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High Spontaneous Mutation Frequency in Shuttle Vector Sequences Recovered from Mammalian Cellular DNA

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The recombinant shuttle vector pSV_2gpt was introduced into V79 Chinese hamster cells, and stable transformants expressing the *Escherichia coli gpt* gene were selected. Two transformants carrying tandem duplications of the plasmid at a single site were identified and fused to simian COS-1 cells. Plasmid DNA recovered from the heterokaryons was used to transform a Gpt^- derivative of *E. coli* HB101, and the relative frequency of plasmids carrying a mutation in the *gpt* gene was determined. The high frequency of Gpt^- plasmids (ca. 1%) was similar to that observed when plasmid was recovered from COS-1 cells which had been transfected with pSV_2gpt. Most of the mutant plasmids had rearrangements in the region containing the *gpt* gene.

Experimental systems utilizing the *Escherichia coli lacI* gene and the bacteriophage lambda *cI* gene have generated a great deal of information concerning both the base specificity and site specificity of mutagens in *E. coli* (16). In these systems a gene is introduced into *E. coli*, mutants in the gene are selected, the gene is recovered, and the mutation is identified by DNA sequencing and genetic techniques. A system of this type, capable of determining the base and site specificity of mutagens, does not currently exist in mammalian cells. The recent construction of shuttle vectors, capable of expressing and replicating genes in both mammalian cells and *E. coli* (17), has raised the possibility that such vectors could be used to study mutagen specificity in mammalian cells.

Initial attempts to devise such a system involved the transfection of simian COS cells with simian virus 40 (SV40)-based shuttle vectors carrying selectable bacterial genes (4, 20). It was observed, however, that very high spontaneous mutation frequencies, on the order of 1 to 10%, were observed in the selectable genes.

Intact plasmid DNA also has been rescued from mammalian cells transformed with SV40-based shuttle vectors after fusion of the transformants with COS cells (3, 6). In one study (6) it was observed that when transformants carrying tandem duplications of plasmid DNA were fused with COS cells, all of the rescued plasmid molecules had a normal restriction digest pattern. If intact plasmid could be rescued from transformants of this type, it might be possible to treat such transformants with mutagen, transform bacterial cells with plasmid rescued by COS cell fusion, isolate bacterial transformants containing the mutant gene, recover the mutant gene, and characterize the mutation.

In an attempt to develop such a system, we have transformed a V79 Chinese hamster cell line with the SV40-based shuttle vector pSV_2gpt (17), which contains the *E. coli* gene (*gpt*) for xanthine-guanine phosphoribosyltransferase. Transformants which carry a tandem duplication of the *gpt* gene at a single site in hamster DNA were identified and fused to COS cells. The plasmid rescued from the fused cells was used to transform a Gpt^- strain of *E. coli*, and transformants carrying mutant *gpt* genes were selected and characterized. Our results indicate that plasmid molecules rescued in this fashion have a very high frequency of mutation.

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MATERIALS AND METHODS

Cell culture. The Chinese hamster cell line 743R is a hypoxanthine phosphoribosyltransferase-deficient derivative of the V79 cell line (9) and was provided by Stephen Warren. Simian COS-1 cells (10) were provided by Raju Kucherlapati. The basic cell culture medium was Dulbecco modified Eagle medium supplemented with 10% fetal calf serum.

Transformation of mammalian cells. Culture dishes (35 mm) were inoculated with 1×10^5 to 2×10^5 743R cells. Twenty-four hours later the dishes were treated with 75 ng of pSV_2gpt DNA by the calcium-phosphate precipitation method of Graham and van der Eb (11) as modified by Lowy et al. (14). On the next day, the cells from each 35-mm dish were placed in a 100-mm dish containing medium supplemented with 0.1 mM hypoxanthine, 0.4 mM aminopterin, and 16 μM thymidine (HAT medium [13]). Approximately 2 weeks later a single colony was isolated from each dish and grown into a mass culture in HAT medium.

