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Recombination between Irradiated Shuttle Vector DNA and Chromosomal DNA in African Green Monkey Kidney Cells

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An autonomously replicating shuttle vector was used to investigate enhancement of plasmid-chromosome recombination in mammalian host cells by gamma irradiation and UV light. Sequences homologous to the shuttle vector were stably inserted into the genome of African green monkey kidney cells to act as the target substrate for these recombination events. The shuttle vector molecules were irradiated at various doses before transfection into the mammalian host cells that contained the stable insertions. The homologous transfer of the bacterial ampicillin resistance gene from the inserted sequences to replace a mutant ampicillin sensitivity gene on the shuttle vector was identified by the recovery of ampicillin-resistant plasmids after Hirt extraction and transformation into *Escherichia coli* host cells. Gamma irradiation increased homologous shuttle vector-chromosome recombination, whereas UV light did not increase the frequency of recombinant plasmids detected. Introducing specific double-strand breaks in the plasmid or prolonging the time of plasmid residence in the mammalian host cells also enhanced plasmid-chromosome recombination. In contrast, plasmid mutagenesis was increased by UV irradiation of the plasmid but did not change with time. The ampicillin-resistant recombinant plasmid molecules analyzed appeared to arise mostly from nonconservative exchanges that involved both homologous and possibly nonhomologous interactions with the host chromosome. The observation that similar recombinant structures were obtained from all the plasmid treatments and host cells used suggests a common mechanism for plasmid-chromosome recombination in these mammalian cells.

Various chemicals and types of radiation act upon DNA to induce structural changes (lesions) that may be converted to mutations or chromosome aberrations in the cellular genome (21, 24, 42, 52, 63). Ionizing radiation (e.g., gamma and X rays) produces lesions in the DNA by direct action and the indirect action of radicals formed in aqueous solution (19, 23). The lesions produced are predominantly single- and double-strand breaks, modified bases, alkali-labile bonds, and DNA-protein cross-links (19, 23). UV radiation reacts with DNA almost entirely by direct reaction, producing primarily the cyclobutane form of the pyrimidine dimer (18, 51) and the 6-4 photoproduct, which occurs at about 1/10 the frequency of the cyclobutane dimer (18).

Extrachromosomal molecules are often 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, and there is not the complication of direct effects of the modifying agent on the cell. Treatment of plasmid DNA with chemical carcinogens (34) or UV radiation (10, 41) before transformation into *recA*$^+$ *Escherichia coli* host cells induces high levels of homologous plasmid-chromosome recombination, which can transfer sequences from the bacterial chromosome to the plasmid molecule. Gamma irradiation of bacterial plasmid DNA also induces plasmid-chromosome recombination events at high frequencies (J. S. Mudgett and W. D. Taylor, submitted for publication).

Shuttle vectors have been used to demonstrate that homologous recombination between plasmid molecules can occur in mammalian cell extracts (11, 22, 26, 29, 40, 44, 57) and transfected mammalian host cells (1, 3–7, 14, 28, 31, 33, 40, 47–49, 54, 57, 61, 70). The frequency of these inter- and intraplasmid exchanges has been reported to be dependent on the length of substrate homologies available for the recombination events (3–5, 7, 31, 48, 54, 61, 70). However, it has also been observed that nonhomologous plasmid recombination, the efficient joining of free ends (27, 38, 70, 73, 74), can occur at frequencies greater than or comparable to the frequencies of interactions between homologous regions (7, 47, 61, 70). Extrachromosomal shuttle vector DNA has been reported to integrate at homologous chromosomal locations (32, 56, 64) or to rescue homologous sequences from the chromosome of the transfected mammalian cells (25, 55, 60, 64, 66, 67). In comparison with homologous extrachromosomal recombination, which can occur at frequencies approaching 50 to 100% (4, 14), homologous plasmid-chromosome recombination was detected at frequencies of between 10^{-8} and 10^{-3} (25, 32, 55, 56, 60, 64, 66, 67). Nonhomologous plasmid-chromosome interactions were more frequent, however, occurring at levels 10^2-fold (64) to 10^5-fold (32) higher than frequencies of comparable homologous insertions.

Mammalian cells have been shown to exhibit recombination activities in several different systems, but their function in comparison to that of the well-characterized recombination pathways of the bacterium *E. coli* remains unclear, particularly in the area of DNA repair. To address the question of recombinational responses to DNA damage in mammalian cells, the ability of radiation damage to induce plasmid-chromosome recombination in mammalian cells was investigated, since it had been previously shown to occur at high levels in *E. coli* bacterial host cells transformed with damaged plasmid molecules. To examine mammalian plasmid-chromosome recombination, the transfer of plasmid sequences stably integrated in the genome of CV-1P African green monkey kidney cells to an irradiated homologous

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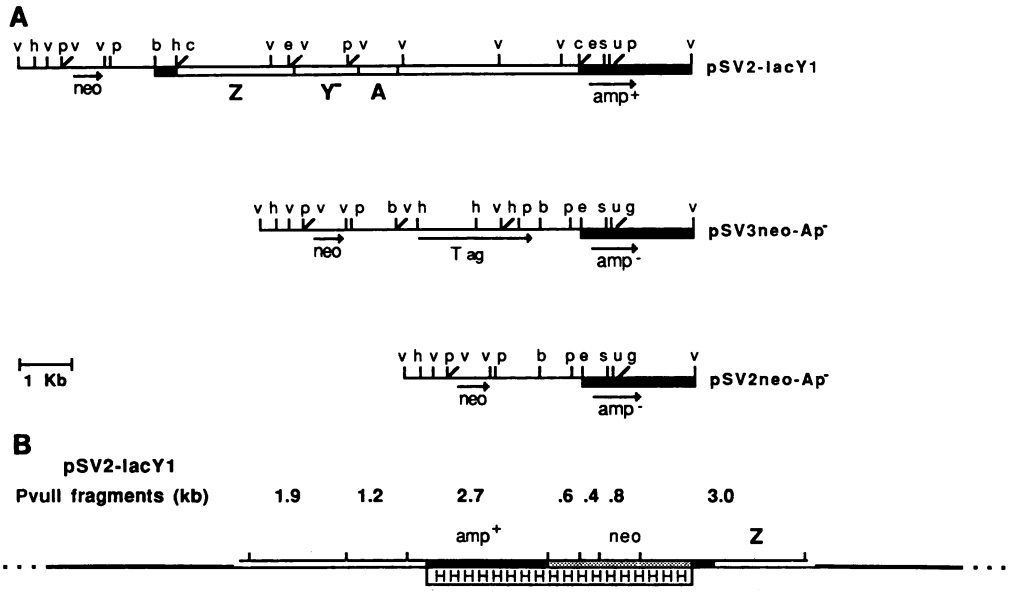

