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Mutagenesis of a Shuttle Vector Plasmid in Mammalian Cells

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Recently we and others have reported a high frequency of mutagenesis of shuttle vector plasmids after passage in mammalian cells (Razzaque et al., Proc. Natl. Acad. Sci. U.S.A. **80**:3010–3014, 1983; Calos et al., Proc. Natl. Acad. Sci. U.S.A. **80**:3015–3019, 1983). The mutation frequency was determined by monitoring the integrity of a bacterial marker gene on the plasmid by standard microbiological procedures. Mutant plasmids contained deletions, insertions of cell DNA, and point mutations. The observed mutation frequency of 1% is much higher than that of cellular markers and could be due to the induction of a mutagenic environment by infection with a replicating plasmid. Alternatively, the hypermutagenesis may be due to some critical transient or persistent difference between the DNA in the plasmid and the cellular chromosome. We performed a number of experiments designed to distinguish between these alternatives, with particular reference to deletion mutagenesis. We conclude that mutagenesis was specific to the plasmid and propose that the majority of the deletion and insertion mutants were generated very early in the infection, before replication of the vector. However, some deletion mutagenesis also occurred after plasmid replication had begun.

Transfection of DNA into animal cells has been widely used in studies of gene expression and regulation, recombination, and gene isolation. Implicit in these experiments is the assumption that the sequence of the DNA is unaffected during the course of introduction and stabilization of the sequences in the nuclei of the recipient cells. However, rearrangement and point mutagenesis of transfected sequences have been described (20, 24), although no quantitative estimate of the frequency of these events has been possible. Recently, we (19) and another group (5) have reported the high frequency of spontaneous mutagenesis of shuttle vector plasmids as a result of passage in mammalian cells. Both studies employed plasmids which contained bacterial marker genes, *galK* or *lacI*, and were dependent on simian virus 40 (SV40) T antigen and an SV40 origin of replication for replication in mammalian cells. Progeny plasmids were extracted from the cells and introduced into the appropriate bacterial strains; colonies with mutations in the marker genes were identified by standard microbiological procedures. In our experiments, we found that about 1% of the progeny plasmids carried a defective galactokinase gene. The mutant plasmids contained deletions, insertions of cell DNA, and point mutations. This mutation frequency is much higher than the spontaneous mutation frequency of cellular markers which ranges from 10^{-4} to 10^{-7}, and threatens the validity of this approach for studying eucaryotic mutagenesis at a biologically relevant frequency.

There are at least two classes of explanations for the hypermutagenesis of the shuttle vector plasmid. The first is that infection of the cells with the shuttle vector induces a mutagenic environment and that both plasmid and cell genes are mutagenized. The explanations of the second class propose that the mutagenesis is specific to the plasmid DNA because of critical differences between the vector and genomic DNA. Some of these differences reflect the introduction of purified DNA grown in bacteria (with procaryotic, not eucaryotic, modifications and without a chromatin structure) and the exposure of the DNA to the cytoplasm of the recipient cells. In this view, once the plasmid acquires a chromatin structure and has replicated, it will be as eucaryotic as possible and would be no more susceptible to mutagenesis than a cell marker. On the other hand, it is possible that the plasmid is susceptible at any time during the infection, perhaps because it is extrachromosomal or contains poison sequences (13) or lacks some protective sequences (5). In the experiments reported here, we have attempted to assess the merits of these explanations.

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MATERIALS AND METHODS

Cells. The African green monkey kidney cell lines BSC1 and CV1 and the Cos-1 line of Gluzman (9) were used as eucaryotic hosts of the shuttle vector and were grown in minimal essential medium supplemented with 10% fetal calf serum. The *Escherichia coli* strains (and relevant genotypes) were: HB101 [(*recA13*) *hsdS20* (r_B^- m_B^-) *galK2*], GM33 (*dam-3*), GM48 (*dcm-6 dam-3*), and LE392 (M^+ *dcm*$^+$ *dam*$^+$).