Preparation of cellular DNA and blot analysis. Cells grown in HAT medium were harvested from two 150-mm culture dishes, and high-molecular-weight DNA was prepared by the method of Blin and Stafford (2). DNA (5 μg) from each sample was digested to completion with restriction enzymes according to the recommendation of the manufacturer (New England Biolabs). The digested DNAs were electrophoresed through a 0.7% agarose gel along with lambda *Hin*dIII markers. The gel was then stained with ethidium bromide and visualized under UV light before denaturation, neutralization, and transfer to nitrocellulose (21). ^{32}P-labeled pSV_2gpt DNA was prepared by nick translation to a specific activity of 3×10^8 cpm/μg of DNA. Approximately 1×10^7 to 5×10^7 cpm of this probe was added to a filter which had been prehybridized for 24 h at 65°C in 6× SSC (1× SSC, 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–100 μg of sheared and denatured salmon sperm DNA per ml. The filter was hybridized overnight at 65°C in the above prehybridization solution, followed by three 1-h washes at 65°C in 2× SSC–0.1% sodium dodecyl sulfate and a single 15-min wash in 0.1× SSC–0.1% sodium dodecyl sulfate. The

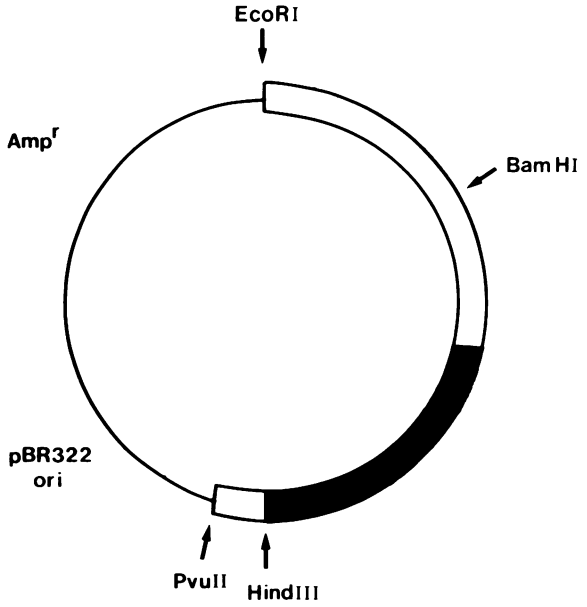

FIG. 1. Restriction map of pSV$_2$gpt. SV40 sequences are indicated by the open boxes, the *E. coli* region containing the *gpt* gene is indicated by the solid box, and pBR322 sequences are indicated by the solid line.

filter was dried and analyzed by autoradiography with Kodak XAR film.

COS cell fusion. Fusion of 743R transformants to COS-1 cells was carried out with polyethylene glycol and dimethyl sulfloxide as described by Breitman et al. (3). At 48 h after the fusion, low-molecular-weight DNA was extracted by the method of Hirt (12). The Hirt supernatants were treated with RNase A and proteinase K, extracted with phenol and chloroform, concentrated by ethanol precipitation, and resuspended in 10 mM Tris (pH 8.0)–1 mM EDTA.

Bacterial transformation and preparation of plasmid DNA. The bacterial strain DT-2, a xanthine-guanine phosphoribosyltransferase- and hypoxanthine phosphoribosyltransferase-deficient derivative of strain HB101 was provided by Oliver Smithies. Transformation was carried out by a modification of the method of Mandel and Higa (15). Transformants were selected on minimal salt plates containing thiamine, glucose, Casamino Acids, and 50 μg of ampicillin per ml. Selection of Gpt$^-$ transformants was carried out on the plates described above supplemented with 54 μM 6-thioguanine (TG). Colonies which grew on the TG-containing plates were always retested for growth on TG before further analysis.

Small-scale plasmid DNA preparations were prepared from 5-ml overnight cultures (1). Approximately 1 μg of these DNAs was digested for 2 h with 10 to 20 U of restriction enzymes and electrophoresed in a 1.0% agarose gel. The gel was stained with ethidium bromide, and the DNA was visualized by UV light.

Selection of mutants and revertants from hamster cell transformants. For the selection of TG^r variants, 743R *gpt* transformants were grown in HAT medium for at least 10 days before TG selection. The transformants were then transferred to medium containing 0.1 mM hypoxanthine and 16 μM thymidine (HT medium) 2 days before selection. To determine the frequency of TG^r variants, four 100-mm culture dishes were inoculated with 10^3 cells in medium containing 72 μM TG. To determine the plating efficiency in the absence of drug, three 100-mm dishes were inoculated with 100 cells in growth medium alone. After 7 to 10 days, the dishes were fixed and stained, and colonies of more than 100 cells were counted. To determine the frequency of HAT^r revertants of the TG^r variants, variant cells growing in TG were transferred to medium without TG 2 days before the start of HAT selection. To determine the frequencies of HAT^r revertants in these cell populations, four 100-mm culture dishes were inoculated with 10^6 cells in HAT medium. Plating efficiency was determined as described earlier. Colonies were stained and counted ca. 10 days after plating. The calculated frequencies of both TG^r and HAT^r variants were corrected for plating efficiency in the absence of drug.