FIG. 1. (A) Restriction maps of plasmids pSV2-lacY1, pSV3neo-Ap⁻, and pSV2neo-Ap⁻. Plasmids were constructed as described in Materials and Methods. Symbols: ▬, pBR322 sequences (35); ——, SV40 shuttle vector sequences (58); □, *E. coli lac* and 3′ noncoding sequences (41). pSV2-lacY1 was successfully inserted into the genome of CV-1P host cells to act as a target sequence for homologous recombination as described in the text. Plasmid pSV2neo-Ap⁻ is as pSV3neo-Ap⁻ but with the SV40 T-antigen region removed to prevent shuttle vector replication. Abbreviations for restriction sites: v, *Pvu*II; h, *Hin*dIII; p, *Pst*I; b, *Bam*HI; c, *Cla*I; e, *Eco*RI; s, *Sca*I; u, *Pvu*I; g, *Bgl*I. The *amp* gene was mutated as described in Materials and Methods to convert its *Pst*I site to a *Bgl*I site; the *Bgl*I site shown is only one of several in each plasmid. Plasmids pSV2-lacY1, pSV3neo-Ap⁻, and pSV2neo-Ap⁻ are 13.2, 8.8, and 5.7 kb, respectively, in size. (B) Apparent structure of the lacY1#5 pSV2-lacY1 plasmid insert along with its *Pvu*II restriction sites and fragment sizes. The lacY1#8 insert structure was similar but lacked the complete 1.9- and 3.0-kb fragments. The insert maps were determined by Southern analysis as described in the text. Symbols: ■, pBR322 sequences; ▩, SV40 shuttle vector sequences; =, *E. coli lac* and 3′ noncoding sequences. Sequences homologous to the shuttle vector pSV3neo-Ap⁻ are indicated by boxed H's.

shuttle vector was investigated. The bacterial ampicillin resistance (Amp^r) gene was used as the recombination marker to allow for detection and analysis of recombinant plasmids in recombination-deficient bacterial cells.

MATERIALS AND METHODS

Cell strains and media. CV-1P African green monkey kidney cells (P. Berg, Stanford University) were grown in Dulbecco modified Eagle medium (DMEM; GIBCO Laboratories) supplemented with 2.5 mM L-glutamine, 100 μg of streptomycin per ml, 60 μg of penicillin per ml, and 10% fetal bovine serum (GIBCO). The cells were routinely maintained at 37°C in an atmosphere of 5% CO_2 in a humidified incubator.

Recombination-deficient *E. coli* DH1 (17) host cells were used to assay extracted background plasmid yield and plasmid-chromosome recombination by plating on V-medium agar plates (41) supplemented with kanamycin sulfate (50 μg/ml) and ampicillin (50 μg/ml), respectively.

Plasmid constructions. Plasmid pSV2-lacY1 (Fig. 1A) was constructed by inserting the *E. coli lac* and associated pBR322 sequences from plasmid pBRM4 (41), which had undergone recombination in AB1157 host cells to acquire the host *lacY1* sequence mutation (41), into pSV2-neo (58). The unique *Bam*HI site and the unique *Sca*I site of each plasmid were used as cloning sites according to standard procedures (35). The Amp^r gene was mutated by cutting pBR322 with *Pst*I, removing the single-stranded bases from the ends, and ligating the ends to create a 4-base-pair deletion and a *Bgl*I site (35). This ampicillin sensitivity (Amp^s) mutant gene was subcloned into autonomously replicating pSV3-neo to create pSV3neo-Ap⁻. The nonreplicating plasmid pSV2neo-Ap⁻ was subcloned from pSV3neo-Ap⁻ by removing the *Bam*HI fragment containing the simian virus (SV40) large-T (tumor) antigen sequences required for autonomous replication (58).

Establishment of CV-1P(pSV2-lacY1) cell lines. CV-1P mammalian cells were stably transformed with pSV2-lacY1 plasmid DNA by the calcium phosphate coprecipitation method (12) for selection of geneticin (G418)-resistant colonies (58). The plasmid inserts were found to be stable for more than 80 generations under nonselective conditions. High-molecular-weight genomic DNA was isolated by using the protocol of Folger et al. (15), with the substitution of proteinase K for pronase, digested to completion with restriction endonucleases, electrophoresed on agarose gels, transferred to nitrocellulose filters (35), and hybridized with nick-translated pSV2-lacY1 (68).

Irradiation of plasmid DNA. Plasmid DNA (20 μg/ml) in TE (10 mM Tris, 1 mM EDTA [pH 8.0]) was irradiated with UV light (254 nm) at room temperature, using a germicidal lamp (General Electric G8T5). The fluences were measured by using a UV intensity meter (Blak-Ray 225; UV Products, Inc.).

Gamma irradiation of plasmid DNA (20 μg/ml in TE) was performed at room temperature in a 1.5-ml plastic microfuge tube lowered into the radiation field of a Gammacell 200 ^{60}Co gamma source (Atomic Energy of Canada, Ltd.). Doses were estimated by the activity of the gamma source (~5.4 krad/min) and the time of plasmid irradiation. Samples of UV- or gamma-irradiated plasmid DNA were used immediately to transfect the mammalian host cells.

