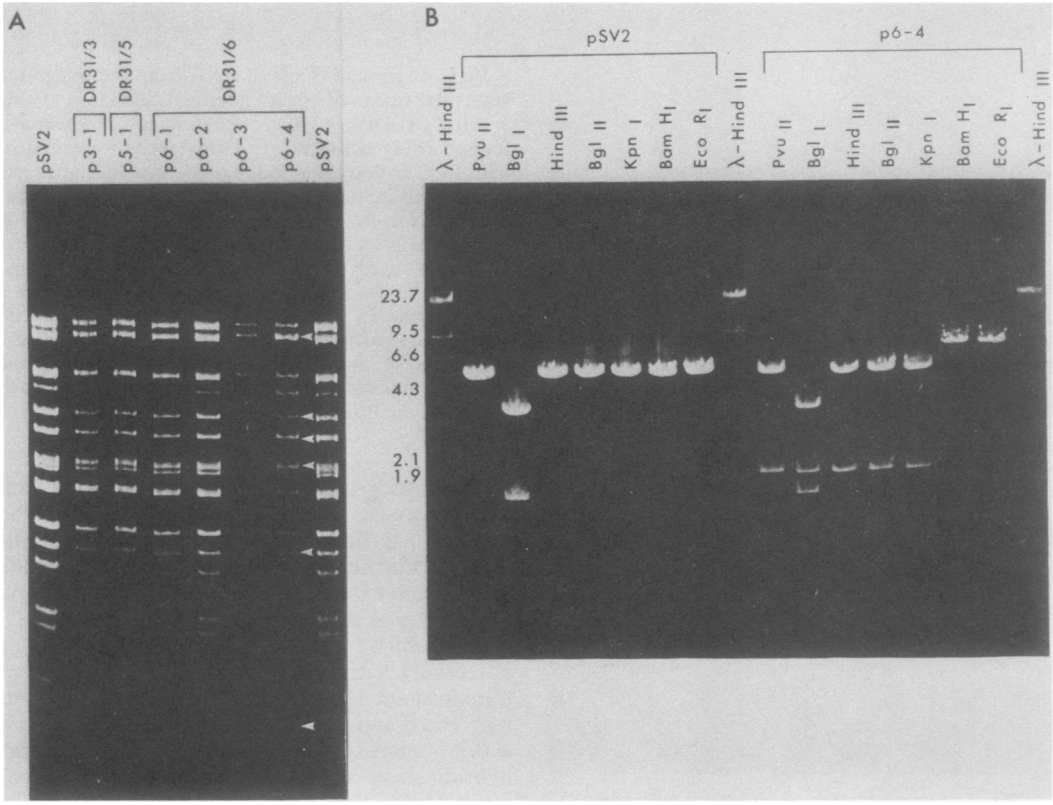

FIG. 5. Analysis of pSV2 DNAs recovered from *gpt* transformants. Four days after fusion of *gpt*-transformed DR31 cells to COS-1 cells, low-molecular-weight DNAs were purified, and pSV2 molecules were reestablished as bacterial plasmids in *E. coli*. (A) Representative plasmids recovered from three *gpt*-transformed cell lines were purified, digested with restriction endonuclease *Alu*I, and analyzed on a 5% polyacrylamide gel. In this gel, 18 of 22 *Alu*I digestion fragments of parental pSV2 were resolved. Arrows indicate restriction fragments that are contained within the duplicated segment of p6-4 and show an increased intensity of ethidium bromide staining. (B) Comparative restriction analyses of pSV2 and p6-4. Plasmid DNAs were digested with the indicated restriction endonucleases and electrophoresed through a 0.7% agarose gel. Markers (in kilobases) correspond to the *Hin*dIII digestion fragments of bacteriophage λ.

weight DNA which comigrated with supercoiled pSV2, as well as trace amounts of other pSV2-related molecules that were both larger and smaller than the native vector. No such molecules were recovered from heterokaryons of DR31 and COS-1 cells. These experiments show that integrated copies of pSV2 can be rescued from the high-molecular-weight DNA of *gpt*-transformed cell lines.