RESULTS

Isolation of *gpt* transformants. The recombinant plasmid pSV$_2$gpt (Fig. 1) was introduced into a TG^r V79 Chinese hamster cell line by the DNA-calcium phosphate precipitation technique. The recipient cell line is a presumptive deletion mutant for hypoxanthine phosphoribosyltransferase, as it was isolated after X-ray mutagenesis, and no revertants of this mutant have been selected in HAT medium (S. Warren, personal communication). As this cell line is deficient in hypoxanthine phosphoribosyltransferase, transformants which express the *E. coli gpt* gene can be selected in HAT medium. In two separate transformations, HAT-resistant colonies were obtained at an average frequency of 55 colonies per μg of DNA/10^6 cells. Six independently derived colonies were isolated and used for further study.

Structure of plasmid DNA in the transformants. Previous studies have demonstrated that the physical structure of the integrated DNA is important in determining the structure of the excised DNA when plasmids containing the SV40 origin of replication are rescued by COS cell fusion (6). To determine the structure of the integrated pSV$_2$gpt DNA in the transformants, high-molecular-weight cellular DNA was prepared and subjected to blot hybridization analysis. The autoradiogram (Fig. 2) shows a blot analysis of DNA isolated from the recipient line (743R) and from five transformants (C5, C6, C7, D1, and D3) which were digested with the restriction endonuclease *Sac*I, an enzyme which does not cut pSV$_2$gpt DNA. Lane 7 (Fig. 2) is a reconstruction in which 5 pg of pSV$_2$gpt DNA was added to 5 μg of 743R DNA before digestion to simulate the presence of one copy of plasmid DNA per cell.

Each of these five transformants contained a single, high-molecular-weight (10 to 22 kilobases [kb]) hybridizing band. No free plasmid DNA was found in any of the transformants. The intensity of the hybridizing bands suggests that one to five copies of the plasmid have integrated into high-molecular-weight DNA in the transformants. The fact that a single band was observed after digestion with *Sac*I, an enzyme which does not cut the plasmid DNA, suggests that the plasmid DNA is integrated at a single site in the cellular DNA in each of the transformants. The various sizes of the bands in each of the transformants suggest that the site of integration is different in each line. A single hybridizing band for each of these five transformants was also observed in a similar experiment in which the DNAs were cut with *Xba*I, another enzyme which does not cut the plasmid DNA (data not shown). A sixth transformant, D2, was analyzed in a similar manner and was found to contain two hybridizing bands after digestion with either *Xba*I or *Sac*I (data not shown).

Blot analysis of the transformants was also carried out

after digestion with *Hin*dIII, an enzyme which cuts the plasmid DNA once. Digestion of DNA from three of the transformants (C6, D2, and D3) produced bands which migrate with the linear form of the plasmid DNA (Fig. 3). Unit-length fragments were also produced by digestion with two other single-cut enzymes, *Pvu*II and *Bam*HI (data not shown). Unit-length fragments were not produced by digestion of DNA from the remaining three transformants (C5, C7, and D1) with the single-cut enzymes *Hin*dIII (Fig. 3), *Bam*HI, *Pvu*II, and *Eco*RI (data not shown). We conclude that transformants C6, D2, and D3, which generate unit-length fragments, contain tandem duplications of all or most of the plasmid sequences, whereas transformants C5, C7, and D1 do not.

Fusion of *gpt* transformants to COS cells. A previous study demonstrated that when a transformant containing a tandem duplication of an SV40-based shuttle vector was fused to COS cells, the excision products recovered were identical to the input plasmid DNA, as judged by the restriction digest pattern of the recovered DNA (6). Two of the *gpt* transformants described above, C6 and D3, were shown by blot analysis (Fig. 2 and 3) to have a tandem duplication of pSV_2gpt integrated at a single site in the chromosomal DNA. Therefore, these two cell lines were chosen as fusion partners with a COS cell line to recover intact plasmid DNA.