Plasmids and mutant selection. The plasmid pGS3 has been described (19) and is diagrammed (Fig. 1). The plasmids pGSCI and pGSCII are similar to pGS3 in that they contain pML (12) and the bacterial gene *galK*. In addition, they contain the entire SV40 genome, in either orientation, joined at the *Bam*HI site. Linear plasmid molecules were prepared by cleavage with *Eco*RI followed by filling of the overhang with T4 DNA polymerase. DEAE-dextran was used as a facilitator for the mammalian cell transfections (17). Plasmids were harvested from infected monkey cells by Hirt extraction (11), purified, treated with *Dpn*I (18), and introduced into HB101 by standard procedures. Bacterial colonies with plasmids carrying a mutant galactokinase gene were identified by colony color on MacConkey galactose agar or by resistance to 2-deoxygalactose (2, 19). Analysis of mutant plasmids by agarose gel electrophoresis, blotting, and hybridization was as described (19).

Selection of 6TG-resistant cells. CV1 and Cos-1 cells were seeded at densities of 5×10^5 cells per 100-mm dish, and 12 h later, the medium was replaced with medium containing 60 μM 6-thioguanine (6TG). The medium was changed every 3 days. After 2 weeks, the resistant colonies with healthy, growing cells were clearly visible. In other experiments, CV1 cells were incubated with DNA (the nonreplicating plasmid pBR322) and DEAE-dextran for 1 h as done before

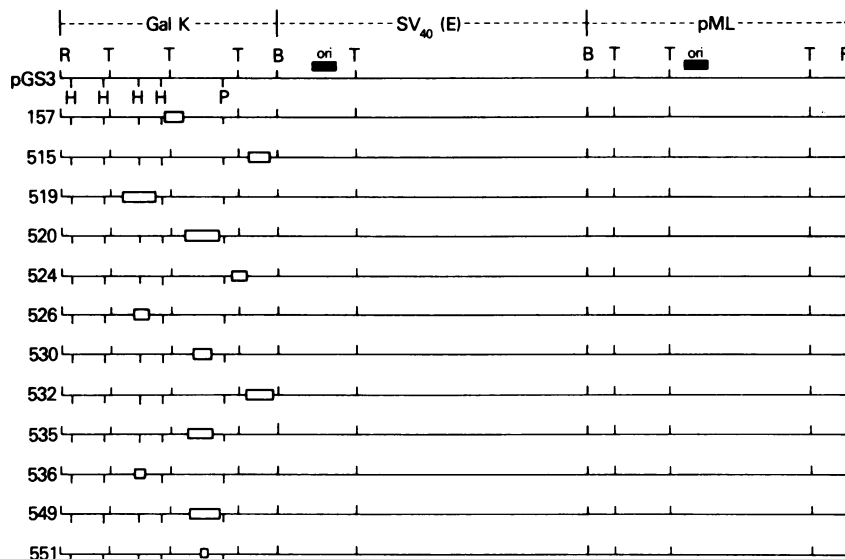

FIG. 1. Restriction mapping of deletion mutant plasmids from standard infections. BSC1 cells were infected with superhelical pGS3 and mutant plasmids isolated as described (18). Those with deletions of less than 500 bp were analyzed by restriction digestion with a variety of enzymes; some of the restriction enzyme sites are shown. T, *Taq*I; H, *Hae*II; B, *Bam*HI; P, *Pvu*I; R, *Eco*RI.

(19), and then the cells were washed and fed with normal medium. The cells were permitted to undergo 4 to 5 population doublings over the course of 1 week. Then they were seeded at a density of 5×10^5 cells per 100-mm dish and placed in 6TG-selective medium as done before.

Chloroquine treatment. BSC1 cells were infected with pGS3 and treated with chloroquine diphosphate (Sigma Chemical Co., St. Louis, Mo.), exactly as described previously (14).

Enzyme. Restriction enzymes were from Bethesda Research Laboratories (Gaithersburg, Md.) and New England Biolabs (Beverly, Mass.).