Considerable variation was observed in the amount of plasmid DNA recovered from the different transformants. Relative yields were generally consistent from experiment to experiment, but no correlation was observed between the amount of rescued pSV2 and the number of insertions in high-molecular-weight DNA (cf. DR31/6 and DR31/7 in Fig. 2). Typically, for most cell lines, 0.1 to 2.0 ng of pSV2 DNA was recovered per 10-cm dish. These yields are approximately 10- to 100-fold lower than those observed when wild-type SV40 DNA is rescued by comparable fusions between SV40-transformed cells and uninfected CV-1 cells (12). The transformant with only one insertion, DR31/1, consistently rescued 20- to 100-fold less efficiently than the other *gpt*-transformed cell lines (Fig. 4A and B). Furthermore, upon fusion to COS-1 cells, this cell line generated a heterogeneous population of pSV2-related DNAs. This heterogeneous pattern of rescue probably reflects random and imprecise excision of the single integrated copy of plasmid DNA in DR31/1.

Analysis of pSV2 molecules rescued from *gpt*-transformed cell lines. To examine the structure of the replicating low-molecular-weight DNAs recovered after cell fusion, several molecules were reestablished as bacterial plasmids by transformation of *E. coli* C600 and selection of ampicillin-resistant colonies. Plasmid DNAs were then purified and analyzed with different restriction enzymes. Figure 5A shows the data for six representative plasmids derived from three *gpt*-transformed cell lines after digestion with *Alu*I, a restriction enzyme that produces 22 cuts in pSV2 DNA. Since a similar restriction