Transfection and extraction of plasmid DNA. CV-1P-de-

rived host cells were seeded at 4×10^6 cells per 100-mm-diameter plate 24 h before transfection. Plasmid DNA was added to Tris-buffered (50 mM; pH 7.4) DMEM without serum to a concentration of 200 ng/ml for all plasmids. This concentration was determined to be in the linear range of transfection for plasmid yield. DEAE-dextran (molecular weight, 500,000; 20 mg/ml in DMEM; Pharmacia, Inc.) was then added as the transfection facilitator (36) to a final concentration of 200 μg/ml. The cells were washed twice with 5 ml of DMEM without serum, and 3 ml of the transfection mixture described above was added to each plate. Two plates of cells (6 ml of mixture) were prepared per dose in each experiment. The cells were incubated at 37°C in 5% CO_2 for 4 h, after which they were gently washed once with 5 ml of DMEM without serum. The cells were incubated with 10 ml of complete medium for 12 to 96 h posttransfection as indicated for each experiment. The medium was replaced with 10 ml of complete medium at 48 h to maintain cell viability.

Low-molecular-weight plasmid DNA was extracted from the transfected mammalian host cells at the specified times by a modification of the method of Hirt (20). The cells were washed twice with 5 ml of phosphate-buffered saline, and 1.8 ml of Hirt lysis buffer (0.6% sodium dodecyl sulfate, 10 mM EDTA [pH 7.5]) containing 200 μg of proteinase K was added per 100-mm-diameter dish. The lysed cells were incubated at 37°C for 2 h, after which the lysates from both plates per dose were scraped into one 15-ml tube, 0.9 ml of 5 M NaCl was added, and the contents were gently mixed by inversion. After precipitation at 4°C for approximately 16 h, the extractions were centrifuged at 21,000 × *g* for 90 min at 4°C in an SW50.1 rotor. The supernatants containing the low-molecular-weight DNA (4.5 ml) were precipitated with 2 volumes of 95% ethanol at −75°C. The precipitated DNAs were centrifuged at 12,000 × *g* for 30 min at 4°C. The DNAs were treated with RNase, precipitated, and washed with 70% ethanol before resuspension in TE.

Detection and analysis of recombinant and mutant plasmids. Samples (15 μl) of the extracted DNAs were digested with the restriction endonuclease *Dpn*I in a final volume of 20 μl or used undigested to transform DH1 host cells by the method of Hanahan (17). Digestion with *Dpn*I cleaves the shuttle vector GATC sites that were methylated in the bacterial cells, thus eliminating input DNA from the Hirt extraction assays. The *Dpn*I digestion buffer did not affect the DH1 transformation efficiency, and controls of undigested and *Dpn*I-digested plasmid DNA were included with every Hirt extraction assay to confirm that the *Dpn*I digestions were effective. Surviving kanamycin-resistant (Kn^r) background and ampicillin-resistant (Ap^r) plasmid colonies were detected on agar plates as described above. At least three separate bacterial assays were performed for each experiment, and multiple (two to four) experiments were performed for each datum point.

Plasmid DNA from the resultant transformants was isolated by the alkaline extraction method (35). Preparative quantities of plasmid DNA were prepared by scaling up the alkaline extraction procedure and purifying the DNA by cesium chloride-ethidium bromide centrifugation (35).

RESULTS

Characterization of CV-1P(pSV2-lacY1) cell lines. The pSV2-lacY1 insert structures were determined by using *Bam*HI, *Hin*dIII, and *Pvu*II, which cleave pSV2-lacY1 once, twice, and 10 times, respectively (Fig. 1A). Analysis of the resultant autoradiograms demonstrated that eight of eight cell lines, designated lacY1#1 to -8, contained less than one complete insertion of plasmid pSV2-lacY1. Cell line lacY1#5, which was used in these studies, gave two hybridizing *Bam*HI fragments that were both different in size from native pSV2-lacY1 fragments. Digestion with *Hin*dIII gave three hybridizing fragments, one of which was identical in size to the *Hin*dIII fragment that contained the *neo* gene (Fig. 1A); the other two were different in size from pSV2-lacY1 fragments. Band intensities were consistent with a single-copy insertion (16). These restriction fragment patterns were also indicative of a single-copy insertion (16) containing the small pSV2-lacY1 *Hin*dIII fragment and its internal *Bam*HI site (Fig. 1A), with 5′ and 3′ insertion points and no head-to-tail arrays. A more precise determination of the 5′ and 3′ insertion points were made by using *Pvu*II. By this analysis, the pSV2-lacY1 insert in lacY1#5 contained the complete 3.0-, 2.7-, 1.9-, 1.2-, 0.8-, 0.6-, and 0.4-kilobase-pair (kb) *Pvu*II fragments, indicating that the plasmid break and chromosomal insertion points were somewhere between the *lacZ* and *lacA* genes (Fig. 1A and B). The apparent pSV2-lacY1 insert structure of lacY1#5 host cells is diagramed in Fig. 1B.

Time dependence of pSV3neo-Ap^- plasmid yield. pSV3neo-Ap^- was transfected into lacY1#5 host cells and extracted at various times posttransfection as described in Materials and Methods. Extracted shuttle vector yield (as determined by *E. coli* transformation) was found to increase up to 48 h posttransfection, presumably due to plasmid replication, after which it decreased with increasing time (Fig. 2A). Plasmid yield was independent of *Dpn*I digestion except for the 12-h time point, indicating that most of the detectable plasmid had successfully replicated by 24 h posttransfection. LacY1#5 host cells were transfected with 200 ng of pSV3neo-Ap^- per ml, the usual nonsaturating concentration used, or with 20 ng of pSV3neo-Ap^- per ml. The results indicated that the plasmid yield decrease was time dependent, not concentration dependent, since host cells containing 1/10 as much vector DNA decreased their plasmid yield at exactly the same time (Fig. 2A).

Inactivation and recombination of UV-irradiated pSV3neo-Ap^-. UV-irradiated pSV3neo-Ap^- was transfected into lacY1#5 host cells, extracted at various times posttransfection, and assayed in DH1 bacterial cells. Surviving plasmids, i.e., viable transformants, were measured for each UV dose and time. Relative survival for each posttransfection time was calculated as the survival at a given UV dose divided by the survival at zero dose (for that posttransfection time). Relative plasmid survival increased with increasing posttransfection time up to 72 h, when further incubation had no more effect (Fig. 3A). Low-dose shoulders were evident at 72 and 96 h, potentially indicative of plasmid repair.

