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Repair of Deletions and Double-Strand Gaps by Homologous Recombination in a Mammalian In Vitro System

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We have designed an in vitro system using mammalian nuclear extracts, or fractions derived from them, that can restore the sequences missing at double-strand breaks (gaps) or in deletions. The recombination substrates consist of (i) recipient DNA, pSV2*neo* with gaps or deletions ranging from 70 to 390 bp in the *neo* sequence, and (ii) donor DNAs with either complete homology to the recipient (pSV2*neo*) or plasmids whose homology with pSV2*neo* is limited to a 1.0- to 1.3-kbp *neo* segment spanning the gaps or deletions. Incubation of these substrates with various enzyme fractions results in repair of the recipient DNA's disrupted *neo* gene. The recombinational repair was monitored by transforming *recA Escherichia coli* to kanamycin resistance and by a new assay which measures the extent of DNA strand transfer from the donor substrate to the recipient DNA. Thus, either streptavidin- or antidigoxigenin-tagged beads are used to separate the biotinylated or digoxigeninylated recipient DNA, respectively, after incubation with the isotopically labeled donor DNA. In contrast to the transfection assay, the DNA strand transfer measurements are direct, quantitative, rapid, and easy, and they provide starting material for the characterization of the recombination products and intermediates. Accordingly, DNA bound to beads serves as a suitable template for the polymerase chain reaction. With appropriate pairs of oligonucleotide primers, we have confirmed that both gaps and deletions are fully repaired, that deletions can be transferred from the recipient DNA to the donor's intact *neo* sequence, and that cointegrant molecules containing donor and recipient DNA sequences are formed.

Several models have been proposed to explain the repair of double-strand breaks by homologous recombination: a helicase-mediated recombination model (54), a discontinuous-heteroduplex model (39), the single-strand annealing model (27, 28), and the double-strand break repair (DSBR) model suggested by Szostak et al. (52) and Stahl (46). In the DSBR model, a displaced strand from an intact homologous duplex is used as a template for the repair of the missing sequence. This proposal derives its strength from studies with *Saccharomyces cerevisiae*, particularly of aberrant segregation of genetic markers (52), from studies on the mechanism of mating-type switching (37, 49), and from experiments in which gapped duplex DNA is repaired and integrated at the homologous chromosomal locus (36, 37).

Double-strand break repair by homologous recombination in mammalian cells has only recently begun to be studied. Several investigations have shown that cotransfection of a gapped DNA along with an intact homologous DNA into mammalian cells results in efficient repair of the gaps (for reviews, see references 24 and 50). Furthermore, there are several reports that recombination is enhanced by specific double-strand breaks (1, 2, 5, 6, 27, 28, 35, 39, 44, 47, 54), although in some cases the recombination frequencies are reduced when one or both DNA substrates are linear (23). More recently, a strategy for gene targeting based on the DSBR model was applied successfully in our laboratory (19, 20). Despite the growing interest in the biochemistry of recombination in higher eucaryotes (3, 7, 9–12, 14, 16, 17, 21, 22, 26, 29–31, 34, 38, 53), including some reports dealing with gap repair (14, 26, 29, 30), very little is known about the enzymes and detailed mechanisms of these processes. In this work, we describe a mammalian cell-free system that catalyzes the repair of deletions and double-strand breaks by using DNAs with homology to the interrupted regions.

Our studies have used two kinds of assays for recombinational repair. One relies on measurements of the repaired products after transfection into *Escherichia coli*. But because this assay is time consuming and complicated by the potential ability of *E. coli* to catalyze some of the reactions needed to complete the repair, we have developed a different kind of assay. This one measures the amount of DNA transferred to a gapped or deletion substrate from a ^{3}H-labeled DNA which has homology to the region being repaired. This assay relies on a specific and rapid means for separating the two DNA partners in the recombination at the end of the reaction. This procedure also allows for more direct analysis of the products formed during the reaction. For example, the polymerase chain reaction (PCR) has been used to verify that gaps and deletions in the substrate DNA are fully repaired and to detect other recombination products generated in the reaction. These recombinational processes are probably mediated by a high-molecular-weight complex that has been partially purified from mammalian nuclear extracts.

