Rearrangement and mutagenesis of a shuttle vector plasmid after passage in mammalian cells

(replication/insertion)

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ABSTRACT A shuttle vector plasmid that contains sequences from simian virus 40, pBR322, and a bacterial marker gene, galactokinase, has been constructed. After replication in cells permissive for virus progeny, plasmid DNA was introduced into a galactokinase-deficient bacterial strain and the relative frequency of colonies with plasmids but without galactokinase activity was determined. This assay showed that 1% of the plasmids were defective after passage in the mammalian cells. Individual mutant plasmids were examined and found to contain deletions, duplications, point mutations, and insertions of cell DNA.

Rearrangements and alterations in the structure and sequence of genes in eukaryotic cells may have important consequences for gene activity and expression. In some cases such changes are associated with gene activation, while in other situations gene function is lost (1-4). One way to study these events involves the identification and isolation of cells in which these events have occurred, followed by analysis of the genomic DNA using standard methods (5, 6). Molecular cloning of genomic sequences of interest may be necessary to complete the description of changes.

Another approach to these problems is provided by current technology for construction of shuttle vector plasmids and their introduction into both eukaryotic and bacterial cells. These molecules carry information that permits replication in both eukaryotic and bacterial hosts (7, 8). The utility of these vectors has been greatly enhanced by the isolation of derivatives of the standard plasmid, pBR322, that lack "poison sequences" (9). Vectors that contain the pBR322 derivative, pML2, and sequences from viruses such as simian virus 40 (SV40) or bovine papillomavirus have been shown to replicate in cells normally permissive for the intact virus. Progeny DNA can be extracted and used to transform bacterial cells with reasonable efficiency (7-9). This shuttling capacity permits experiments in which plasmids carrying marker genes can be allowed to replicate in mammalian cells and be recovered, after which the activity of the marker gene on individual progeny plasmids can be assayed in bacteria by using classical techniques. These procedures will report any event that inactivates the marker gene and in the process yield cloned plasmids for further study. We have constructed such a shuttle vector that contains pML2, sequences from SV40, and the bacterial gene for the enzyme galactokinase (galk). Here, we describe a collection of mutant plasmids that were isolated after replication of the vector in African green monkey kidney (AGMK) cells in culture.

MATERIALS AND METHODS

Plasmid Construction. The pBR322 derivative, pML2, was obtained from the laboratory of M. Botchan (9). The bacterial gene for galactokinase was taken from plasmid pKO482 [from the laboratory of M. Rosenberg (10-12)] by cutting at the single Acc I site and then digesting ≈200 base pairs (bp) with BAL-3I to remove any poison sequence. BamHI linkers were attached and the EcoRI/BamHI fragment of ≈2,200 bp was inserted between the EcoRI and BamHI sites of pML2. The early gene coding region and origin of replication from SV40 virus contained in the large HpaII/BamHI fragment (now with BamHI termini) was obtained from G. Khoury and inserted in the BamHI site of the pML-galK plasmid to form the shuttle vector pGS3 (see Fig. 1).

Cells, Transfection, and Purification of Progeny Plasmid DNA. Escherichia coli strain SA 820 was obtained from S. Adhya. A GMK primary cells (Microbiological Associates), the BS-C-1 cell line, and COS 1 cells (13) were all grown in minimal essential medium (GIBCO) supplemented with antibiotics and 10% fetal calf serum. Cells were transfected with plasmid DNA by using either the DEAE-dextran (14) or the protoplast fusion (15) method. At appropriate times after infection, the cells were extracted by the method of Hirt (16) and the DNA in the supernatant fraction was treated with proteinase K and RNase, phenol extracted, and ethanol precipitated. The DNA was further incubated with Dpn I to eliminate input DNA (17).

Bacterial Transformation and Isolation of Mutant Plasmids. Purified plasmid DNA was introduced into E. coli strain HB101 (which is galk-) by using standard techniques. Aliquots of the transformation mixture were spread on agar plates with ampicillin and on plates with ampicillin/2-deoxygalactose (18). Cells that grow on the 2-deoxygalactose/ampicillin plates contain a plasmid that carries a defective galk gene, which can be confirmed by streaking on MacConkey agar with galactose/ampicillin (19). Plasmids were prepared from overnight cultures as described (20). Plasmid DNA was labeled by nick-translation (9) and used for hybridization to filter-bound DNA by using published procedures (6). Plasmids with inserts of monkey α satellite (21) and Alu sequence (22) were obtained from M. Singer.