RESULTS

Generalized mutagenesis. In our first series of experiments, we considered questions posed by the hypothesis: infection with the shuttle vector induces a mutagenic condition affecting both cellular and plasmid genes. The putative induction may be due to exposure of the cells to the facilitiating agent, DEAE-dextran, to the SV40 T antigen, or to the presence of a high-copy-number plasmid. We tested the first two of these proposals by measuring the frequency of 6TG-resistant cells in the appropriate population of cells. In the initial experiment, the comparison was made between control cells and cells treated with a DNA–DEAE-dextran mixture. Because DEAE-dextran and other agents (calcium phosphate, polyethylene glycol) used in standard transfection procedures are all toxic, it seemed reasonable to consider the possibility that these treatments are responsible for the mutagenesis. However, both the experimental and control cultures had the same frequency of 6TG-resistant cells, 1×10^{-6} to 2×10^{-6}, and we concluded that the exposure of the cells to the DNA–DEAE-dextran mixture was not significantly mutagenic. In the second series of experiments, we asked whether T antigen was involved. Geissler and colleagues have proposed that cell genes are mutagenized by SV40 infection and have suggested that T antigen is responsible (12). The Cos-1 cells of Gluzman (9) constitutively express a replication-competent T antigen and thus are the equivalent of a shuttle vector-infected cell without the plasmid DNA. We repeated the 6TG resistance study and compared the frequency of resistant cells in cultures of Cos-1 and CV1 cells. In two separate experiments, we found no difference between the two lines. As before, resistant cells were present at a frequency of about 10^{-6} in both cultures. The third possibility, that mutagenesis was induced by the presence of multiple copies of the vector, was not readily tested. However, if this were true, the induction could occur only after replication began (see below).

We next turned our attention to explanations which are based on some crucial difference between cellular and plasmid genes.

Influence of chloroquine treatment on plasmid mutagenesis. The entry of the plasmid into the nucleus requires passage through the cytoplasm. Recently, Luthman and Magnusson described a modification of the DEAE-dextran procedure in which the efficiency of infection was substantially increased by treatment of the cells with chloroquine diphosphate (14). They argued that most of the DNA in a transfection is degraded by lysosomes and that the enhanced efficiency is due to the inhibition of the lyososomal hydrolases by the change in lysosomal pH resulting from the chloroquine treatment. We asked whether chloroquine treatment would affect the yield of plasmid DNA and whether there was any effect on the mutation frequency. The results were disappointing. We found no change in yield, and furthermore, the mutation frequency tripled (Table 1). Analysis of the mutant plasmids indicated that about 40% were molecules with no change in molecular weight. Customarily, we have observed that about 20 to 25% of the mutant plasmids are of this type (19). We and others have shown that many of this class of mutant plasmid are point mutants (5, 19). Chloroquine (perhaps because it is an intercalator) appears to increase the frequency of point mutations.

Effect of methylation. An obvious difference between the input plasmid DNA and the cellular DNA is the nature and location of methylated bases. We prepared plasmids from a variety of bacterial strains which differed in their capacity for cytosine or adenine (or both) methylation (Table 1). The

mutation frequency of the progeny plasmids, regardless of methylation state, was always about 1%, and the pattern of mutant plasmids was also unaffected. These results suggest that procaryotic modification patterns did not influence the mutagenesis.

Effect of additional sequences from SV40. Recently, Calos et al. (5) described experiments similar to those reported by us (19). They have found that the mutation frequency of a shuttle vector plasmid with the entire SV40 genome is 10-fold lower than that of several other plasmid constructions which contain only the origin of replication from SV40. They have suggested that the additional SV40 sequences stabilize the plasmid and partially protect it from mutagenesis. The plasmid used in our studies, pGS3, contains the early region of SV40 but not the late genes. We constructed vectors with the entire SV40 genome (pGSC) in either orientation and determined the mutation frequency in the plasmid populations after passage in either BSC1 or Cos-1 cells. In all experiments, the mutation frequency was ca. 1%, the same as with the original construction (Table 1).