Recombinant Ap^r transformants were counted as CFU per milliliter, and the ratios of these recombinants to total survivors (in CFU per milliliter) at a given dose and time were calculated. Plasmid recombination (Ap^r transformant) frequencies were determined for pSV3neo-Ap^- at 48, 72, and 96 h posttransfection (Fig. 3A); no recombinants were detected at 24 h posttransfection. No enhancement of plasmid recombination was observed with increasing UV dose to pSV3neo-Ap^- (Fig. 3A). Recombinants were detected at a frequency of 8.7×10^{-5} at 48 h posttransfection. The recombination frequencies detected at 72 h posttransfection were about 12 times those observed at 48 h and were another twofold greater at 96 h (Fig. 3A and Table 1). Plasmid

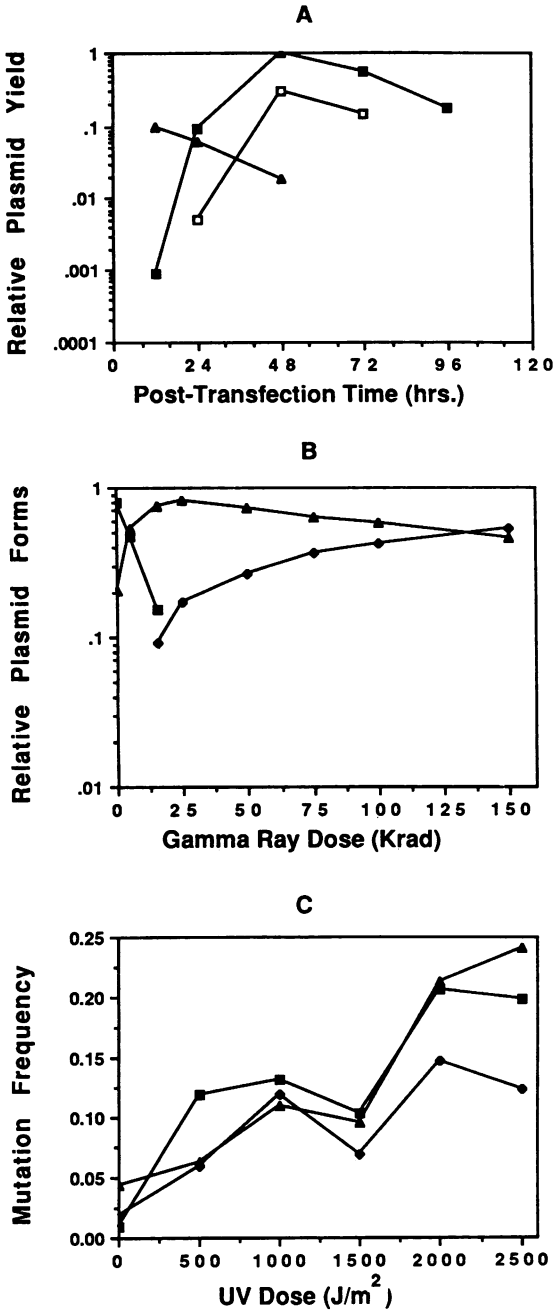

FIG. 2. (A) Relative plasmid yield of unirradiated pSV3neo-Ap$^-$ and pSV2neo-Ap$^-$ in lacY1#5 host cells. The experiments were performed as described in the text. Unirradiated pSV3neo-Ap$^-$ was transfected into lacY1#5 host cells at a concentration of 200 (■) or 20 (□) ng of plasmid DNA per ml. Nonreplicating pSV2neo-Ap$^-$ (▲) was transfected into lacY1#5 at 200 ng/ml. After extraction at various times posttransfection, the extracted plasmid DNA was transformed into DH1 bacterial cells for determination of surviving Kn^r transformants (plasmid yield). The data plotted are the ratios of Kn^r DH1 transformants for a given plasmid and time relative to the number of Kn^r colonies observed with 200 ng of pSV3neo-Ap$^-$ per ml at 48 h. (B) Interconversion of pSV3neo-Ap$^-$ forms upon treatment with various doses of gamma radiation. pSV3neo-Ap$^-$ was irradiated with gamma rays, electrophoresed on agarose gels, stained with ethidium bromide, and photographed with Polaroid 665 film. The negatives were scanned with an LKB laser densitometer, and the relative amounts of plasmid forms RFI (■), RFII (▲), and RFIII (◆) were plotted. (C) Mutation of UV-irradiated pSV3neo-Ap$^+$ in lacY1#5 host cells. pSV3neo-Ap$^+$ plasmid DNA was irradiated with UV light, transfected into lacY1#5, extracted at various times posttransfection, and transformed into DH1 host cells. The data plotted are the ratios of Kn^r Ap^s DH1 mutants to total surviving transformants for each dose. Posttransfection times were 48 (◆), 72 (▲), and 96 (■) h.

survival and recombination frequencies were not affected by *Dpn*I digestion.

Inactivation and recombination of gamma-irradiated pSV3neo-Ap$^-$. Gamma-irradiated pSV3neo-Ap$^-$ was transfected into lacY1#5 host cells, extracted at various times posttransfection, and assayed in DH1 bacterial host cells. Relative survival of gamma-irradiated pSV3neo-Ap$^-$ was unlike that observed with UV-irradiated plasmid in that no increase in plasmid survival with increasing incubation time or low-dose shoulders were observed (Fig. 3B). The absence of these indicators of (gamma-irradiated) plasmid repair suggests that the mechanisms of ionizing radiation repair, if any, were different from those that might be acting on the UV-irradiated plasmid. In contrast to the results for UV irradiation, shuttle vector recombination frequencies were enhanced by increasing gamma-ray doses to the plasmid (Fig. 3B). This increase was between 8- and 10-fold at 72 and 96 h and was around 3-fold at 48 h posttransfection (Fig. 3B). As before, no recombinants were detected at 24 h, background recombination frequencies increased with posttransfection time, and both survivors and recombinants were *Dpn*I resistant.

To determine the relative amounts of potentially recombinogenic linear (replicative form III [RFIII]) pSV3neo-Ap$^-$ transfected into the lacY1#5 host cells, the structural forms of the plasmid were separated and assayed (Fig. 2B). There appeared to be a rapid conversion of supercoiled (RFI) to relaxed (RFII) molecules due to single-strand breaks between 0 and 25 krad (Fig. 2B). After 25 krad, the plasmid consisted mostly of relaxed molecules until 150 krad, when the amounts of relaxed and linear (RFIII) molecules (due to random double-strand breaks) were almost equal (Fig. 2B).