The transformants were fused to COS-1 cells, and low-molecular-weight DNA was prepared from the fused cells. Undigested DNA from the Hirt supernatant was fractionated on a 0.7% agarose gel, bloted on nitrocellulose, and probed with ^{32}P-labeled pSV_2gpt. The excision products from the two fusions gave similar banding patterns (Fig. 4). Strong hybridizing bands were observed at positions where supercoiled, circular, and multimeric plasmid DNA would be expected to run. Although no other distinct bands were observed, a smear of hybridizing material was seen in both lanes.

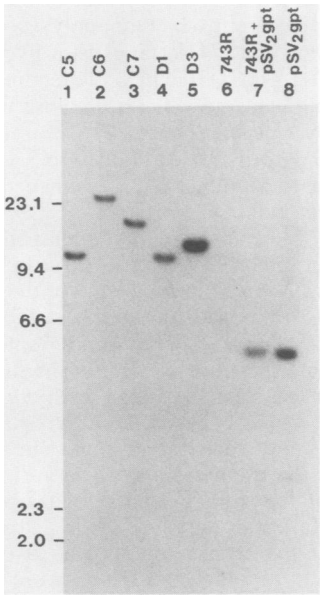

FIG. 2. Blot hybridization analysis of the 743R *gpt* transformants. High-molecular-weight DNA (5 μg) from each of the transformants was digested with *Sac*I, electrophoresed on a 0.7% agarose gel, and blot hybridized with pSV_2gpt as a probe. The sample in lane 7 contained 743R DNA to which 5 pg of pSV_2gpt was added before digestion. Lane 8 contains 5 pg of uncut pSV_2gpt DNA.

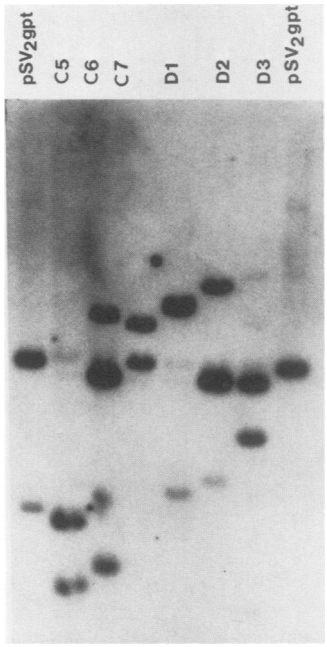

FIG. 3. Blot hybridization analysis of the 743R *gpt* transformants. DNA (5 μg) from each of the transformants was digested with *Hin*dIII and blot hybridized with a pSV_2gpt probe. Uncut pSV_2gpt (5 pg) was run in the first lane as a marker.

Mutation frequency of the excised plasmid. The biological activity of the rescued plasmids was examined by transforming an *E. coli* strain with the Hirt supernatants and testing for *gpt* activity in the bacteria. The bacterial strain DT-2, a Gpt^- derivative of strain HB101, was transformed, and samples of

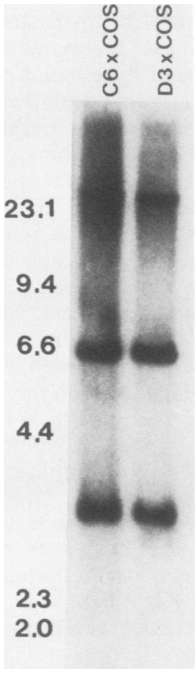

FIG. 4. Blot hybridization analysis of the low-molecular-weight DNA from C6 × COS and D3 × COS fusions. Low-molecular-weight DNA was extracted from two 100-mm tissue culture dishes of fused cells from each of the fusions. One-tenth of this material was fractionated on a 0.7% agarose gel and blot hybridized with a pSV_2gpt probe.

