A Shuttle Vector System for Studying Ionizing Radiation-Induced Mutagenesis in Mammalian Cells

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A shuttle vector system was developed to quantitate and analyze ionizing radiation-induced mutation in mammalian host cells, COS-1 and CV-1. The shuttle vector pSV2-lacY, which was constructed to detect both point mutations and deletions, was irradiated \textit{in vitro} with $^{60}$Co gamma rays before introduction into unirradiated host cells. The plasmid was then isolated and reintroduced into HB101 (lacY\textsuperscript{-}) bacterial host cells for identification of mutated lacY marker genes. Gamma-irradiation produced a decrease of the survival (recovery) and an increase of mutation of the shuttle vector. The mutated shuttle vector molecules were examined for structural changes by means of restriction endonuclease digestion and agarose gel electrophoresis. A dose dependent increase was observed in the percentages of gross alteration events of total mutations in mammalian host. This system will be useful for studies of ionizing radiation-induced mutagenesis.

\section*{INTRODUCTION}

Extrachromosomal molecules, modified \textit{in vitro}, have often been used as probes for cellular responses to DNA damage. By this approach, an accurate determination of the number and types of DNA lesions can be made, without complication of direct effects of the modifying agent on the mammalian cells. Extrachromosomal shuttle vectors have been used for determination of recombination induced by ultraviolet (UV)\textsuperscript{1,2}, ionizing radiation\textsuperscript{3-4} and mutagenesis induced by UV\textsuperscript{5}. These shuttle vectors are typically constructed with elements of plasmid replication and selection in the bacteria, and those of viral replication and selection in the mammalian cells and a marker gene to allow for identification and characterization of mutation or recombination.

To reduce the level of spontaneous mutation, particularly deletion, shuttle vectors have
been constructed with small marker genes (e.g., supF) placed between the genetic elements essential for replication and selection in the bacteria\textsuperscript{5,6}. These modified shuttle vectors have been used for studies of mutagenesis by UV light radiation and chemical agents, which are known to induce primarily point mutations\textsuperscript{5}. Only recently a few studies have been made to examine the process of ionizing radiation-induced mutation\textsuperscript{5,7}. Since the most prevalent type of ionizing radiation-induced genetic mutation is caused by deletion and rearrangement\textsuperscript{8,9}, these restrictive shuttle vectors with small sized marker genes would not be effective for detecting and analyzing these types of mutation. This study was initiated to construct a shuttle vector which could be used as a simple and easy extrachromosomal system for detection and characterization of ionizing radiation-induced mutation.

MATERIALS AND METHODS

Shuttle vector system and experimental procedure

African green monkey kidney cell lines COS-1 and CV-1 (ATCC) were grown as described\textsuperscript{2}.

pSV2-lacY1 has 13.2 kb in full length and contains lacZ, lacY, and lacA. The construct pSV2-lacY had 7.9 kb in length and only lacY sequences. The \textit{E. coli} lacY marker gene was inserted downstream to the pBR322 P1 promoter using the unique pBR322 EcoRI site, and this active lacY gene was subcloned into the unique BamHI site of pSV2neo using BamHI linkers. This 1.7 kb marker gene (including promoter) was at least 1.5 kb away from the bacterial ampicillin resistance gene (amp) and origin (SVori) of replication. pSV2-lacY contained the neo gene, which encodes for kanamycin resistance in bacterial host cells. It also contained the SV40 origin of replication (SVori) gene, which enabled pSV-lacY to replicate in the permissive monkey cell line COS-1, but not in the parent monkey cell line CV-1\textsuperscript{10}. This allowed the effects of shuttle vector replication on mutagenesis to be determined in a similar genetic background. The size of target gene in new plasmid is convenient for detecting both gross rearrangement and point mutation on plasmid DNA induced by ionizing radiation (see also Fig. 1).