RESULTS

Construction of pGS3. The shuttle vector plasmid pGS3 was constructed by combining three functional elements as shown in Fig. 1. Approximately 2,600 bp are contributed by sequences from pML2, which confers the capability of replication in bacteria under ampicillin selection (9). The vector is able to replicate in mammalian cells permissive for SV40 virus because it contains ≈3,000 bp of DNA from the virus, including the origin of replication and the early region coding for large and small tumor antigens. The third component is a marker gene coding for the enzyme galactokinase (≈2,200 bp). By including 2-deoxygalactose in the appropriate medium (18), it is possible
FIG. 1. Map of shuttle vector plasmid pGS3. The vector contains sequences coding for galactokinase, the large and small tumor antigens from the SV40 early region, the origin of replication (ori) from SV40, and the β-lactamase gene (amp), which confers ampicillin resistance. The direction of transcription for each is shown. There are two BamHI sites and a single EcoRI site.

to select for bacteria that do not have galactokinase activity. In practice, we plate transformation mixtures on medium containing 2-deoxygalactose and confirm each mutant by streaking on MacConkey galactose/agar indicator plates.

Replication in Permissive Cells. In our initial experiments, we studied replication of the plasmid in BS-C-1 cells. The DEAE-dextran procedure (14) was used to infect the cells with pGS3. At 24, 48, 72, and 96 hr after infection, the plasmid DNA was extracted by the method of Hirt. As part of the purification protocol, the DNA was treated with Dpn I, a restriction enzyme that cleaves the input DNA because it contains methyladenine at the recognition site; the progeny DNA is resistant to the enzyme because it lacks this modification (17). Aliquots from each sample were analyzed by agarose gel electrophoresis followed by transfer to nitrocellulose and hybridization with 32P-labeled pGS3 (Fig. 2). The plasmid DNA reaches a maximum concentration at 48 hr after infection. After this time, the plasmid level declines so that, by 96 hr, there is about as much DNA present in the extracts as at 24 hr. Similar results have been reported (23, 24). The decay of the plasmid in the population of cells could be due to an actual degradation of the plasmid, coupled, perhaps, with a decrease in replication rate, or to a loss of infected cells from the monolayer. In similar experiments with wild-type SV40 DNA, Peden et al. (17) found a steady increase in the amount of viral DNA throughout the 96 hr of their experiment. If infected cells were lost from the population during the time of the experiment, the results with the plasmid and wild-type viral DNA should be similar. Since they are clearly different, we favor the interpretation that plasmid replication eventually stops or is reduced (for unknown reasons) and the plasmid DNA is degraded. In the case of the wild-type SV40 DNA, efficient encapsidation would protect the viral DNA (25). From the intensity of hybridization of the 48-hr sample and our measurement that about 1% of the cells are infected, we estimate that there are 50,000–100,000 copies of plasmid in a cell at this time (see ref. 9). Another feature of interest is the apparent integrity of the plasmid. Across the time course, the progeny DNA appears very similar to the starting material. Molecules having altered electrophoretic mobility, which characterize serial passage stocks of SV40, are not visible (26).

Isolation of Mutant Plasmids. Progeny plasmid DNA was isolated from BS-C-1 cells 48 hr after infection and used to transform HB101. Colonies carrying mutant plasmids were isolated. The mutation frequencies from three independent experiments are shown in Table 1. In contrast to the expectation provided by the data in Fig. 2, 1 to 2% of the colonies harbored mutant plasmids.

We were concerned that the mutations might be generated in HB101 because of some inherent instability of the plasmid or some component in the Hirt extract (cell DNA, for example). However, HB101 cells were transformed with pGS3 (without prior replication in BS-C-1 cells) many times during the course of these experiments, ~106 colonies were screened, and no mutants were obtained. To test the possibility that some factor in the Hirt extract might provoke mutagenesis in the bacteria, a reconstruction experiment was carried out. We prepared pGS3 from the dam− strain CM 33. Plasmids grown in this strain do not contain methyladenine and are resistant to Dpn I. The dam− pGS3 was mixed with a Hirt extract of uninfected cells and the standard Dpn I treatment and purification protocol was then carried out. No mutants were obtained when this preparation was introduced into HB101.