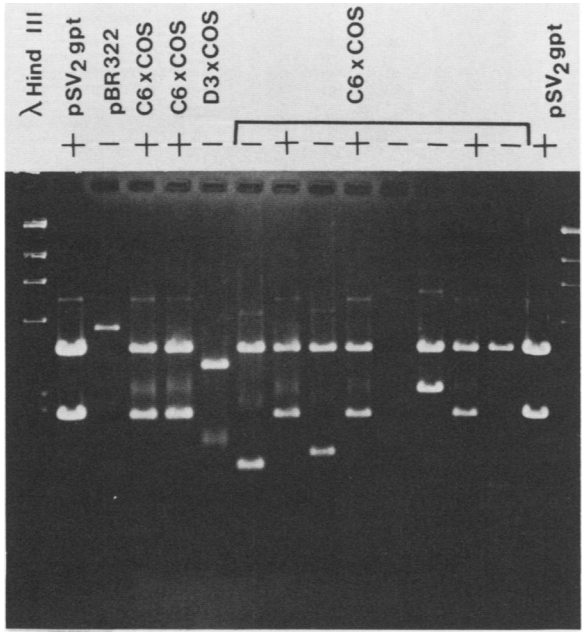

FIG. 5. Restriction digests of plasmids rescued from COS cell fusions. Small-scale plasmid DNA preparations from various DT-2 transformants were digested with *Hin*dIII and *Bam*HI. The Gpt$^+$ or Gpt$^-$ phenotype of the transformant and the origin of each plasmid used to transform DT-2 are indicated above the appropriate lane.

transformed cells were plated on minimal plates supplemented with either ampicillin (MA plates) or ampicillin and TG (MATG plates). Transformants which are Amp^r and which express the *gpt* gene of pSV$_2$gpt will be killed by TG and, thus, will not grow on MATG plates. Transformants which are Amp^r but have a mutation in the *gpt* gene will grow on both plates. An estimate of the Gpt$^-$ mutant frequency among the recovered plasmid molecules is given by the number of colonies on MATG plates divided by the number of colonies on MA plates.

In control experiments, strain DT-2 was transformed with pBR322 and pSV$_2$gpt and plated on both MA and MATG plates. Equal numbers of pBR transformants, which should be Amp^r and Gpt$^-$ were obtained on both MA and MATG plates. The pSV$_2$gpt transformants, however, did not grow on the MATG plates. Samples of pSV$_2$gpt-transformed cells capable of forming ca. 5,600 colonies on MA plates did not form a single colony on MATG plates.

When Hirt supernatants from three separate C6 × COS cell fusions were used to transform strain DT-2, samples of cells which produced a total of ca. 3,200 colonies on MA plates produced 24 colonies on MATG plates. Thus, almost 1% of the Amp^r transformants were TG^r, and presumably Gpt$^-$. In one experiment, the Hirt supernatant from a D3 × COS fusion produced 1 colony on MATG plates and 414 colonies on MA plates.

Structure of the rescued Gpt$^-$ plasmids. Plasmid DNA was prepared from a number of Gpt$^+$ and Gpt$^-$ bacterial transformants isolated from MA and MATG plates. These DNAs were digested with *Hin*dIII and *Bam*HI, and their restriction patterns were compared with that of pSV$_2$gpt. Digestion of pSV$_2$gpt DNA with these enzymes produces a 3.4-kb band and a 1.9-kb band which contains the *gpt* gene (Fig. 5). All five of the Gpt$^+$ plasmids had both the 3.4- and 1.9-kb bands. In contrast, none of the Gpt$^-$ plasmids had an intact 1.9-kb band. Many of the Gpt$^-$ plasmids had an apparently normal 3.4-kb band, whereas the band containing the *gpt* gene was smaller or missing completely. Sixteen Gpt$^-$ plasmids rescued from C6 × COS fusions were tested in this way, and at least 11 different restriction patterns were observed. In cases in which apparently identical restriction patterns were observed, the plasmids were always obtained from the same bacterial transformation and could possibly be sibs. Thus, the mutations producing the Gpt$^-$ phenotype seem to be primarily, if not exclusively, rearrangements, and most frequently deletions, and apparently arose from a number of independent events.

Mutant frequency from COS transfection. Some recent studies (4, 20) have shown that when COS cells are transfected with SV40-based shuttle vectors, a very high mutation frequency is observed among the recovered plasmid. To compare both the frequency and types of mutations generated after COS cell fusion to transformants with those of mutations generated after transfection, COS-1 cells were transfected with pSV$_2$gpt, and the plasmid products were analyzed. Low-molecular-weight DNA was extracted 48 h after the COS-1 cells were transfected by the DNA-calcium phosphate precipitation method. This DNA preparation was then used to transform DT-2 cells, and samples of the transformants were plated on MA and MATG plates. A total of 21 colonies were obtained on MATG plates, and the ratio of MATG-MA colonies was 0.017. This estimated mutant frequency of 1 to 2% was very similar to that obtained in other studies in which COS cells were transfected with other SV40-based shuttle vectors (4, 20) and was roughly twice that seen with the plasmids rescued by fusion of COS cells with transformants.