The preceding experiments permitted us to eliminate, or at least reduce the likelihood of, a number of possible explanations for the mutagenesis of the shuttle vector. However, they failed to answer questions that are fundamental to an understanding of this phenomenon: What is the mechanism of the mutagenesis? When does the mutagenesis occur? In the following experiments, we addressed these questions as they concern one class of mutant plasmids, the deletions. Deletion mutations have been studied in both bacterial and eucaryotic systems, and it has been suggested that recombination or slipped mispairing, during replication, between homologous sequences at either end of a deletion would generate the deletion (1, 7). In contrast, other workers in experiments with transfected DNA have concluded that free ends of DNA in mammalian cells can be ligated to any other free end (26). Plasmids with deletions could be the consequence of recircularization of a molecule that had been cleaved by endonucleases at different sites or been digested by a combination of endo- and exonucleases. Such events would be independent of replication.

With restriction digestion, we analyzed mutant plasmids with deletions of less than 500 base pairs (bp) to see whether there was any preferred location for the deletions in the galactokinase gene as would be predicted by models which invoke sequence homologies. The results of the analysis with 12 such mutant plasmids are shown (Fig. 1). We could find no specificity in the location of the deletions.

We then turned to the arguments based on free-end ligation. We reasoned that if a linearization event were a key step, it would be possible to influence both the frequency and location of deletions by infecting with a plasmid linearized by restriction enzyme cleavage at a particular site. It is important to note that the mutation frequency would be influenced only if a significant fraction of deletion events occurred before plasmid replication. If deletion mutagenesis necessarily occurred on replicating molecules, then no effect of linearization would be noted. (We assume that a circular, or recircularized, molecule is the template for replication.) The plasmid was cleaved with *Eco*RI which makes a single cut a few hundred bases from the promoter for either galactokinase or β-lactamase. Mild exonuclease digestion in the BSC1 cells in either direction followed by recircularization would not impair the activity of either gene. We found that the frequency of mutant plasmids in the population harvested from cells infected with the linear molecules was about 10%. The profile of some of these mutants is shown (Fig. 2A). The gel pattern shows the now-familiar collection of plasmids larger and smaller than the original pGS3. However, the frequency of mutant plasmids with no change in size, ca. 25% of the total in infections with superhelical molecules, was only 6% (5 of 83). Plasmids with deletions accounted for 54%, whereas the remaining 40% of the mutants were of increased size. A total of 19 plasmids with deletions were analyzed by restriction digestion as done before (Fig. 2B). The majority of the deletions (17 of 19) lost the fragment containing the *Eco*RI site. The simplest interpretation of these data is that the majority of the deletion mutants were derived from linear molecules which had suffered additional endo- or exonucleolytic cleavage before recircularization. A few of the mutants arise from molecules which recircularize before the deletion event, as the site of the deletion is located outside restriction fragments carrying the *Eco*RI site (21, 22). These data are reminiscent of results obtained by other workers who infected cells with linearized SV40 DNA and found progeny molecules with deletions of variable lengths at the site of the original cleavage (6, 22, 26).

In previous work, we have found that some of the mutant plasmids contained insertions of cell DNA. The results of the analysis with the deletion mutants led us to examine the mutant plasmids that were larger than pGS3. A number of these were spotted onto nitrocellulose and hybridized to ^{32}P-labeled BSC1 DNA (20 are shown in Fig. 3). This assay revealed the presence of repeated DNA sequences in the mutant plasmids, but not single- or low-copy-number sequences. The results showed that the majority of the putative insertion mutants did contain repeated DNA from the monkey cell genome (17 of 20; Fig. 3). Mutant plasmids containing cell sequences were analyzed by restriction digestion. A few plasmids (3 of 22) retained the region of the vector which contained the *Eco*RI site and had additional fragments but were missing other fragments from the *galK* region (data not shown). These molecules, as noted in the analysis of deletion mutants, must have recircularized before mutagenesis. The majority (19 of 22) of the mutants had lost the fragment containing the *Eco*RI site. In addition, all of these were missing additional sequences from either the

TABLE 1. Mutation frequency of different plasmid preparations

Condition	Mutation frequency (%)
Plasmid grown in[a]:	
HB101 (*hsdS20*)	1.5
GM33 (*dam-3*)	1.7
GM48 (*dcm-6 dam-3*)	1.2
LE392 (M^+ dcm^+ dam^+)	1.2
Plasmid with entire SV40[b]:	
pGScI (BSC1)	1.6
pGScI (Cos-1)	1.0
pGScII (BSC1)	1.0
pGScII (Cos-1)	1.2
Chloroquine-treated cells	3.0
Infection with linear plasmid	10.0

[a] BSC1 cells were infected with pGS3 grown in bacterial strains with differing capacities for DNA methylation. After extraction from the BSC1 cells, the progeny plasmids were introduced into HB101 for determination of mutation frequency.