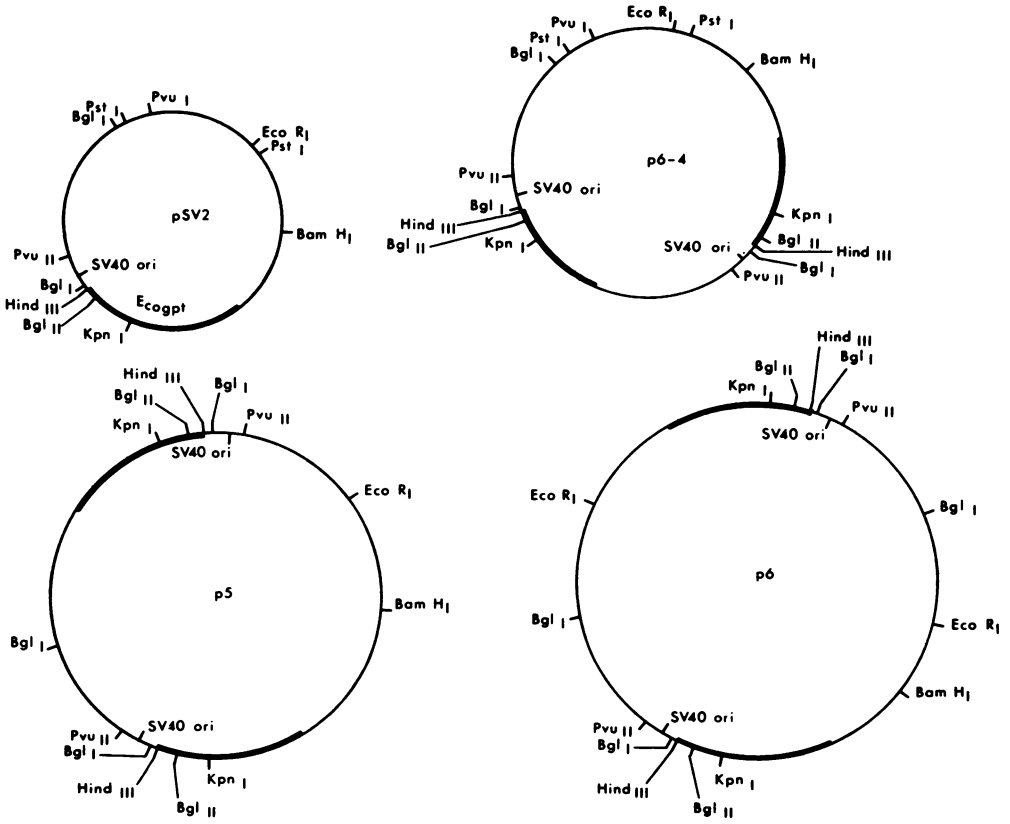

FIG. 6. Restriction map of p6-4 and partial tandem duplications of pSV2 recovered from COS-1 cells. p5 and p6 were reestablished in *E. coli* from the low-molecular-weight DNAs purified from COS-1 cells 4 days after transfection with 10 ng of circular pSV2. Restriction analysis of plasmids was performed as described for p6-4 in the text. p5 and p6 contain partial tandem duplications of 3.2 and 4.6 kbp, respectively.

pattern was observed for pSV2 and the transformant-derived plasmids, we conclude that most pSV2 molecules did not undergo detectable rearrangement during or after excision from high-molecular-weight DNA. However, one isolate, p6-4, was found to contain a 1.98-kbp tandem duplication after digestion with several restriction enzymes (*Pvu*II, *Hin*dIII, *Bgl*I, *Bgl*II, *Kpn*I, *Bam*HI, *Eco*RI) (Fig. 5B). As indicated in Fig. 5A, this duplication correlates with an increased intensity of ethidium bromide staining of six *Alu*I restriction fragments. Detailed mapping of the duplication was carried out by restriction analyses of p6-4 with *Hin*fI, *Sau*3A, and *Hae*III (data not shown). The duplication in p6-4 spans the SV40 origin of DNA replication as well as the entire bacterial segment harboring *E. coli gpt* (Fig. 6). Analysis of the integrated forms of pSV2 in DR31/6 cells (see below) suggested that p6-4 was generated either by imprecise excision or during replication at some stage after rescue from high-molecular-weight DNA. To investigate the possibility that p6-4 arose during replication, COS-1 cells were transfected with circular pSV2. Four days later, low-molecular-weight DNAs were purified, and several pSV2-related molecules were reestablished as bacterial plasmids in *E. coli*. Restriction analyses of several of these plasmids revealed that 2 of 25 contained partial tandem duplications similar to that observed for p6-4 (see Fig. 6). From these studies, we suggest that p6-4 arose at some stage after excision from the high-molecular-weight DNA of DR31/6 cells.

Although restriction analyses suggested that most of the rescued plasmids were identical to pSV2, transformation studies were carried out to establish biological activity. Cultures of CGSC 6186, a *purE gpt* strain of *E. coli*, were transformed with different isolates rescued from the *gpt*-transformed cell lines and tested for growth on minimal medium plates containing guanine. In all cases, Gpt^+ colonies were detectable within 24 h. No such colonies were observed in control cultures treated with pBR322 DNA. Furthermore, treatment of mouse L929 cells with the rescued plasmids led to the recovery of colonies resistant to medium containing HAT, mycophenolic acid, and xanthine. Since no drug-resistant colonies developed in control cultures, the above studies indicate that an active bacterial gene has been introduced into and rescued from the high-molecular-weight DNA of Chinese hamster cells.

Integrated structures of pSV2 DNA in *gpt*-transformed cell lines. Rodent cells transformed by SV40 frequently contain tandem head-to-tail copies of integrated viral DNA (2, 4, 5, 7, 15, 21, 23). To investigate whether similar arrangements of pSV2 sequences were present in *gpt*-transformed cell lines, cellular DNAs were digested with several restriction enzymes (*Pvu*I, *Pvu*II, *Hin*dIII, *Bam*HI, *Eco*RI) that produce a single cut in pSV2 DNA. Restricted DNAs were then electrophoresed through 0.6% agarose gels and analyzed for the presence of 5.2-kbp unit-length (form III) pSV2 DNA by blot hybridization with a [^{32}P]SV2 probe. Figure 7 shows results obtained for three different cell lines. A 5.2-kbp monomer of pSV2 DNA was observed for each transformant after digestion with several of the above enzymes. Similar analyses carried out on a total of eight transformants showed that for all cell lines except DR31/1, at least two single-cut restriction enzymes could generate form III pSV2 DNA. These results indicate the presence of tandem duplications of pSV2 in most *gpt*-transformed cell lines.