To analyze the plasmid products from the transfected cells, plasmid DNA was prepared from Gpt$^+$ and Gpt$^-$ plasmids, digested with *Hin*dIII and *Bam*HI, and subjected to electrophoresis on an agarose gel. As shown above, the Gpt$^-$ plasmids all contained both the 3.4- and 1.9-kb bands of pSV$_2$gpt (Fig. 6). Also similar to the above results, most of the Gpt$^-$ plasmids had altered restriction patterns. Of the 19 plasmids that were analyzed, 16 had an altered restriction pattern, whereas 3 could not be distinguished from pSV$_2$gpt. Among the 16 rearranged plasmids, 15 different restriction patterns were observed. Although all of the rearranged plasmids had an altered 1.9-kb band, several had alterations in the size of the 3.4-kb band as well.

Generation of TG^r variants in the hamster cell transformants. During the course of our work we observed that many of the Chinese hamster Gpt$^+$ transformants lost their *gpt* activity (as judged by their ability to form colonies in TG) at a very rapid rate. Measurements of the frequency of TG^r colonies in populations of transformants C6 and D3 were made 2 to 4 days after the cells were transferred from HAT medium to nonselective medium. These values, after correcting for plating efficiency, were 1.7×10^{-2} for C6 and 3.6×10^{-2} for D3. The similarity of these values to the approximate frequency of TG^r plasmid rescued from C6 raised the possibility that the mutations that produced the TG^r phenotype in the rescued plasmids may have occurred in the Chinese hamster cell transformants before the COS cell fusion.

To determine the mechanism by which TG^r variants were generated in hamster cell transformants, six TG^r colonies were isolated from each of the C6 and D3 transformants. After the colonies had been expanded in TG-containing medium, they were transferred to nonselective medium, and 2 days later the frequency of HAT^r colonies was measured.

Among the TG^r variants derived from C6, HAT^r colonies were obtained at relatively high and very uniform frequencies, ranging from 1.8×10^{-4} to 3.2×10^{-4}. Similar results were obtained with the TG^r variants derived from D3. Four of the variants had reversion frequencies in the same range as those observed for the C6 variants. For the remaining two D3 TG^r variants, one had a slightly higher reversion frequency (5.3×10^{-4}), whereas the other had a significantly lower reversion frequency (1.3×10^{-5}).

High-molecular-weight DNA was prepared from all six of the C6 TG^r variants and from three of the D3 variants. These DNAs were digested with *Hin*dIII, blotted, and probed with a ^{32}P-labeled pSV$_2$gpt probe. All six of the TG^r colonies had three bands of hybridizing material which were identical in size to those seen in the parental C6 line (Fig. 7). Similar results were obtained from blot analysis of the D3 TG^r variants. All three variants contained bands equal in size and number to those of D3 (data not shown).

One of the C6 TG^r variants, C6-S1, was fused to COS cells to rescue plasmid DNA. The Hirt supernatant from the fused cells was used to transform DT-2 cells, and Amp^r colonies were selected on MA plates. The Amp^r colonies were moved by toothpick onto MATG plates to test the functional state of the *gpt* gene. None of the 22 Amp^r colonies tested were able to grow on MATG plates, demonstrating that only Gpt$^+$ plasmids were isolated from the TG^r cells.

The results of three groups of experiments all suggest that the mechanism by which TG^r variants were generated in the hamster cell transformants is different from the mechanism by which mutations were generated in the rescued plasmids. The reversion analysis showed that all of the TG^r variants reverted to HAT^r at a relatively high and uniform rate which would not be expected if this phenotype was the result of genetic rearrangement. Also, the blot analyses showed that the structure of the integrated *pSV$_2$gpt* in all of the TG^r variants was identical to that of the parental transformants. Finally, only Gpt$^+$ plasmid was rescued from one of the TG^r variant transformants. Taken together, these results suggest that the TG^r phenotype in the hamster cells was generated by a nongenetic mechanism and that the mutations generating the Gpt$^-$ phenotype in the rescued plasmids took place subsequent to COS cell fusion.