[b] The pGSC plasmids (with the entire SV40 genome in either orientation) were used to infect either BSC1 cells or Cos-1 cells.

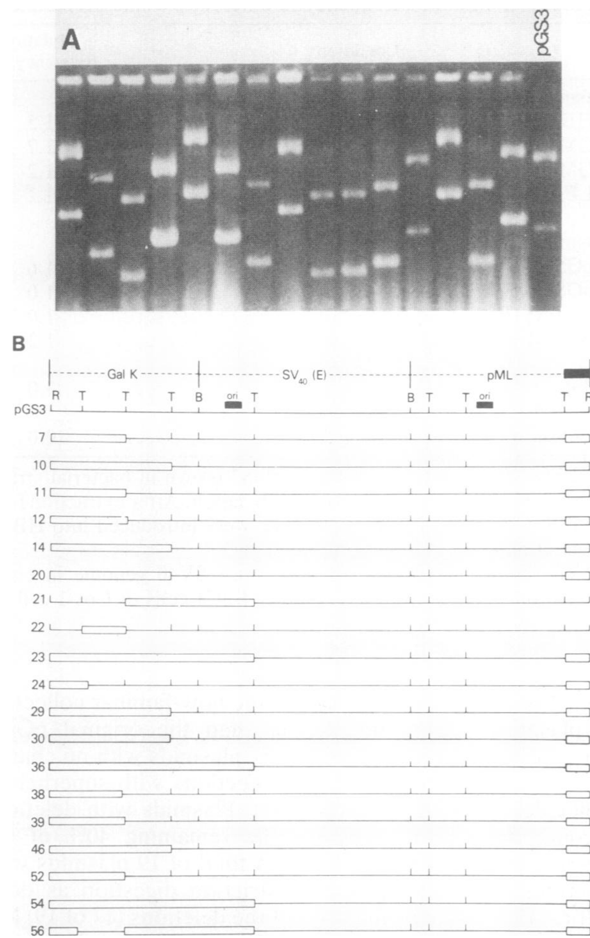

FIG. 2. (A) Agarose gel pattern of mutant plasmids from the infection with the linear plasmid. (B) Restriction mapping of deletion mutant plasmids from infection with the linear plasmid. Restriction enzyme sites are indicated as in Fig. 1. The position of the 344-bp fragment from pML, used as a probe in the experiment of Fig. 3B, is indicated by the heavy line at the top right of the figure. Fragments missing from the *Taq*I digest of the mutant plasmids are indicated.

promoter or coding region of the galactokinase gene. Thus, all of the insertion mutants in this study had suffered deletions, in addition to acquiring cellular sequences. We then asked whether there was a relationship between the region of the genome carrying the site of linearization and the inserted cell DNA. The fragments from a *Taq*I restriction digestion were transferred by sandwich blotting them to nitrocellulose. The BSC1 DNA was used as a probe for one filter, while the 344-bp *Taq*I-*Eco*RI fragment from the β-lactamase gene side of the *Eco*RI site was the probe for the other (Fig. 2B). The autoradiograms were then compared to determine whether there were mutant plasmids with individual fragments that hybridized to both probes (Fig. 3B and C). A total of 17 samples had fragments which showed detectable hybridization to the BSC1 probe, and 12 of these showed hybridization of both probes to the same fragment. Thus, in 65% of these mutants, the BSC1 DNA was contiguous with the fragment adjacent to the *Eco*RI site. This percentage is an underestimate because only repeated DNA sequences were detected in this analysis, and linkage of a low-copy-number sequence to the 344-bp *Taq*I-*Eco*RI fragment would not be detected. Since the free end of the linearized plasmid appeared to be a participant in the generation of the majority of both deletion and insertion mutations and since it is not likely that the linear molecule was a template for replication, we conclude that the majority of the mutagenesis in this experiment occurred before replication of the vector. This suggests that the majority of the deletion (and insertion) mutants from infections with the supercoiled plasmid may also have arisen from linearized molecules, possibly before the onset of plasmid replication (see below).