Plasmid DNA used for radiation experiments was prepared as follows. \textit{E. coli} HB101 cells carrying pSV2-lacY were cultured in V-media (1% Bacto tryptone, 0.5% yeast extract, 0.12 M NaCl, 0.02 M KCl, and 0.002 M MgSO\textsubscript{4}\textcdot7H\textsubscript{2}O) and harvested. Cells were lysed by a mixture of 0.2% NaOH and 10% SDS. Thereafter, closed circular DNA was purified by using standard cesium chloride equilibrium centrifugation method.

pSV2-lacY was dialyzed against 10 mM potassium phosphate (pH 7.0) overnight, and then diluted to 50 \(\mu\)g/ml with the same buffer. The diluted shuttle vector DNA was irradiated at room temperature at a dose rate of 2.38 Gy/min with a \(^{60}\)Co source. Irradiated pSV2-lacY (5 \(\mu\)g) was introduced into \(4 \times 10^6\) monkey cells (in 0.8 ml phosphate buffered saline) by electroporation using a Gene Pulser (Bio-Rad, Inc.) at 300 volts and 960 \(\mu\)FD. The cells were then incubated at 37\(^\circ\)C for 48 hr in 100 mm dishes. The extrachromosomal shuttle vector molecules were extracted (from the two plates for each dose) as described\textsuperscript{2}, resuspended in a final volume of 20 \(\mu\)l TE (10 mM Tris, 1 mM EDTA pH 8.0), and purified through (200 \(\mu\)l) a CL6B
Sepharose column.

Identification and analysis of mutant shuttle vectors

Purified shuttle vector molecules were introduced into E. coli host cells HB101 (recA<sup>-</sup>, lacY1) by electroporation at 2,500 volts using the Gene Pulser, according to manufacturer's instructions. Since HB101 cells have a mutated lacY gene, wild-type lacY<sup>+</sup> shuttle vector molecules complemented the bacterial mutation and yielded Lac<sup>+</sup> transformants, while mutant lacY<sup>-</sup> shuttle vector molecules yielded Lac<sup>-</sup> transformants. The transformed HB101 cells were plated on MacConkey agar (Difco) supplemented with ampicillin or kanamycin sulfate for selection of transformation and identification of Lac phenotypes as described<sup>11</sup>.

Relative survival was calculated as ratio of the number of kanamycin resistant transformants at a given dose to the number of transformants at zero dose, normalized to 1 for zero dose. Mutation frequencies by radiation was calculated as the percentage of whole lacY<sup>-</sup> mutants against kanamycin resistant total transformants.

To analyze the molecular nature of mutation in pSV2-lacY gene as determined by lacY<sup>-</sup> phenotype, restriction endonuclease analysis was performed. For this, at least sixty individual lacY<sup>-</sup> mutant colonies were isolated and examined. Cells from each colony were cultured overnight in the medium containing kanamycin. Then, DNA was extracted, purified and analyzed for point mutation by means of restriction endonuclease (PstI) digestion and agarose gel electrophoresis. Nonmutated pSV2neo-lacY yielded upon PstI digestion four fragments consisting of 3.7 (lacY gene), 2.4, 0.9 and 0.9 kb in size (See, also Fig. 1). DNA from point mutated mutants showed the migration pattern which was indistinguishable from that obtained with original pSV2neo-lacY digested by PstI. Other DNA samples from mutant lacY<sup>-</sup> cell colonies showed frequently gel electrophoresis patterns which were different those obtained with original pSV2neo-lacY digested by PstI. The alteration involved deletion, rearrangement, and others of which the molecular nature was still not well identified. The percentages of gross alteration were expressed in ratio of the number of mutant colonies the DNA of which showed alterations in gel electrophoreogram to the total number of mutant colonies examined for DNA.

To confirm that pSV2-lacY replicated in the COS-1 host cells, plasmid DNA extracted from bacterial cells after replication was treated with DpnI to digest nonreplicated input DNA because replicated DNA did not have methylated adenine.

Comparison of DNA breaks induced by irradiation and endonuclease and their subsequent role in mutation induction

Studies were made to compare the nature of DNA breaks induced by irradiation or endonuclease digestion, and their subsequent roles in mutation induction. pSV2-lacY was irradiated with 20 Gy gamma radiation and the molecular forms were separated on agarose gels. The DNA was analyzed on agarose gels to confirm the induction of single-strand breaks (conversion to RFII) and double-strand breaks (conversion to RFIII).

To test the effect of specific double-strand breaks, the 7.9 kb liner RFIII molecules were purified by agarose gel electrophoresis method when necessary. Unirradiated pSV2-lacY was linearized by ClaI digestion, which introduced specific double-strand breaks in pSV2-lacY.
molecules. The linearized and 0 and 20 Gy irradiated shuttle vector preparations were transfected into COS-1 cells by incubating for 48 hrs. Then DNA was extracted and tested with _E. coli_ system. The mutation frequencies and the percentage of gross alteration were determined as described in the preceding section. The average size of mutant DNA was calculated according to the standard method by measuring of the migration distance of DNA band separated by agarose gel electrophoresis.