Additional analyses were carried out on the integrated structures of DR31/6. As shown in Fig. 2, this cell line contains two discrete insertions of pSV2 DNA, one of which produces a hybridizing signal that is considerably weaker than that expected for a single-copy insertion. To examine the basis of this difference in band intensities, DNA fragments containing the two insertions were eluted from a gel after *Xba*I digestion and subjected to further restriction analyses with *Eco*RI, *Bam*HI, and *Hin*dIII (single-cutters of pSV2). The insertion producing a strong hybridizing signal (upper band of DR31/6 in Fig. 2) gave rise to form III pSV2 DNA after digestion with all three enzymes (Fig. 8). Interestingly, the insertion corresponding to the weakly hybridizing band also generated form III pSV2 DNA when digestion was carried out with *Eco*RI or *Bam*HI. Digestion with *Hin*dIII did not produce unit-length pSV2 DNA. Since these results indicate that both insertions in DR31/6 contain tandem duplications of pSV2, the one producing a weak hybridizing signal must be present in only a small fraction of the cell population.

As shown earlier, one of the plasmids rescued from DR31/6 cells, p6-4, was found to carry a partial tandem duplication. In an attempt to determine the origin of this plasmid, we investigated whether p6-4 sequences were tandemly duplicated in the high-molecular-weight DNA of DR31/6 cells. The two restriction fragments generated by *Hin*dIII digestion of p6-4 (a 5.2-kbp fragment corresponding to the unit length of pSV2 and a 1.98-kbp fragment corresponding to the duplicated sequence in this plasmid) are shown in the far right-hand lane in Fig. 7. Since a 1.98-kbp fragment was not observed after digestion of DR31/6 DNA with *Hin*dIII (Fig. 7), we conclude that recovery of this plasmid does not coincide with the presence of a tandem duplication of p6-4 sequences in cellular DNA.

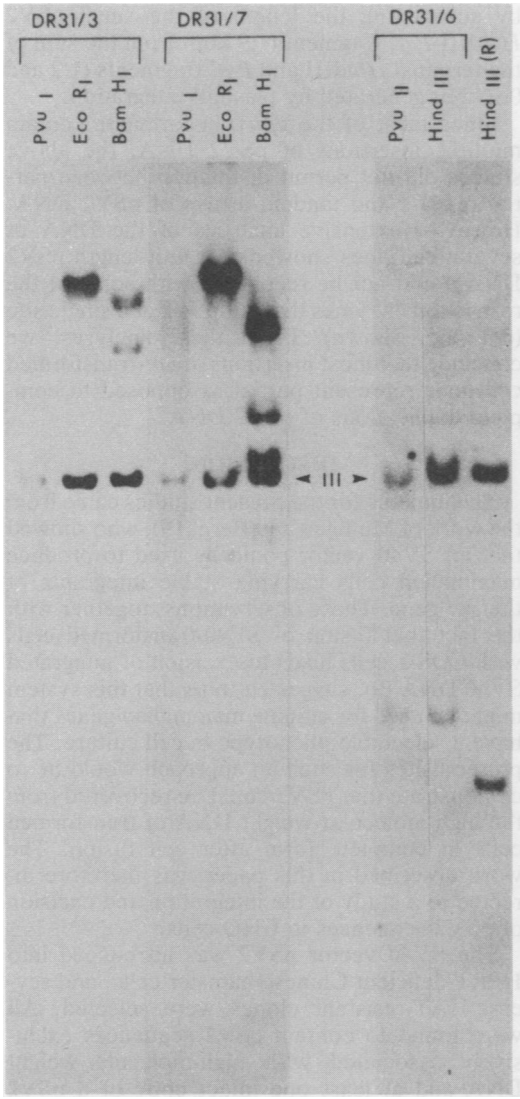

FIG. 7. Restriction analyses of pSV2 DNA in *gpt*-transformed cell lines. Transformant DNAs (5 μg) were digested with various restriction enzymes (*Pvu*I, *Eco*RI, *Bam*HI, *Pvu*II, *Hin*dIII) that cut pSV2 at a single site, electrophoresed through 0.6% agarose gels, transferred to nitrocellulose filters, and hybridized with a [^{32}P]pSV2 probe. Form III of pSV2 (5.2 kbp) was included in the gels as a marker. The lane marked *Hin*dIII(R) corresponds to a reconstruction experiment in which 10 pg of p6-4 was mixed with 5 μg of DR31 DNA and digested to completion with *Hin*dIII restriction endonuclease. The two *Hin*dIII digestion fragments of p6-4 correspond to the 5.2-kbp unit length of pSV2 and the 1.98-kbp duplicated sequence contained within this pSV2-related molecule.