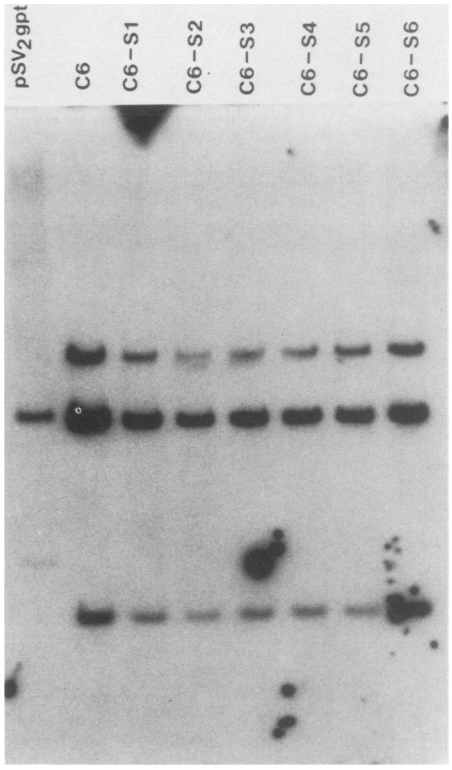

FIG. 7. Blot hybridization analysis of C6 TG^r variants. DNA (5 μg) from C6 and each of six TG^r variants (C6-S1 to 7) was digested with *Hin*dIII, fractionated on a 0.7% agarose gel, and blot hybridized with a pSV$_2$gpt probe.

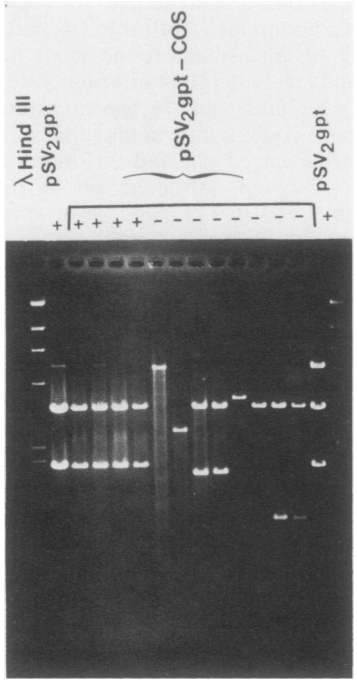

FIG. 6. Restriction digests of plasmids recovered from COS cell transfections. Small-scale plasmid DNA preparations from DT-2 transformants were digested with *Hin*dIII and *Bam*HI. The Gpt$^+$ or Gpt$^-$ phenotype of the transformant and the origin of the plasmid used to transform DT-2 are indicated above the appropriate lane.

DISCUSSION

Work by Conrad et al. (6) has shown that the structure of SV40-based shuttle vectors rescued from mammalian cell transformants by COS cell fusion is determined by the structure of the integrated plasmid. In their experiments, plasmid molecules rescued from transformants having less than a single copy or a partial tandem duplication of the plasmid in their high-molecular-weight DNA had a high frequency of rearrangements. Presumably, these rearrangements resulted from imprecise excision of the integrated plasmid. Alternatively, when plasmid was rescued from a transformant having a tandem duplication of the plasmid, all 64 of the plasmid molecules rescued had a normal restriction digest pattern. This result suggested a method of recovering shuttle vector sequences from mammalian cells that might avoid the problem of high spontaneous mutant frequencies observed in other studies (4, 20).

In our experiments, hamster cell *gpt* transformants were isolated, and the structures of the integrated plasmids were determined by blot hybridization analyses. Two transformants (C6 and D3) which have a tandem duplication of

pSV_2gpt at a single site were identified (Fig. 2 and 3). Fusion of these transformants to COS-1 cells appeared to produce predominantly intact plasmid (Fig. 4), as judged by blot analysis, in agreement with the results of Conrad et al. (6). However, transformation of a Gpt^- *E. coli* strain with plasmid rescued from a C6 × COS cell fusion demonstrated that, although the great majority (ca. 99%) of the recovered plasmid does appear to be intact pSV_2gpt, there is a relatively high spontaneous mutation frequency (ca. 1%).