In other experiments, we have found that purification of progeny plasmid DNA on CsCl density gradients prior to transformation of HB101 has no effect on the mutation frequency. We conclude that the plasmid is stable in bacteria, even after cotransformation with the constituents of a Hirt extract of the mammalian cells, and that mutagenesis of the marker occurs as a consequence of passage in the BS-C-1 cells. Furthermore, this assay appears to be more sensitive than hybridization for the detection of mutant plasmids.

Mutation Frequency Is Independent of Method of Infection, Cell Type, and Time of Harvest. One possible explanation for the appearance of mutants in the progeny plasmid population is that the DEAE-dextran treatment of the BS-C-1 cells might induce an intracellular environment that is mutagenic. An additional concern was the potential for DNA damage

Table 1. Frequency of mutant plasmids after replication in BS-C-1 cells

<table>
<thead>
<tr>
<th>Exp.</th>
<th>Colonies, no.</th>
<th>Mutants, no.</th>
<th>Mutation frequency</th>
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<tbody>
<tr>
<td>1</td>
<td>770</td>
<td>13</td>
<td>1.7</td>
</tr>
<tr>
<td>2</td>
<td>3,800</td>
<td>54</td>
<td>1.3</td>
</tr>
<tr>
<td>3</td>
<td>10,200</td>
<td>108</td>
<td>1.0</td>
</tr>
</tbody>
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Progeny plasmid DNA was purified after replication in BS-C-1 cells and introduced into HB101. Aliquots of the transformation mixtures were plated on ampicillin-containing plates with or without 2-deoxygalactose and the number of colonies on each plate was used to calculate the mutation frequency.
during the actual uptake of the plasmid, damage that would be the primary cause of mutation. To test these possibilities, the experiment was repeated using the protoplast fusion method of infection in which the plasmid DNA is introduced into the recipient cells within hours of its synthesis. The results of this experiment were similar to those obtained previously (Table 2).

We have attributed the decline in plasmid concentration at later times after infection to the presence of an efficient degradative process coupled with inactivation of the replication process. Consequently, we wondered whether the mutagenesis of the plasmid might be linked to the degradation. Since the total plasmid population is decreasing at later times, the relative proportion of mutants might increase. To test this possibility, the mutation frequency of the plasmid population from each of the time course samples of Fig. 2 was determined. The mutation frequency was constant across the time course, indicating that the relative proportion of mutant plasmids is not affected by the overall decrease in plasmid concentration (Table 2). In another experiment, the effect of DNA concentration during infection was examined. The amount of plasmid DNA was reduced from 100 ng per plate to 1 ng per plate. Although the yield of progeny DNA was substantially reduced, mutant plasmids were obtained at a similar frequency. Finally we asked whether the mutagenesis was peculiar to BS-C-1 cells. Mutant plasmids were obtained at the same frequency after replication in primary AGMK cells as were obtained from the SV40-transformed permissive line COS 1 (13) (Table 2). We conclude from this series of experiments that the generation of mutant plasmids is not a function of the specific method of infection nor a consequence of the manipulation of DNA in vitro. This phenomenon does not appear to be necessarily linked to intracellular degradation of the vector late in the infection. Instead, it seems likely that plasmid is susceptible to mutagenic activities during its residence in permissive cells.

Description of Mutants. We have examined >300 mutant plasmids by agarose gel electrophoresis. Representative profiles of several mutants are shown in Fig. 3. A variety of mutant plasmids appear: deletions, molecules with no apparent molecular weight change, and molecules larger than pGS3. The results are summarized in Table 3. Members from each class have been examined in more detail and some of the conclusions from those studies are described below.

Deletions. Deletions of various sizes comprise ~50% of all mutants. The deletions affect the galK gene and range from as little as 10–20 bp (from analysis of restriction digests on polyacrylamide gels) to as large as 5,000 bp. Such very large deletions represent the limit that can be detected in the assay because a functional bacterial origin of replication and β-lactamase gene are absolute requirements. We have analyzed, by restriction digestion, a number of mutants with deletions of <300 bp and can find no preferred location in the galK gene.