In contrast to the other transformants, transfer hybridization analyses of the single insertion in DR31/1 failed to detect a tandem duplication of pSV2 sequences. Thus, a band corresponding to form III of pSV2 was not observed when DR31/1 DNA was digested with *Eco*RI, *Bam*HI, *Hin*dIII, or *Pvu*II (Fig. 9). Additional restriction analyses with *Pst*I and *Bgl*I showed that integration of pSV2 had occurred through the small *Bgl*I fragment, resulting in an intact SV40 origin and *E. coli gpt* gene in chromosomal DNA. Figure 10 shows the integrated structure of

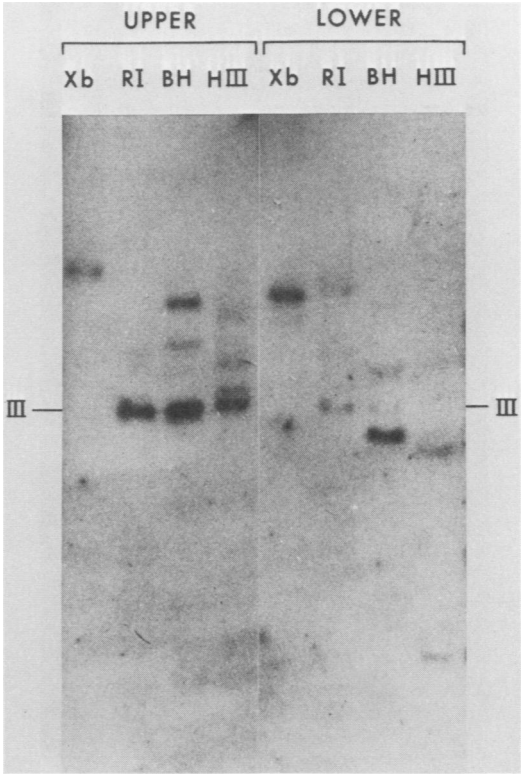

FIG. 8. Restriction analyses of individual pSV2 insertions in *gpt*-transformed DR31/6 cells. Approximately 50 μg of DR31/6 DNA was digested with restriction endonuclease *Xba*I and electrophoresed through a 0.6% agarose gel. With the *Hin*dIII digestion fragments of bacteriophage λ used as reference markers, *Xba*I fragments containing the two discrete pSV2 insertions in DR31/6 cells were located in the gel, electroeluted from appropriate gel slices, and purified by phenol-chloroform extraction and ethanol precipitation. The *Xba*I fragments were then electrophoresed through a 0.7% agarose gel directly (Xb) or first subjected to further digestion with *Eco*RI, *Bam*HI, or *Hin*dIII (RI, BH, HIII). After electrophoresis, the DNA fragments were transferred to nitrocellulose and hybridized with ^{32}P-labeled pSV2. Lanes marked upper and lower correspond to analyses of the *Xba*I fragments containing the "upper" and "lower" insertions of pSV2 DNA in DR31/6 cells (see Fig. 2). Form III of pSV2 (5.2 kbp) was included in the gel as a marker. *Eco*RI digestion was incomplete for the *Xba*I fragments containing the lower insertion of pSV2 DNA.