Effects of specific double-strand breaks on pSV3neo-Ap$^-$ recombination. To examine the recombinogenic potential of linearized plasmid DNA, specific double-strand breaks were made at either the *Pvu*I or the *Eco*RI site of pSV3neo-Ap$^-$ (Fig. 1A). Linear DNA was transfected into lacY1#5 host cells, extracted at various times posttransfection, and assayed in DH1 host cells. The extracted plasmids were *Dpn*I resistant, suggesting that the plasmid molecules recircularized and then replicated. The frequencies of plasmid-chromosome recombination detected were greater when linearized substrates were used (Table 1). *Pvu*I-digested pSV3neo-Ap$^-$ appeared to be slightly more recombinogenic than *Eco*RI-digested plasmid at the later times (Table 1). The relative increases in recombination for linear substrates were between six- and sevenfold for linear pSV3neo-Ap$^-$ at 48 h and between two- and fourfold at 72 and 96 h (Table 1). No recombinants were detected at 24 h posttransfection.

Restriction endonuclease analysis of recombinant plasmids. Recombinant plasmids from each of the posttransfection times, radiation (UV and gamma) doses, and plasmid forms were digested with *Pvu*II for determination of restriction fragment sizes. Whereas background Kn^r plasmids never showed different restriction patterns (0 of 60), most Ap^r recombinants were extensively rearranged (Fig. 4). Novel *Pvu*II fragments were observed in almost all of the recombinants analyzed (Fig. 4A), and native pSV3neo-Ap$^-$ *Pvu*II

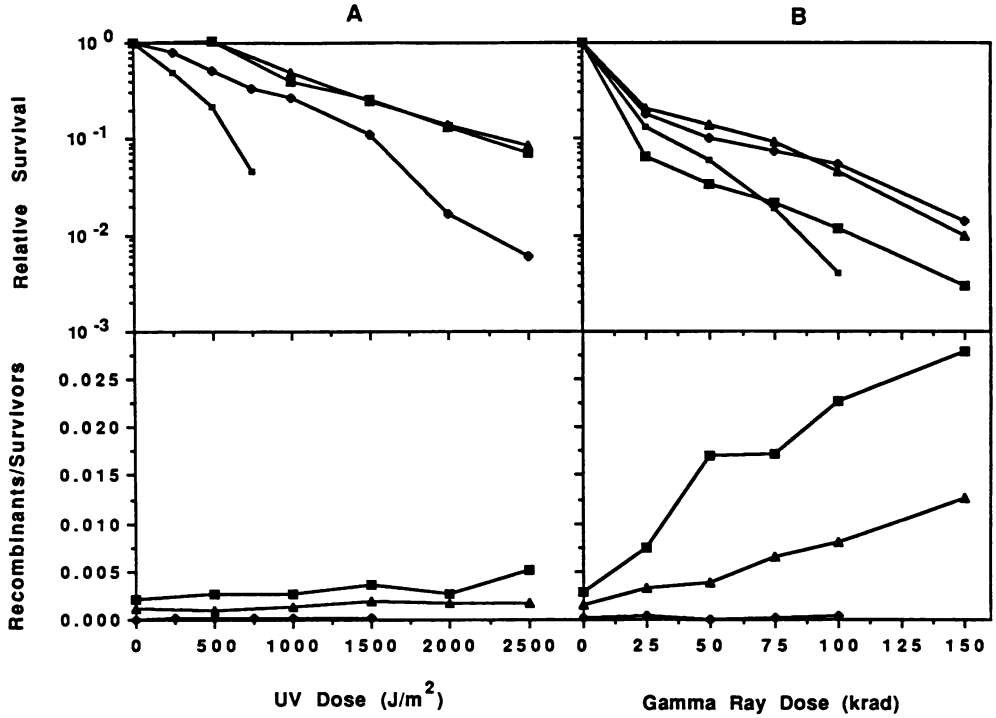

FIG. 3. Relative survival and recombination of UV- or gamma-irradiated pSV3neo-Ap$^-$ in lacY1#5 host cells. The experiments were performed as described in the text. pSV3neo-Ap$^-$ plasmid DNA was irradiated with UV light (A) or gamma rays (B), transfected into lacY1#5 host cells, extracted at various times posttransfection, and transformed into DH1 host cells. The survival data plotted are the ratios of Kn^r DH1 transformants at a given dose relative to the transformants at zero dose for each time. The recombinant data are the ratios of Ap^r DH1 transformants to total surviving transformants for each dose and time. Posttransfection times were 24 (●), 48 (◆), 72 (▲), and 96 (■) h. No recombinants were detected at 24 h for either type of irradiation.

fragments were preferentially lost 5′ of and including the Amp^s gene region (Fig. 1A). The recombinants that retained the native pSV3neo-Ap$^-$ *Pvu*II fragment containing the Amp^s gene were usually conserved plasmids that had not undergone rearrangements detectable by restriction enzyme analysis (Fig. 4A). Conserved plasmids were observed at a frequency of about 10% (31 of 317). The majority (~71%) of the rearranged recombinants obtained the pSV2-lacY1 2.7-kb *Pvu*II fragment containing the Amp^r gene from the chromosome (Fig. 1B and 4A). Thus, most of the recombinants replaced their native *amp*-containing *Pvu*II fragment with the 2.7-kb chromosomal insert fragment. These recombinational exchanges also transferred the Ap^r *Pst*I site, even if the recombinants did not contain the complete 2.7-kb *Pvu*II fragment (data not shown). Since the 5′ end of the 2.7-kb chromosomal insert fragment was nonhomologous to the pSV3neo-Ap$^-$ plasmid substrate (Fig. 1B), the recombinants apparently obtained nonhomologous sequences along with Amp^r gene. This was shown by Southern analysis using the 5′ *Pvu*II-to-*Cla*I region from the pSV2-lacY1 2.7-kb fragment (Fig. 1B) and the pSV2-lacY1 1.2-kb fragment (Fig. 1B) as probes (Fig. 4B). The *Pvu*II-to-*Cla*I probe hybridized with the pSV2-lacY1 2.7-kb fragment and the 2.7-kb fragments in the recombinants but not with pSV3neo-Ap$^-$ (Fig. 4B). Recombinants that did not contain the complete 2.7-kb fragment often had random-size fragments that hybridized with the *Pvu*I-to-*Cla*I probe (Fig. 4B). When the 1.2-kb fragment was used as a probe, only fragments from pSV2-lacY1 and the recombinants that contained the complete 2.7-kb fragment hybridized (Fig. 4B); some recombinants contained the entire 1.2-kb fragment. These results indicate that more than 71% of the recombinants contained fragments derived from nonhomologous (*lac*) sequences which flank the Amp^r marker gene and that sequences more distal to the marker were transferred less often, as expected.