Examination of the structures of the Gpt^- plasmids generated by COS cell fusion (Fig. 5) showed that the mutant plasmids had undergone major rearrangements in structure. None of the 16 Gpt^- plasmids examined had a normal restriction digest pattern. The most common rearrangement seemed to be a deletion in the *Hin*dIII-*Bam*HI fragment containing the *gpt* gene. The larger *Hin*dIII-*Bam*HI fragment was intact in many of the mutants. This result was not surprising since the function of three sites in this fragment (the Amp^r marker and the origins of replication for pBR322 and SV40) was selected for in either mammalian cells or bacteria. The endpoints of several of the deletion plasmids are currently being determined to ascertain whether any common sequences are involved in the generation of the deletions.

A high spontaneous mutant frequency (1.7%) was also observed after transfection of COS-1 cells with pSV_2gpt. This result is in the range of mutant frequencies generated by the transfection of COS cells with other SV40-based shuttle vectors (4, 20). As was the case with the plasmid rescued by COS cell fusion, most of the mutant plasmids had rearrangements in their structure (Fig. 6).

Both the site and mechanism of the mutagenic events which produce the Gpt^- plasmids remain to be elucidated. As has been observed for many mammalian cell transformants (7, 18), the hamster cell transformants used in this study, C6 and D3, lose their Gpt activity spontaneously at a high rate. Several results suggest, however, that this loss of Gpt activity in the hamster cells does not contribute to the generation of the Gpt^- plasmids isolated after COS cell fusion. Although the Gpt^- plasmids seem to result largely and possibly exclusively from DNA rearrangements, the structure of the integrated plasmid in the TG^r hamster cell variants appears to be unchanged, as judged by blot analysis (Fig. 7). Also, all of the TG^r variants spontaneously reverted to HAT^r at a relatively high and extremely uniform rate, and plasmid rescued from a TG^r variant was found to be Gpt^+ when transformed into *E. coli*. These results suggest that the TG^r hamster cell variants arose by an epigenetic event rather than by a change in DNA sequence. Changes in chromatin structure and methylation patterns have been proposed as mechanisms by which expression of transformed genes can be altered in mammalian cells (5, 8).

The possibility that the mutations arise in *E. coli* subsequent to transformation with the Hirt supernatants cannot be ruled out. However, replication of the plasmid in bacteria does not appear to be an error-prone process, as no TG^r transformants were observed when DT-2 was transformed with pSV_2gpt which had been isolated from *E. coli*. It is also possible that COS cells have an intrinsically high rate of mutation. However, it has been shown that spontaneous mutation frequencies for cellular genes do not appear to be unusually high in COS cells (19; C. Ashman, unpublished data). Another possibility is that the observed high mutation frequencies are due in part to preferential replication of the rearranged plasmids. However, other studies have shown that plasmid mutant frequency does not increase with time after transfection, as would be expected if there was preferential replication of mutant plasmids (20). Also, our restriction digests of the mutant plasmids (Fig. 5 and 6) showed that a variety of different rearrangements were produced after both COS cell transfection and COS cell fusion. Most of the mutants thus apparently arose from independent mutational events rather than from replication of a small number of mutants.

It has been proposed (4) that some or all of the mutations generated during COS cell transfection may result from DNA damage which occurs in the cytoplasm before the uptake of plasmid into the nucleus. In our experiments, however, pSV_2gpt was rescued by COS cell fusion from Chinese hamster cell transformants which contained a functional *gpt* gene integrated into chromosomal DNA. Therefore, the high observed mutation frequency presumably resulted from DNA damage which occurred subsequent to the uptake of the plasmid and its integration into cellular DNA. Although our results do not rule out DNA damage during uptake of the plasmid as a cause of some of the mutational events, they strongly suggest that other mechanisms are operating as well.

Although it is not possible to rule out imprecise excision as a possible mechanism for the generation of mutants after COS cell fusion, the similarities in both the frequencies and types of mutations generated during COS cell transfection and rescue by COS cell fusion suggest that the mutations arise by the same mechanism during a common step. Recently, evidence has been presented that much of the DNA damage during COS cell transfection occurs before the onset of DNA replication (19). If replication errors do not play a large role in the generation of these mutants, a likely explanation for the high mutation rate is that the plasmid molecules are subject to high levels of nucleolytic degradation (4, 19). It is possible that this degradation is directed toward specific sequences in the vectors. If this is the case, it may be possible to construct vectors which are more capable of stable replication in mammalian cells. Alternatively, the instability of these vectors may be a general property of autonomously replicating DNA molecules in mammalian cells.

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