MATERIALS AND METHODS

Four different types of mammalian cells were used for preparation of nuclear extracts: COS1, simian virus 40-transformed monkey cells (15); BHK-21, spontaneously transformed hamster kidney cells (48); HeLa; and JM, human T lymphocytes (19). COS1 and JM cells were grown in Dulbecco modified Eagle medium containing 5 to 10% fetal calf serum. HeLa cells were kindly provided by R. Tjian (University of California, Berkeley), and BHK-21 cells were provided by W. Doerfler (University of Cologne, Cologne, Federal Republic of Germany); the cells were

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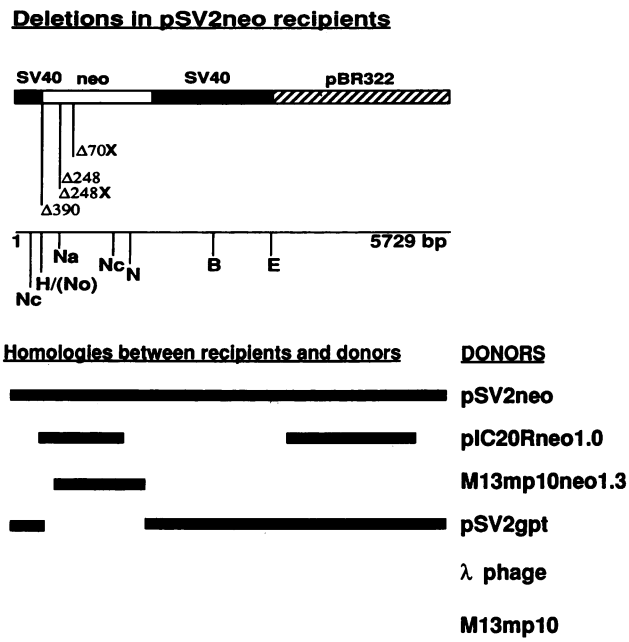

FIG. 1. Constructs used. The recipient substrates are derived from pSV2*neo* by the introduction of deletions of 70, 248, and 390 bp at the sites indicated in the *neo* gene. These are designated Δ70X, Δ248, Δ248X, and Δ390. Deletions marked X contain an *Xho*I linker (8 bp) within the deletions. Another version of Δ248 contains a *Nar*I site at the deletion, and Δ390 has a *Not*I site at the deletion. Donors are either the original pSV2*neo* fragments of the *neo* gene cloned into different vectors, pSV2*gpt*, which except for the *neo* gene is homologous to pSV2*neo*, or totally nonhomologous DNAs such as M13mp10 and λ DNAs. The regions of shared homology between recipients and donors are shown as dark bars. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nru*I; Na, *Nar*I; Nc, *Nco*I; No, *Not*I. SV40, Simian virus 40.

grown in suspension in spinner culture medium (Joklik modified minimal essential medium) with 5% fetal calf serum.

Recombination substrates. A series of modified plasmids, derived from pSV2*neo* (45), provided the substrates for recombinational repair of gaps and deletions. One set, the recipients, contains deletions of 70 to 390 bp in the *neo* coding sequence (Fig. 1). Recipient plasmids marked with an X contain a linker sequence carrying the *Xho*I restriction site at the deletion site. The Δ248 construct has a *Nar*I site at the deletion, and the Δ390 construct has a *Not*I site (which was created by an incomplete fill-in reaction during cloning) at the deletion site. Therefore, Δ248 and Δ390 do not contain any extraneous sequences in the *neo* gene. The gapped substrates were generated by cleavage within the deletion by appropriate restriction enzymes. The other recombination partners, the donors, contain the *neo* sequences missing from the recipients linked to sequences with various degrees of homology to the recipient. Complete homology to the recipient is provided by pSV2*neo*, but the homology in pIC20R*neo*1.0 includes only a 1.0-kbp segment of the *neo* gene and part of the vector backbone sequence. The homology in M13mp10 *neo*1.3 is limited to the 1.3 kbp in the *neo* gene. Plasmid pSV2*gpt* (33) lacks any *neo* sequence but shares complete homology with the vector parts of pSV2*neo*. M13mp10 and λ phage, which lack any homology to the recipient DNAs, served as controls for the in vitro reactions.

Nuclear extracts and partial purification of recombinational activity. All procedures were performed at 4°C. About 10^8 actively growing cells were harvested from either dishes or suspension cultures. The cells were washed three times with phosphate-buffered saline (PBS; 140 mM NaCl, 3 mM KCl, 8 mM NaH_2PO_4, 1 mM K_2HPO_4, 1 mM $MgCl_2$, 1 mM $CaCl_2$), resuspended in 2 to 3 ml of hypotonic buffer A (10 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol), and kept on ice for 10 to 15 min. Phenylmethylsulfonyl fluoride was added to 1 mM, and the cells were broken by 5 to 10 strokes in a Dounce homogenizer, pestle B. The released nuclei were centrifuged at 2,600 rpm in a Beckman TJ-6 centrifuge for 8 min. The supernatant was removed carefully and stored in 10% glycerol–100 mM NaCl at −70°C (cytoplasmic fraction). The nuclei were resuspended in 2 ml of buffer A containing 350 mM NaCl, and the following proteinase inhibitors were added: pepstatin to 0.25 μg/ml, leupeptin to 0.1 μg/ml, aprotinin to 0.1 μg/ml, and phenylmethylsulfonyl fluoride to 1 mM (all from Sigma Chemicals). After 1 h of incubation at 0°C, the extracted nuclei were centrifuged at 70,000 rpm in a Beckman TL-100/3 rotor at 2°C. The clear supernatant was adjusted to 10% glycerol–10 mM β-mercaptoethanol and frozen immediately in liquid nitrogen prior to storage at −70°C (fraction I). The average yield per 10^8 cells was 15 to 20 mg of protein, and the concentration was 4 to 10 mg/ml. Protein concentrations were determined according to the method of Warburg and Christian (55) or Bradford (4).