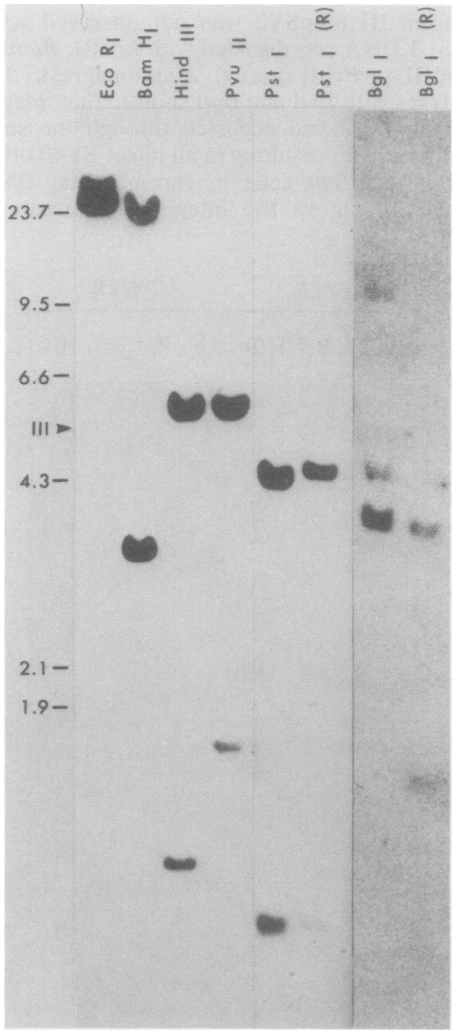

FIG. 9. Transfer hybridization analysis of the single pSV2 insertion in *gpt*-transformed DR31/1 cells. Samples containing 5 μg of DR31/1 DNA were digested with restriction endonucleases *Eco*RI, *Bam*HI, *Hin*dIII, *Pvu*II, *Pst*I, and *Bgl*I, electrophoresed through a 0.6% agarose gel, and blot hybridized with a [^{32}P]pSV2 probe. Markers (in kilobases) correspond to the *Hin*dIII digestion fragments of bacteriophage λ and form III of pSV2. In reconstruction experiments, 10 pg of pSV2 was mixed with 5 μg of DR31 DNA and digested with *Pst*I or *Bgl*I [lanes marked PstI(R) and BglI(R)].

pSV2 DNA in this cell line. From these studies we are unable to exclude the possibility that a small tandem duplication of pSV2 sequences occurs near the boundaries of plasmid and cellular DNA. However, an upper limit of about 0.2 kbp can be assigned to the size of a duplication if, in fact, it exists. This estimate was obtained by subtracting the length of the small pSV2 *Hin*dIII-*Pst*I fragment (1.9 kbp) from the sum of the terminal *Hin*dIII and *Pst*I fragments (1.2 and 0.9 kbp) generated by plasmid integration.

Since most of the *gpt* transformants contain multiple insertions of pSV2 DNA, the above studies did not permit distinction between partial repeats and tandem arrays of pSV2 DNA. However, extensive analyses of the DNA of several cell lines showed that unit-length pSV2 DNA could not be recovered with some of the restriction enzymes that cut pSV2 at a single site (data not shown). From these analyses, we conclude that most insertions in *gpt*-transformed cell lines represent partial as opposed to complete duplications of pSV2 DNA.

DISCUSSION

The impetus for the present studies came from the work of Mulligan and Berg (19), who showed that an SV40 vector could be used to produce mammalian cells carrying stable integrants of the *gpt* gene. These observations, together with the fact that fusion of SV40-transformed cells with COS-1 cells leads to excision of integrated SV40 DNA (9), suggested to us that this system might be used for cloning mammalian genes that have a selectable phenotype in cell culture. The prerequisites for such an approach would be to demonstrate that pSV2 could be recovered from the high-molecular-weight DNA of transformed cells in complete form after cell fusion. The work described in this paper was therefore directed to a study of the integration and excision of pSV2 sequences in CHO cells.

The SV40 vector pSV2 was introduced into HPRT-deficient Chinese hamster cells, and several HAT-resistant clones were selected. All were found to contain pSV2 sequences exclusively associated with high-molecular-weight DNA and at least one intact copy of a pSV2 fragment spanning the origin of SV40 DNA replication, the bacterial segment, and the SV40 regulatory signals required for expression of the *E. coli gpt* gene in mammalian cells. In addition, all of the pSV2 cell lines maintained stable expression of *gpt* in the absence of selection.