No Size Change. Forty mutant plasmids for which no molecular weight change could be detected by analysis of restriction digests on polyacrylamide gels were used to transform the bacterial strain SA 820, which lacks genes for the galactose operon and has an amber suppressor (tyrosine) tRNA. Suppression of the galactokinase-lacking phenotype carried by the mutant plasmids was found with six of the mutants. The most likely explanation for this result is that a mutation in the galK gene resulted in the formation of an amber codon. Consequently, we conclude that at least some of the mutants in this class are point mutants.

Higher Molecular Weight. The plasmids in this class range from a few hundred bp to ~8,000 bp more than pGS3. Restriction analysis of 25 of these indicated that 5 contained duplications of sequences in pGS3. These may be dimers of molecules that have deletions. Another explanation for the presence of mutants of this class is that cellular DNA has been inserted into the galK gene. We tested this possibility by nick-translating selected mutants and hybridizing them to dot blots of BS-

<table>
<thead>
<tr>
<th>Table 2. Effects of experimental conditions on mutation frequency</th>
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<tbody>
<tr>
<td>Condition of experiment</td>
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<tr>
<td>-------------------------</td>
</tr>
<tr>
<td>Protoplast fusion infection</td>
</tr>
<tr>
<td>Time course</td>
</tr>
<tr>
<td>24 hr</td>
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<tr>
<td>48 hr</td>
</tr>
<tr>
<td>72 hr</td>
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<td>96 hr</td>
</tr>
<tr>
<td>Cell type</td>
</tr>
<tr>
<td>AGMK</td>
</tr>
<tr>
<td>COS 1</td>
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<td>BS-C-1</td>
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**Table 3. Characterization of mutant plasmids**

<table>
<thead>
<tr>
<th>M, changes*</th>
<th>Plasmids, no.</th>
<th>% total</th>
</tr>
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<tbody>
<tr>
<td>None</td>
<td>78</td>
<td>24</td>
</tr>
<tr>
<td>Deletion</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;1,000</td>
<td>110</td>
<td>34</td>
</tr>
<tr>
<td>&gt;1,000</td>
<td>75</td>
<td>23</td>
</tr>
<tr>
<td>Increase</td>
<td>59</td>
<td>18</td>
</tr>
</tbody>
</table>

* Relative to pGS3.
C-1 DNA. In our first experiment, 20 mutants were chosen and 12 gave positive hybridization. This type of assay is diagnostic for the presence in the plasmids of repetitive DNA sequences from the monkey genome. Of the positive mutants, one, no. 161, showed very strong hybridization (Fig. 4A). For comparison, we have included the results of hybridization of two other plasmids containing inserts of highly repeated sequences from the cellular DNA, α satellite, and the Alu sequence (21, 22). Restriction enzyme digestion analysis suggests that both deletion and insertion of plasmid sequences has occurred (Fig. 4B). The data indicate that mutant 161 is 10.2 kb long and must contain a minimum of 2.4 kb of additional sequences. The HindIII digest of mutant 161 is shown in Fig. 4C. The 2.4-kb fragment that contains the galK gene in pGS3 does not appear, instead, four new fragments are visible. One of these, ≈180 bp, is quite intense. We estimate that there are ≈13 copies of this sequence in the mutant. The size of this fragment is highly suggestive because there is a 172-bp HindIII repeat unit in the α-satellite component of the monkey genome. 32P-Labeled mutant 161 DNA was hybridized to a Southern blot of a HindIII digest of BS-C-1 DNA (Fig. 4D). For comparison, plasmid pCal004, which contains the α-satellite insert (the 172-bp HindIII repeat unit) (21), was used as a probe in a parallel hybridization.

From the results of these experiments, we conclude that mutant 161 contains an insert of cellular DNA that includes ≈13 copies of the 172-bp repeat of α-satellite DNA. The presence of these sequences in defective SV40 viruses has been described by other workers (27).