TABLE 1. Plasmid-chromosome recombination frequencies of uncut and linear pSV3neo-Ap$^-$

Time (h) post-transfection	Avg Ap^r recombination frequency (10^5) with given plasmid		
	Uncut[a]	*Pvu*I digested[b]	*Eco*RI digested[b]
48	8.7 ± 4.7	61.5	55.8
72	104.2 ± 40.3	442.7	167.4
96	203.9 ± 80.6	788.9	410.6

[a] Averages and standard deviations from eight experiments.
[b] Averages from two experiments.

Recombinant plasmids from the different radiation doses (including zero dose) for both gamma and UV radiation all had similar distributions of molecular structures. Likewise, posttransfection time did not alter the distribution of structures observed. The Ap^r recombinants from both enzyme-linearized plasmid substrates were also found to have molecular structures similar to those previously described, except that no conserved molecules were observed. Background (Kn^r) plasmids from the RFIII transfections were analyzed at random, and almost two-thirds were found to have sequence deletions detectable by agarose gel electrophoresis near the sites of linearization.

Recombination control experiments. To confirm that the Ap^r plasmids were the result of interactions between the

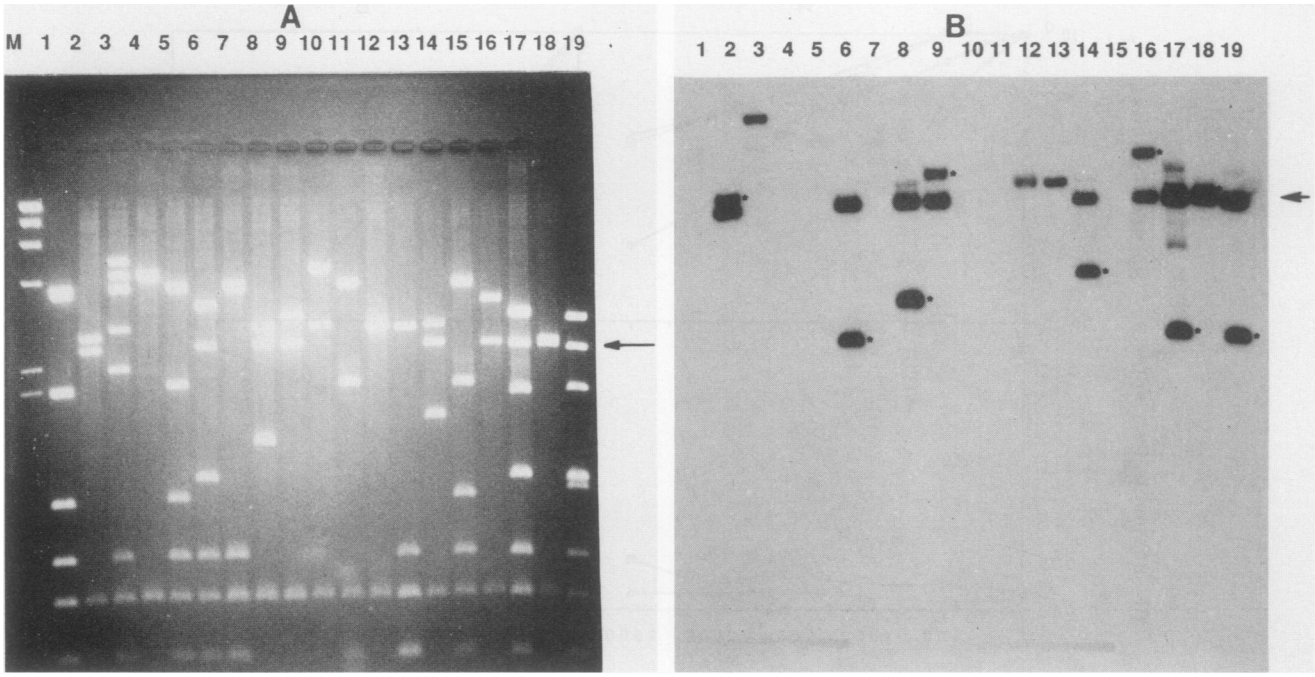

FIG. 4. (A) Restriction endonuclease analysis of recombinant pSV3neo-Ap$^-$ plasmids. Recombinant isolates shown are representative of all experimental conditions and times. Lanes: M, markers of 23.13, 9.42, 6.56, 4.36, 2.32, 2.02, and 0.56 kb; 1, *Pvu*II digest of pSV3neo-Ap$^-$ plasmid DNA; 2 to 18, *Pvu*II digests of random Ap^r recombinant plasmid DNAs; 19, *Pvu*II digest of pSV2-lacY1 plasmid DNA. Lanes 5 and 15 contain conserved Ap^r recombinant pSV3neo-Ap$^-$ plasmids. The arrow indicates the 2.7-kb band containing the pSV2-lacY1 Amp^r gene. (B) Southern analysis was performed by using the 5′ *Pvu*II-to-*Cla*I region from the 2.7-kb pSV2-lacY1 fragment and the 1.2-kb pSV2-lacY1 fragment. The results shown are from a mixed probe, but this analysis was also performed with separate probes (data not shown). Fragments that hybridized with the 1.2-kb probe are marked (*); fragments that hybridized with the *Pvu*II-to-*Cla*I probe are not marked. The arrow indicates the 2.7-kb band containing the pSV2-lacY1 Amp^r gene.