One of the transformants, DR31/6, was found to contain an insertion of pSV2 sequences that was not present in every cell. In addition, passage of this cell line in nonselective medium led to the appearance of new pSV2-hybridizing bands as well as to a decrease in copy number of one of the initial insertions of pSV2 DNA. These observations suggest an instability and rearrangement of integrated pSV2 sequences on cell passage or, alternatively, that several "pekelasomes" of pSV2 and carrier high-molecular-weight DNA were present in the initial transformant and its immediate descendants. In the

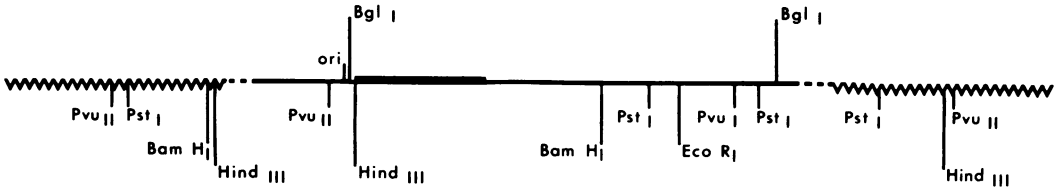

FIG. 10. Map of the single pSV2 insertion in DR31/1 cells.

latter case, heterogeneity would arise if the pekelasomes were diluted out or randomly integrated during cell passage. Subcloning and subsequent DNA analyses would be required to identify the mechanism which led to heterogeneity in DR31/6 cells. In any event, we found no evidence of free pSV2 in any of the *gpt* transformants, and since pSV2 does not code for SV40 T antigen, it is unlikely that heterogeneity results from a mechanism similar to T antigen-mediated excision of integrated SV40 DNA (3).

Fusion of *gpt* transformants with permissive COS-1 cells led to the excision and replication of a population of pSV2-related DNAs. Detailed analysis of the molecules recovered from several transformants revealed a strong correlation between the pattern of rescue of pSV2 DNA and the arrangement of pSV2 sequences integrated within these cell lines. Our observations allowed the following conclusions. First, molecules that are homogeneous in size and indistinguishable in structure and biological function from native pSV2 can be rescued from most *gpt*-transformed cell lines. These arise by precise excision from transformants containing tandem duplications of integrated pSV2 DNA. Second, transformants which carry insertions that lack appreciable sequence duplication (e.g., DR31/1) rescue imprecisely, generating a heterogeneous population of pSV2-related molecules. The above conclusions are consistent with previous proposals based on observations of the integrated state and rescue upon fusion of cell lines containing integrated SV40 sequences (1, 3, 12, 23). Third, in all the earlier studies the work on integration and excision involved viruses (or vectors) encoding the T antigen of SV40. Our studies show that SV40 T antigen is not required to achieve an integrated state that will effect precise rescue upon fusion with permissive cells. Hence, it should theoretically be possible to rescue precisely any circular DNA molecule that contains an origin of SV40 replication after its introduction into recipient cells. Limitations on the rescue process presumably include the size of the molecule to be recovered as well as the extent of duplication required to effect precise excision. Both of these factors remain to be determined.

Perhaps the largest gap in our understanding of the rescue process is the mechanism by which tandem duplications arise. Such integrated structures are frequently observed upon transformation of nonpermissive cells by SV40, and it has been speculated that they result from limited replication of viral DNA followed by recombination between tandem oligomers of SV40 and the host cell genome (6). Although it is unclear whether T antigen plays a role in these events, the presence of tandem duplications in *gpt*-transformed cell lines suggests that such structures form independently of T antigen. However, since the mechanism of integration which occurs during viral transformation may differ from that which occurs when DNA is introduced into cells as a calcium phosphate coprecipitate, no unequivocal statement can at present be made regarding the possible involvement of T antigen in the integration process.

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ADDENDUM IN PROOF

After this manuscript had been accepted for publication, the authors became aware of a paper by Bullock and Botchan in which similar observations pertaining to insertion and fixation were described (P. Bullock and M. Botchan, p. 215–224, *in* R. T. Schimke, ed., *Gene Amplification*, 1982).

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