Fraction I was adjusted to 60 mM NaCl with buffer EO (10 mM KCl, 10 mM Tris hydrochloride [pH 7.4], 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 10% glycerol), and about 100 mg of protein was loaded onto an 8-ml BioRex70 (Bio-Rad) cation-exchange column, which had been equilibrated with E60 (60 mM NaCl in EO) at a flow rate of 0.2 ml/min. Elution was performed at the same flow rate in five steps: E60 (flowthrough) and E300, E500, E700, and E1000 (300, 500, 700, and 1,000 mM NaCl in EO, respectively). The E500 fraction, which contained 10 to 15% of the proteins in fraction I at a concentration of 1.5 to 2.5 mg/ml, was the most active (fraction II). Judging from measurements of the A_{260}/A_{280} ratio, this fraction still contained about 2% nucleic acids. After dilution of fraction II to 50 mM NaCl with EO, β-lactoglobulin A (Sigma) was added to 100 μg/ml. The adjusted fraction II was loaded onto a 5-ml DEAE column (DE52; Whatman) that had been equilibrated with E60, and elution was performed at 0.2 ml/min in three steps: E60, E300, and E600. Most of the activity freed from nucleic acids was contained in the E600 fraction (fraction III, 3 to 5 mg of protein). After the addition of β-lactoglobulin A to fraction III (to 200 μg/ml), ammonium sulfate was added to 0.6 saturation, the precipitated proteins were collected by centrifugation, and the pellet was dissolved in 200 μl of E200 (fraction IV). Fraction IV (2 to 4 mg of protein) was loaded onto a 24-ml Superose 5 FPLC gel filtration column (Pharmacia) and eluted with E200; 0.2- to 0.3-ml fractions were collected every minute and adjusted to 0.3 mg of β-lactoglobulin A per ml (fraction V). Generally, the fractions were assayed and frozen immediately. Repeated thawing and freezing significantly decreased the activity, which otherwise remained stable for at least several weeks. The overall yield of protein through fraction V is 0.01 to 0.06 mg.

Transfection assay. In this assay, a plasmid containing a *neo* gene lacking varying lengths of its coding sequence (recipient DNA) is incubated with a plasmid containing a DNA segment that spans the recipient DNA's missing sequence (donor DNA). Restoration of an intact functional *neo*

gene is measured by transfection into *E. coli* and its ability to confer resistance to kanamycin.

Intact double-stranded donor and the gapped or deleted recipient DNAs, 0.1 μg each, were incubated with 3 to 5 μg of extract protein in a reaction mixture containing 60 mM NaCl, 2 mM β-mercaptoethanol, 2 mM KCl, 12 mM Tris hydrochloride (pH 7.4), 1 mM ATP, 0.1 mM each deoxynucleoside triphosphate (dNTP), 2.5 mM creatine phosphate, 12 mM $MgCl_2$, 0.1 mM spermidine, 2% glycerol, and 0.2 mM dithiothreitol (total volume, 50 μl). Where fractions II to V were used, creatine phosphokinase (1 μg) was added. After 30 min at 37°C, the reaction was stopped by the addition of EDTA to 25 μM, sodium dodecyl sulfate (SDS) to 0.5%, and 20 μg of proteinase K and incubation for 1 h at 37°C. Prior to this incubation, 1 ng of a multicopy plasmid, which contains the 236-bp operator-promoter fragment of the *E. coli lac* operon (13), was added to provide an internal standard for the following steps and to normalize for variations in the transfection of the *E. coli*. DNA was extracted twice from the reaction mixtures with phenol-chloroform (1:1) and once with chloroform and precipitated with ethanol. The resulting DNA pellet was dissolved in 0.2 M NaCl–10 mM Tris hydrochloride (pH 7.4)–1 mM EDTA and transfected into competent *recA E. coli* cells. One half of each transfection mixture was plated on L plates containing ampicillin (100 μg/ml) and 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal; 1 mg); the other half was plated on plates containing kanamycin (50 μg/ml) and ampicillin (100 μg/ml). The numbers of white, Amp^r Kan^r colonies are a measure of the number of mutant *neo* genes that were repaired. In each experiment, these values were normalized by the numbers of blue, Amp^r colonies, which measure the uptake of the *lac* operator-promoter fragment.