pSV3neo-Ap$^-$ shuttle vector and the pSV2-lacY1 chromosomal insert, control experiments were performed. pSV3neo-Ap$^-$ transformed into DH1 bacterial host cells did not give rise to Ap^r colonies, nor was this plasmid rearranged after bacterial transformation. Unirradiated and UV-irradiated pSV3neo-Ap$^-$ transfected into CV-1P host cells gave survival results identical to those observed with lacY1#5 host cells, but no Ap^r plasmids were obtained. Plasmid DNA derived from pBR322 that contained *lac* sequences cloned into the Amp^r gene was transfected into lacY1#5 host cells, and no Ap^r or Kn^r plasmids were obtained. Thus, lacY1#5 transfections with plasmid DNA containing homologies to the integrated sequences did not induce those sequences to be excised and detected. Recombinant molecules were also shown by Southern blot analysis to often contain SV40 T-antigen sequences even though the recombinant molecules were usually rearranged. These recombinants were therefore derived from pSV3neo-Ap$^-$ and were not the result of induced pSV2-lacY1 excision. Since plasmid yield decreased and recombination increased at 48 h posttransfection, the effect of changing the growth medium at this time was examined. Control experiments determined that this change of medium did not affect plasmid survival or recombination frequencies and structures.

To determine whether the recombination frequencies and structures were unique to the specific cell line lacY1#5, an alternative cell line was used. The insert structure of lacY1#8 was determined to be similar to that of lacY1#5 (Fig. 1B) except that the 3.0- and 1.9-kb *Pvu*II fragments were absent. Plasmid-chromosome recombination frequencies at the different posttransfection times were the same as observed with lacY1#5 host cells, as was the distribution of recombinant structures. As with lacY1#5 host cells, the majority of the recombinants (13 of 21) obtained the 2.7-kb pSV2-lacY1 *Pvu*II fragment from the chromosome.

Recombination of pSV2neo-Ap$^-$. Since plasmid-chromosome recombination was detected only at 48 h posttransfection and later, the time period during which the plasmid yields were decreasing, the possibility that plasmid degradation was giving rise to recombinogenic (linear) molecules was addressed. The nonreplicating shuttle vector pSV2neo-Ap$^-$ (Fig. 1A) was transfected into lacY1#5 host cells, extracted at 12, 24, and 48 h posttransfection, and assayed in DH1 host cells. As expected, the extracted plasmids were sensitive to *Dpn*I. In the absence of replication, pSV2neo-Ap$^-$ yield began to decrease immediately and at the same rate observed for pSV3neo-Ap$^-$ from 48 to 96 h posttransfection (Fig. 2A). No Ap^r recombinants were detected from any of the pSV2neo-Ap$^-$ transfections. Plasmid degradation (in the absence of replication) alone did not appear to enhance plasmid-chromosome recombination.

Mutagenesis of UV-irradiated pSV3neo-Ap$^+$. Mutagenesis of the pSV3neo-Ap$^+$ Amp^r gene was determined by plating background Kn^r transformants on medium containing ampicillin. Plasmid mutagenesis increased almost 10-fold with increasing UV dose, but there did not appear to be any significant increase in plasmid mutagenesis from 48 to 96 h (Fig. 2C). Background mutagenesis levels were around 1%, as has been reported for similar shuttle vectors (2, 8, 30, 37, 39, 45, 46, 50, 53, 69). pSV3neo-Ap$^+$ survival was the same as observed for pSV3neo-Ap$^-$ (data not shown). When the mutant plasmids were analyzed by *Pvu*II analysis, it was

observed that around 68% of the mutants were the result of gross rearrangements, with preferential loss of the *Pvu*II fragment that contained the Amp^r marker gene. Novel band sizes were randomly distributed. Mutant plasmid structures were not affected by UV dose or posttransfection time.

DISCUSSION

This study was performed to investigate the effects of radiation-induced DNA damage on homologous recombination between autonomously replicating shuttle vectors and homologous sequences stably inserted into the chromosomes of CV-1P mammalian host cells. Gamma irradiation enhanced pSV3neo-Ap^- recombination with the lacY1#5 mammalian host cell chromosomal insert, with increases of up to 10-fold observed at 150 krad. This contrasts with intrachromosomal recombination, on which gamma radiation had no effect (72). Either intrachromosomal recombination proceeds by a different mechanism, as suggested previously (71, 72), or differences between the systems used gave contrasting results. When the gamma-irradiated pSV3neo-Ap^- plasmid DNA was analyzed for changes in molecular form, it was determined that gamma irradiation had created an average of one single-strand break per molecule at about 7 krad (D_{37} RFI) and that one-half of the plasmid molecules contained double-strand breaks at 150 krad. These strand breaks could have enhanced recombination. In particular, double-strand breaks and gaps have been shown to increase the frequencies of homologous chromosome-to-plasmid recombination (25, 60). Assuming that the double-strand breaks must be in or near the recombination marker and plasmid homologies to be effective (25, 57) and that breaks within 500 base pairs of the 860-base-pair Amp^s gene would comprise 20% of the (50%) random linear molecules, then only about 10% of the gamma-irradiated molecules might have contained recombinogenic double-strand breaks. If this 10% enhanced recombination 10-fold, then the ratio of linear plasmid to supercoiled plasmid recombination would be about 100. The increase in plasmid-chromosome recombination due to complete linearization of pSV3neo-Ap^- was only sevenfold greater than the value for uncut plasmid. It is therefore doubtful that the small number of random gamma-induced double-strand breaks at or near the recombination marker could account for the increase in plasmid-chromosome recombination. It is assumed that linear molecules produced by gamma irradiation or restriction endonucleases were transfected with similar efficiencies and that the observed (RFIII) end degradation would have eliminated any differences due to terminal structure. Therefore, gamma-induced lesions other than the random double-strand breaks were probably recombinogenic in this system. Alternative lesions for consideration are the gamma-induced single-strand breaks, since these were efficiently induced and have previously been associated with mammalian cell recombination (21, 42, 52).

Plasmid-chromosome recombination was also enhanced by prolonged posttransfection incubation time. No Ap^r recombinants were detected at 24 h even with linear substrates, and the recombination frequency increased about 24-fold from 48 to 96 h. These observations are consistent with reports that chromosome-to-virus (SV40) recombination occurred after 2 days postinfection (25) or at later times (60) and that linearization increased the frequency but not the time of recombination (60). To determine whether this increase in recombination could be correlated with the time-dependent decrease in plasmid yield, cells were transfected with 1/10 less pSV3neo-Ap^-. Recombinant plasmids were detected at the same frequency for both plasmid concentrations. These results agree with reports that interplasmid recombination was not affected by the concentration of plasmid in the nucleus (14).