Although the results of experiments with the linear plasmid support a prereplicative derivation for at least some, if not most, of the deletion mutants from infections with supercoiled DNA, the possibility remains that replicating plasmids and their progeny are also susceptible to deletion mutagenesis. We attempted to answer this by screening our collection of mutant plasmids (from infections with superhelical plasmids) for molecules which had lost the SV40 origin of replication. Our protocol for purification of the plasmid from the infected mammalian cells included treatment with *Dpn*I, an enzyme which cleaves the input DNA but not that which has replicated (18). (*Dpn*I recognizes a site with a methylated adenine and thus cleaves the plasmid grown in bacteria, but not after replication in mammalian cells.) A *Dpn*I-resistant mutant plasmid lacking an SV40 origin of replication must have suffered the deletion after replication began. Mutant plasmids with deletions greater than 2,000 bp were chosen and digested with *Taq*I; the fragments were resolved on an agarose gel and blotted. They were hybridized to ^{32}P-labeled *Eco*RII G fragment from SV40 which contains the origin of replication (Fig. 4A). The plasmids which failed to give a hybridization signal were then used to infect Cos-1 cells, and replication of the plasmid was determined by blot hybridization analysis of the material in the Hirt extract after *Dpn*I treatment (Fig. 4B). We found seven mutant plasmids that were incapable of replication from the collection of about 500 mutants. We concluded that deletion mutagenesis does occur on plasmids after replication has begun although it was not possible to estimate the frequency of these events.

DISCUSSION

At the start of our experiments, we proposed that the hypermutagenesis of the shuttle vector could be due to two classes of explanations: (i) the induction of a mutagenic environment in infected cells or (ii) some critical difference between the plasmid DNA and cellular genes. The first explanation predicted that cell genes would also be mutagenized as a result of the infection. The results of our experiments indicated that a readily monitored cell marker was not mutagenized in cells treated with DNA–DEAE-dextran or containing T antigen. A direct test of the importance of multiple copies of the plasmid in the putative induction was not possible in the SV40-based shuttle vector system (only 1 to 5% of the cells have replicating DNA, and these may not survive the infection). However, if this were the important factor, we would expect mutagenesis to be induced after the rise in copy number, i.e., after replication began. In the light of our previous time course data (19) and the results of the experiment with the linear plasmid, this seems unlikely, certainly in regard to the deletion-insertion mutants.

The second explanation proposed that mutagenesis was confined to the shuttle vector. The critical differences between the vector and a cellular sequence are of two types: those which are transient (exposure to the cytoplasm, bacte-