RESULTS

Figure 1 shows the restriction map of pSV2-lacY with 7.9 kb in whole length used in the present experiments. The SV40 origin of replication (SVori) allowed the plasmid vector to replicate in the monkey cells, whereas the _neo_ and _amp_ genes conferred kanamycin and ampicillin resistance, respectively, on the bacterial host cells and served for selection.

Figure 2 shows the changes in the pattern of agarose gel electrophoresis of the plasmid following gamma-ray irradiation with indicated doses. It can be seen that the irradiation induced

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**Fig. 1.** Restriction map of pSV2-lacY. The letters refer to following restriction sites: B, _BamHI_; C, _ClaI_; E, _EcoRI_; P, _PstI_. Line shows SV40 sequences, open box pBR322 sequences, and black box _E. coli_ _lac_ sequences.

**Fig. 2.** Agarose gel electrophoresis (0.9% in Tris borate buffer) of gamma-irradiated pSV2-lacY DNA. RFI, closed circular; RFI, open circular; RFIII, linear plasmid. Lane 1, pSV2-lacY RFIII control (_ClaI_ digest); lanes 2-7, irradiated with 0, 5, 10, 20, 40, and 60 Gy, respectively; lane 8, size markers of 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, and 0.56 kb.
Table 1. Frequency and nature of pSV2-lacY mutation induced by gamma-rays and restriction endonuclease \textit{ClaI}.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percent Mutation\textsuperscript{b}</th>
<th>Percent Gross Alteration\textsuperscript{c}</th>
<th>Avg. Mutant Size\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 Gy/All Forms</td>
<td>1.5 ± 1.2</td>
<td>86.0 ± 2.0</td>
<td>Not Determined</td>
</tr>
<tr>
<td>20 Gy/All Forms</td>
<td>24.4 ± 11.5</td>
<td>86.9 ± 8.1</td>
<td>Not Determined</td>
</tr>
<tr>
<td>20 Gy/RFlII</td>
<td>29.4 ± 0.01</td>
<td>88.5 ± 7.8</td>
<td>4.4 ± 1.2 kb</td>
</tr>
<tr>
<td>Clal/RFlII</td>
<td>44.0 ± 0.01</td>
<td>91.5 ± 3.5</td>
<td>5.3 ± 1.5 kb</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Replication permissive COS-1 cells were used as described in the text.

\textsuperscript{b} The data is from four separate experiments for the 20 Gy/All Forms and two separate experiments for the RFlII samples, ± the standard errors.

\textsuperscript{c} The data is from four separate experiments for the 20 Gy/All Forms and two separate experiments for the RFlII samples, ± the standard deviations. For each experiment, at least sixty individual mutant colonies were examined for alteration of their DNA.

\textsuperscript{d} The data is from two separate experiments, ± the standard deviations. For each experiment, at least twenty individual mutant colonies were examined for alteration of their DNA.

both single and double strand breaks in the plasmid DNA and the proportion of these breaks increased with increasing radiation dose. Results of comparison of the nature of radiation-induced double strand breaks and those produced by \textit{ClaI} endonuclease will be presented later in Table 1.

Figure 3 shows the changes in the relative survival (transformed HB101) of pSV-lacY as a function of gamma ray dose. The survival was the same for ampicillin as for kanamycin selection (data not shown), and was independent of the potential for shuttle vector replication in the mammalian cell lines used. Survival decreased with increasing gamma ray dose, and shoulders at

![Graph showing relative pSV2-lacY survival vs. gamma dose (Gy) with data points marked by squares.](image-url)

\textbf{Fig. 3.} Inactivation of gamma-irradiated pSV2-lacY in COS-1 (open squares) and CV-1 (closed squares) host cells. The data are the average of four COS-1 and two CV-1 experiments, each assayed three times.
low-dose range, indicative of potential shuttle vector repair, were not observed for experiments involving the two cell lines.

As shown in Figure 4, frequency of shuttle vector mutation, as measured by changes in the number of lacY\(^-\) and ampicillin resistant bacterial colonies, increased with increasing gamma ray dose, with about 40% of the pSV2-lacY molecules being mutated to lacY\(^-\) at 60 Gy. Mutation was induced to a level which was higher between threefold (at 5 Gy) and fortyfold (at 60 Gy) than that of spontaneous background mutation (1.2%), indicating that most of the observed lacY\(^-\) mutants were induced by irradiation. The potential for shuttle vector replication of host mammalian cells (COS-1 versus CV-1) significantly affected gamma ray-induced shuttle vector mutagenesis.