If the plasmid molecules were being degraded more with increasing time, this degradation might have led to an increase in plasmid-chromosome recombination. Plasmid degradation would be expected to create and expand double-strand breaks in the extrachromosomal substrate. Since double-strand breaks have been reported to increase recombination for a variety of substrates in mammalian cells (1, 5, 6, 25, 28, 31, 49, 57, 60, 64, 70) and in this study, degradation may have partially accounted for the time dependence of recombination observed. To test whether plasmid degradation per se could increase plasmid-chromosome recombination, the nonreplicating shuttle vector pSV2neo-Ap^- was used. This plasmid appeared to degrade immediately, but no recombinants were observed. It has been reported that an increase in shuttle vector degradation would probably lead to an increase in plasmid mutagenesis (46), so mutagenesis might be indicative of plasmid breaks and degradation. No increase in shuttle vector mutagenesis with time was observed. These results suggest that plasmid degradation (without replication) per se did not enhance plasmid-chromosome recombination, and the time-dependent increase in recombination might not have been due to a concurrent increase in plasmid breaks and degradation. The possibility exists that other time-dependent factors were required to enhance plasmid-chromosome recombination.

No UV-dependent enhancement of plasmid-chromosome recombination was observed, in contrast to reports that homologous interviral recombination (13), homologous intrachromosomal recombination (72), and nonhomologous plasmid integration (43, 59, 65) were enhanced by UV irradiation. Gamma irradiation and double-strand breaks enhanced pSV3neo-Ap^- recombination, so the observation that UV light increased the frequency of mutant (mostly degraded) plasmids almost 10-fold but did not affect recombination was a bit contradictory. One explanation might be that the former recombinogenic plasmid alterations (strand breaks) were created in vitro before transfection. The plasmid breaks were therefore uncontrolled in origin, and free plasmid ends may have been readily available for plasmid-chromosome recombination. The plasmid deletions (mutations) induced by UV irradiation may have resulted from controlled cellular (repair) mechanisms in which free ends were not available to recombine with the chromosomal insert.

When the recombinants were analyzed by agarose gel electrophoresis, it was observed that 90% of the molecules were rearranged. This rearrangement contrasts with reports that the rescue of integrated sequences by SV40 viral DNA was conservative (25, 67). The SV40 experiments depended on the formation of viable virions by recombination, so extensive alterations of the type observed in this study might not have been detected. The recombinants from all experimental conditions, including all gamma and UV radiation doses, double-strand breaks, and posttransfection times, had similar distributions of molecular structures. Two independent host cell lines with similar insert structures (Fig. 1B) both led to rearrangement and the rescue of the 2.7-kb *Pvu*II fragment. These results suggest a common mechanism of plasmid-chromosome recombination and associated rearrangement. The double-strand break repair and single-strand annealing models of mammalian cell recombination are

suggested, since both depend on degradation occurring at double-strand breaks.

The double-strand break repair model (62) predicts that limited degradation would occur at a break point and that the two plasmid ends would both invade the chromosome at homologous regions so that the plasmid gap would be filled by chromosomal sequences. Thus, breakage and degradation at or near the Amp^s marker gene would lead to removal of the mutant sequences and replacement by the chromosomal Amp^r allele. This model could account for the 10% conserved Ap^r recombinants detected. However, more than 71% of the rearranged recombinants obtained flanking nonhomologous *lac* sequences from the chromosomal insert. The rescue of these pSV2-lacY1 sequences into the recombinant molecule would have required that the shuttle vector interact with the insert plasmid or chromosome sequences even further upstream, where the sequences continue to be nonhomologous (in both cell lines used) (Fig. 1B), thereby preventing a homologous interaction at one of the invading ends. The single-strand annealing model of extrachromosomal recombination (1, 9, 31, 33, 70) was proposed on the basis of observations of nonconservative recombination. According to this model, unwinding or exonuclease activities would expose single-strand regions in both recombinant substrates simultaneously, which would then hybridize to form the recombinant molecule. Intervening or nonhybridizing sequences would be lost. Since incorporation of flanking nonhomologous sequences into the shuttle vector would require hybridization in the 5′ nonhomologous region (Fig. 1B), this model would not predict this flanking region to be rescued.

An alternative recombinational mechanism could be that a plasmid break (and degradation) allowed the shuttle vector to interact with the chromosome at homologous sequences (Fig. 1B). The second plasmid end could then interact with the chromosome at a nonhomologous location to complete the recombinant molecule and rescue nonhomologous sequences. Since it has been reported that nonhomologous plasmid-chromosome interactions occurred 10^2 to 10^5 times more often than did homologous interactions (32, 64), resolution of the recombinant molecule by nonhomologous interactions may be favored. This mechanistic variation is supported by reports that interplasmid recombination between molecules containing partial homologies usually resulted in both homologous and nonhomologous joints (7). Alternatively, the resolving interactions could have involved unidentified recombination hot spots or small regions of sequence homology in the 5′ chromosomal or *lac* sequences. Since no sequence information was obtained for the chromosomal insert region or the recombinant molecules, the possibility that only homologous interactions were occurring cannot be excluded.

In conclusion, it appeared that gamma irradiation (strand breaks), defined double-strand breaks, and increasing posttransfection time were active in enhancing plasmid-chromosome recombination in this system. The time-dependent increase in plasmid-chromosome recombination may have required that recombination begin to occur only after the plasmid molecules had replicated. The results reported here differ sharply with reports that interplasmid recombination occurred within 1 h of the DNA entering the nucleus (14). It has been proposed that chromatin formation may prevent plasmid molecules from interacting to recombine (14, 31). In contrast, the plasmid-chromosome recombination reported here and in other studies (25) was detected only at later times, after replication had occurred. These results may indicate that plasmid-chromosome recombination requires plasmid replication for effective recombination with the host chromosome, as was suggested by studies of adenovirus recombination (75). Since the study described here was designed to investigate the effects of DNA damage in stimulating recombination, the potential requirement for plasmid replication can be answered only by future studies using a different plasmid system.

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