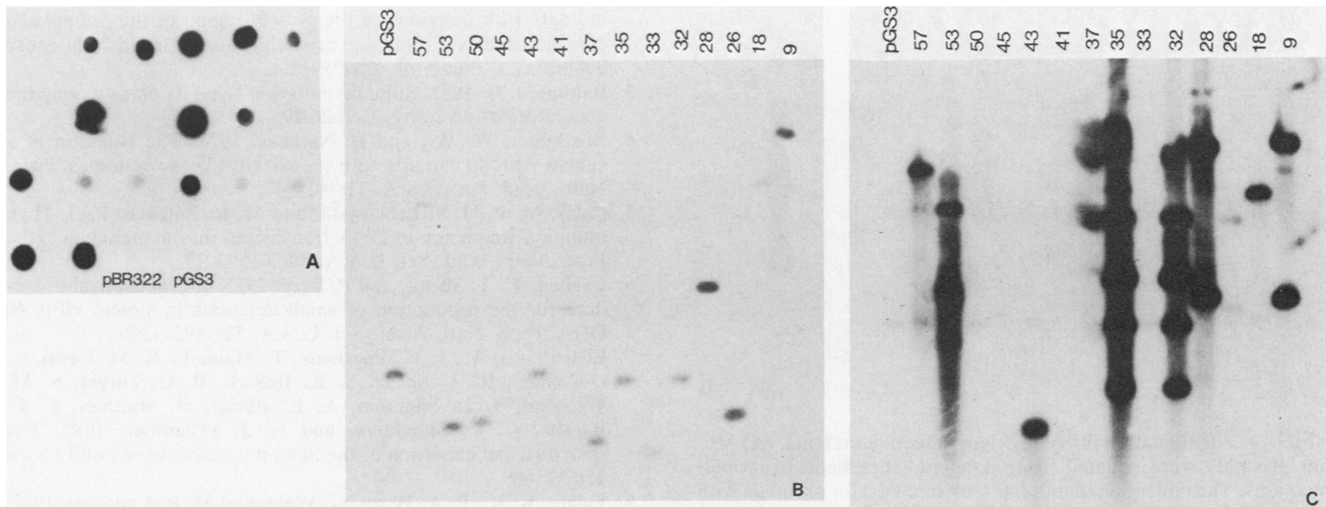

FIG. 3. Characterization of mutant plasmids with insertions of cell DNA. (A) DNA blot analysis. Mutant plasmids from infection with linear plasmids of molecular weight greater than those of pGS3 were spotted onto nitrocellulose (1 μg), and the filter was hybridized to ^{32}P-labeled total BSC1 DNA. The plasmids pBR322 and pGS3 were used as negative controls. (B) Mutant plasmids containing BSC1 DNA inserts were digested with *Taq*I and sandwich blotted. One filter was probed with a ^{32}P-labeled 344-bp *Taq*I-*Eco*RI fragment from pGS3 (Fig. 2B). (C) The second filter from the sandwich blot was hybridized to ^{32}P-labeled BSC1 DNA. Comparison of the autoradiograms in (B) and (C) indicates the plasmids with the colinear 344-bp *Taq*I-*Eco*RI fragment from pGS3 and the BSC1 repeated DNA insert.

rial modification, absence of chromatin structure) and no longer apply to replicating plasmids and their progeny and those which persist through the infection (extrachromosomal replicon, presence or absence of some special sequence). Of the transient differences, bacterial methylation appeared to be unrelated to the mutagenesis. The relevance of the cytoplasmic passage is best tested by direct nuclear injection of a shuttle vector plasmid. Ligation of free ends of linear DNA has been reported in nuclear microinjection experiments (8). If the plasmid were exposed to nucleases in the nucleus, then deletion-insertion mutagenesis would be probable. It has been suggested that the mutagenesis may be due to the presence on the plasmid of poison sequences or the absence of protective sequences (5, 13). We think it is unlikely that specific sequences are responsible for the deletion-insertion mutagenesis. It seems more probable that poison sequences are eliminated by random deletion mutagenesis and that the altered molecules, now free of the constraint on transcription, replication, or both imposed by the poison sequence, possess a replicative advantage.

The characterization of putative critical differences between the vector and cellular genome as transient or persistent provides a heuristic landmark in the scheme of the infection—the initial replication of the plasmid. Progeny plasmids are complete minichromosomes; there is no such certainty, however, about the state of plasmids before replication begins. Thus, we find it useful to view our experiments with the linear plasmid as indicative of the timing of deletion insertion mutagenesis relative to the onset of replication. Replication of the shuttle vector requires T antigen which is encoded by the plasmid. Linear plasmid molecules have been shown to be inefficient templates for transcription (10). Furthermore, it seems likely that supercoiled molecules are the preferred substrate for replication. Therefore, in infection with linear plasmids, recircularization must precede both transcription and replication. Since the site of linearization (i.e., the free end) was involved in the majority of both deletion and insertion mutants from this experiment, we conclude that the majority of the deletion insertion mutagenesis occurred before replication. It also seems clear that the insertion mutants are the result of ligation of the ends of the linear plasmid molecule to ends of available pieces of cellular DNA, much of which is repetitive DNA. Our results also suggest, but do not prove, that deletion-insertion mutagenesis of the shuttle vector in standard (supercoiled) infections proceeds via mechanisms similarly dependent on a double-strand cleavage event, largely, also, in the prereplicative phase of infection. Because the frequency of deletion-insertion mutants arose as a consequence of linearization, it appears that the cleavage event is rate-limiting for this type of mutagenesis. The absence of a eucaryotic chromatin structure may be the critical difference insofar as this class of mutants is concerned.