![Figure 4](image_url)

**Fig. 4.** Mutation frequency of gamma-irradiated pSV2-lacY as a function of radiation dose, as measured in COS-1 (open squares) and CV-1 (closed squares) host cells. Spontaneous mutation frequencies were found to be 1.2 and 0.2% for COS-1 and CV-1 host, respectively. The error bars represent the standard error of means of independent four COS-1 and two CV-1 experiments, each assayed three times.

Mutant (lacY\(^-\)) shuttle vectors were isolated and analyzed by restriction endonuclease (PstI) digestion and agarose gel electrophoresis as described above. As shown in Figure 5, the percentages of mutant pSV2-lacY molecules which had gross alterations containing deletions and rearrangements increased with gamma ray dose from about 55% at 5 Gy to almost 100% above 20 Gy, when CV-1 cells were used as host. The reason for high frequency of gross alterations found for unirradiated DNA in the replicating COS-1 system is not understood well at the present time.

As shown in Table 1, the mutation frequency and percentage gross alteration of RFIII molecules from irradiated DNA were essentially similar to those observed using irradiated total pSV2-lacY molecules. When Clal-digested pSV2-lacY was used, the lacY mutation frequency
increased to a level higher than that of 20 Gy irradiation, indicating the importance of double strand breaks in mutation induction. The percentages of the mutant molecules with gross alteration and their average sized did not show variations characteristic to different treatments.

**DISCUSSION**

A shuttle vector system was constructed for a simple and quick detection and characterization of radiation-induced mutational events. The lacY marker gene was placed to all essential shuttle vector elements to detect gross alterations (e.g., deletions and rearrangements) as well as point mutations. Gamma radiation decreased shuttle vector survival and increased shuttle vector mutagenesis, as expected. These results are consistent with previous studies that utilized gamma-irradiated shuttle vectors\(^2,^4\)). Induced total mutation frequencies of shuttle vectors were found to be higher in replication permissive than non-permissive host.

When the mutant shuttle vector molecules were examined, it was observed that gross alteration increased to levels approaching 100% with gamma-ray irradiation particularly when CV-1 cells were used as mammalian host, and that the altered molecules were reduced to about one half of the original size. This agrees with reports that the irradiation of mammalian cells with ionizing radiation results in primarily gross alteration\(^8,^9\), although effects of small deletions and base changes were not fully examined in the present experiments.

The nature of the spontaneous (zero dose) mutants, however, appeared to be affected by
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The ability of pSV2-lacY to replicate in the mammalian host cells. High percentages of gross alterations were observed for spontaneous mutant particularly in the simian cells, i.e. COS-1 host, possibly because the SV40 origin is preserved in the mutations\(^2\). Further improvements of shuttle vector system may be necessary in this respect. Future studies may enable us to characterize the mutant molecules using simple molecular techniques, such as DNA sequence analysis and determine the role of shuttle vector replication in the mutagenic process, as has been made for UV-induced shuttle vector mutagenesis\(^3\). Southern blotting analysis also may need to characterize the molecular nature of mutated DNA fragments.

A fractionation test was performed to confirm that the mutation frequency was not due to small subpopulations of the transformed cells having a dominant effect. The transfected COS-1 cells were split into four small populations prior to the 48 hr incubation. Four extractions were performed and the extracts assayed separately, and each population was found to have mutation frequencies identical to those of the larger samples. To eliminate the possibility that unreplicated gamma-irradiated molecules extracted from the mammalian cells were mutated during transformation of the HB101 bacterial host, gamma-irradiated pSV2-lacY was introduced directly into HB101 host cells. The lacY mutation levels were between zero and 0.3% for all gamma ray doses, indicating that the bacterial assay did not affect the observed pSV2-lacY mutation frequencies.

Double-strand breaks appeared to account for the majority of the mutations. The nature of the gross alteration was further characterized by determining the average size of the altered mutants; this was accomplished by summing all restriction fragment for each gross altered mutant. It was observed that average size of the spontaneous and gamma-derived mutants was not significantly different from that of the Clai derived mutants. The gamma-derived shuttle vector molecules contained also an excess of single-strand breaks\(^2\), but the Clai-derived molecules did not. These results suggest that the single-strand breaks do not contribute significantly to the frequency or extent of shuttle vector mutations and rearrangements observed in this study. pSV2-lacY would be a useful shuttle vector for future analysis of the ionizing radiation-induced mutagenesis using different qualities and sources of radiation.

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