**DISCUSSION**

The observation that 1% of the shuttle vector molecules are mutant after passage in mammalian cells raises questions about the location and mechanism of the mutagenic events. One possibility is that the plasmid DNA used for infection is damaged during storage and manipulation (depurination, deamination, nicking, etc.) and this is the basis for the mutagenesis. The results of the protoplast fusion experiment, in which the DNA is not manipulated or stored, argue against this possibility and also dismiss the DEAE-dextran as a relevant factor in the process. Another explanation is that mutants are generated in the bacteria. However, the plasmid is stable in HB101 and we were unable to recover mutants in the reconstruction experiments, suggesting that components in the Hirt extract do not provoke mutagenesis in bacteria. There remains the possibility that the mutagenesis is a consequence of the bacterial response to foreign DNA. Our experiments cannot speak directly to this objection. There is a substantial literature on the generation of defective papovavirus genomes in which molecules having deletions, insertions, duplications, and point mutations have been described (26–28). From these studies, it seems clear that replication in bacteria is unnecessary for the production of mutants. Although the alternative explanation may describe the generation of some of the mutant plasmids, we think it more likely that the shuttle vector is reporting mutational activities that function in the mammalian cells. Furthermore, because of the lack of packaging constraints and the variety of molecular events recorded by the vector, experiments with this system should be more informative than those with viral probes.

The deletion and insertion mutants can be viewed as the consequences of recombinational, most likely heterologous, events. A simple explanation for "illegitimate" recombination has been proposed by Wilson et al. (29) (see also ref. 30). They argue that free ends of DNA molecules are capable of recombination and can be ligated in cells to any other free end available. Both deletion and insertion mutants can be generated this way. The extrachromosomal small circular DNA found in many eukaryotic cells and BS-C-1 cells in particular (31) might be the source of the cellular DNA in the insertion mutants. Winocur and Kesht (32) have shown that recombination between two heterologous DNA molecules, SV40 and φX174, will occur in infected cells. It is also possible that some of the insertions are transposable elements as described in other systems (2). Shut-
tle vectors of this type may prove to be useful for the detection and isolation of such sequences. These explanations for the production of the deletion and insertion mutants are certainly reasonable but may not be readily applied to all the point mutants. Some of these mutants will probably be due to the replication of vector DNA containing deaminated cytosine residues. However, our recent results indicate that not all of these mutations can be explained this way. Consequently, it seems likely that some of these mutations arise from replicative errors, perhaps because the input plasmid lacks the appropriate signals (modifications) to direct correct proofreading during replication. We have attempted to consider the role of replication in the mutagenesis by infection with a nonreplicating plasmid (pML-galK) but have been unable to recover enough DNA for successful analysis.

The mutation frequency of $10^{-2}$ for the galK gene on the shuttle vector is dramatically higher than the spontaneous mutation frequency of $10^{-6}$ or $10^{-7}$ that characterizes conventional cell markers such as 6-thioguanine resistance or ouabain resistance (33). Although it is possible that there is something remarkable about the galK gene itself, it seems more likely that the plasmid is exposed to an environment much more mutagenic than that seen by a cellular gene in a normal cell. Some possible explanations for this situation are as follows. (i) The mutagenesis is experienced by input DNA, perhaps because it is "foreign" (DNA modification may be important), or because it does not have the proper eukaryotic chromatin structure, or because it is an unavoidable feature of these methods of introducing DNA into cells. These explanations predict that most of the mutagenesis will occur very early in infection, which is consistent with our time-course data. (ii) Hypermutagenesis might be a property of all extrachromosomal DNA and might also apply to the small circular DNA in normal uninfected cells. If this is true, then the vector might be subject to mutagenic activities at any time during the infection. If these two classes of explanation are correct, then the DNA used in many gene-transfer experiments might be subject to mutagenesis. (iii) The plasmid might induce mutagenic activity in the cell simply because of its presence or as a function of a gene product(s) encoded by the vector. It has been argued that SV40 induces mutations in infected cells (34, 35). Our recent experiments, however, suggest that the hypermutagenesis of the plasmid does not extend to cellular genes. If the viral gene products do induce mutagenesis in our system, it would have to be confined to the vector.

Finally, we note that this shuttle vector system, because of the high spontaneous-mutation frequency, may not be immediately appropriate for studying the effects of mutagens such as radiation or chemical carcinogens. On the other hand, it does provide a sensitive and fairly rapid assay for the influence of DNA structure and sequence, and cellular environment on recombinational and mutagenic events.

Similar results have been obtained by Calos et al. (36).

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### References