Although we favor the hypothesis that the majority of the deletion-insertion mutants arise before replication, our isolation of *Dpn*I-resistant, replication-defective mutants indicates that the vector is susceptible throughout the infection. Defective molecules arise in SV40 viral infections (4), but a quantitative estimate of the frequency of these events in one cycle of infection is not available. The viral molecules and the progeny shuttle vector plasmids may be susceptible to the same mutagenic activities.

In this discussion, we have focused on the deletion-insertion class of mutant plasmids. In doing so, we ignored the point mutants which represented about 20 to 25% of the total in our collection. We characterized two such mutants and found a guanine → adenine transition, whereas the other was a frameshift insertion of a thymine (TT → TTT). Presumably, these mutations were the consequences of replication errors in the eucaryotic cells, but we cannot rule out the formal possibility that they were generated in the bacteria after the introduction of the foreign progeny plasmid DNA. The absence of adenine methylation in bacteria has been linked to mutagenesis (15) and the progeny plasmid DNA lacks this modification. If they were generated in

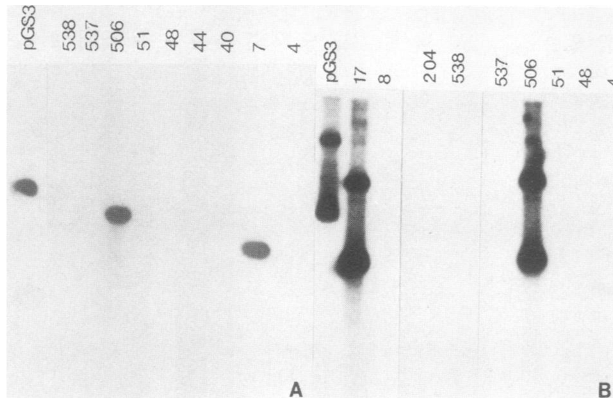

FIG. 4. Identification of SV40 origin-defective mutants. (A) Mutant plasmids were isolated from standard (superhelical plasmid) infections. The collection of mutants was screened for plasmids with deletions of greater than 2,000 bp. These were digested with *Taq*I and electrophoresed; the fragments were blotted. The filter was probed with ^{32}P-labeled *Eco*RII G fragment from SV40 which contained the origin of replication of SV40 (see the position of the SV40 ori in Fig. 2B). (B) Those plasmids which failed to give positive hybridization with the SV40 origin fragment were used to infect Cos-1 cells. After 48 h, the cells were extracted by the Hirt procedure, and the DNA in the extracts was examined by electrophoresis, blotting, and hybridization to ^{32}P-labeled pGS3. The plasmid pGS3 and mutants 17 and 506, which have functional SV40 origins, were used as positive controls.

animal cells, it may be because the plasmid lacked eucaryotic proofreading signals or may simply reflect deamination and depurination of the DNA during transfection. This is an important question, given the significance of localized point mutagenesis in certain problems in developmental biology.

It is appropriate to consider the validity of the shuttle vector system for future studies of mutagenesis and rearrangement of DNA in mammalian cells. The very high spontaneous mutation frequency would appear to severely limit the use of forward mutation vectors in experiments which concern events that occur at frequencies of less than 10^{-2}. Unfortunately, this includes most biologically relevant, spontaneous (23) or induced mutagenesis, transpositions, chromosome translocations, etc. On the other hand, biologically relevant experiments can be done if the background frequency can be reduced or if a particular event can be recognized over the background. Such approaches might include a version of the Ames test (15), in which plasmids with specific mutations in marker genes would be used to assay the reversion to wild-type phenotype. In addition, it is possible to construct plasmids with segments of marker genes arranged so that recombination or gene conversion reconstructs the marker which can be detected in bacteria. These and similar approaches should reduce the background while preserving the advantages of the shuttle vector systems.

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