DNA transfer assay. In this assay, the transfer of ^3H contained in the donor DNA to the recipient DNA serves as a measure of gap repair. This type of assay requires a procedure for the efficient and specific separation of the two DNAs at the end of the incubation (Fig. 2). This is done by labeling the recipient DNA with either digoxigeninylated or biotinylated nucleotides by nick translation and ligation. The recipient and donor DNAs can be separated with an anti-digoxin monoclonal antibody coupled to Sepharose in the case of the digoxigeninylated recipient DNA or with streptavidin agarose in the case of biotinylated recipient DNA.

Biotinylation and digoxigeninylation of recipient DNA. Samples (10 μg) of recipient plasmid DNA were incubated in a standard nick translation procedure (40). The nucleotide concentrations were either 4 μM biotin-dATP (Bethesda Research Laboratories) and 16 μM dATP or 2 μM digoxigenin-dUTP (Boehringer Mannheim) and 20 μM dTTP, as well as 40 μM concentrations of each of the other dNTPs. These concentrations were chosen to introduce a low number of substitutions per molecule of plasmid DNA. However, the introduction of 100 times more biotin into the recipient DNA did not affect the results in the recombination assay. Following the addition of 50 pg of DNase I and immediately 20 U of DNA polymerase I, the mixture was incubated for 2 to 4 h at 16°C. The reaction was terminated by heating for 30 min at 65°C. After cooling, 200 U of T4 DNA ligase and ATP (to 1 mM) were added, and the mixture was incubated for 1 h at 16°C to seal the remaining nicks. After a second heat inactivation, the DNA was purified by passage over two successive Sephadex G-50 columns. To create the gap, the DNA containing the deletion was digested with the appropriate restriction endonuclease (Fig. 1) at the deletion site. The products gave defined bands without evidence of degradation or nicking after analysis by alkaline agarose gel electrophoresis and staining with ethidium bromide. Siliconized reaction tubes were used exclusively in the procedures involving digoxigenin-labeled DNA.

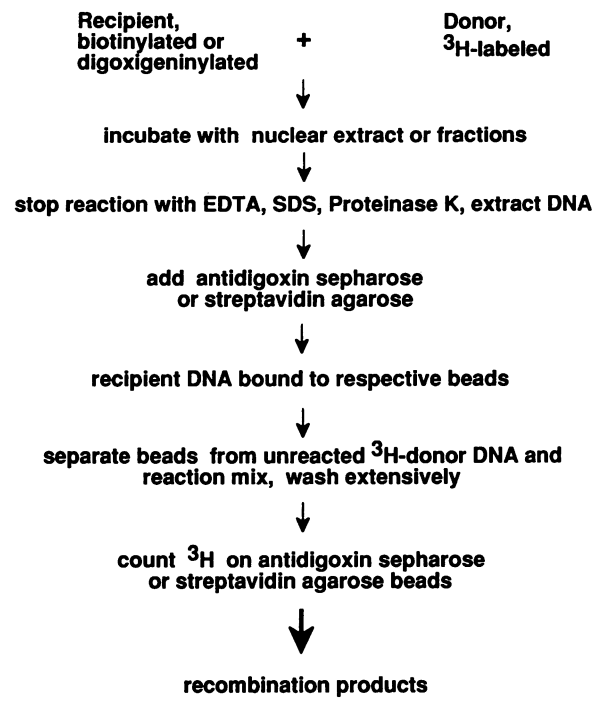

FIG. 2. DNA strand transfer assay scheme. For details, see Materials and Methods.

Labeling of donor DNA. A single colony of the plasmid-harboring strain (AB2487; *recA13 thyA16 deoB11*; obtained through B. Bachmann, Yale University, New Haven, Conn.) was inoculated into a minimal medium containing (per liter) 13.6 g of KH_2PO_4, 3.3 g of $(NH_4)SO_4$, 2.4 g of NaOH, 0.4 g of $MgSO_4 \cdot 7H_2O$, 0.011 g of $CaCl_2 \cdot 2H_2O$, trace $FeSO_4 \cdot 7H_2O$, 10.0 g of glucose, 2.5 g of Difco Casamino Acids, and 0.01 g of thiamine. Labeling of the plasmid was done by adding 1 mCi of [^3H]thymidine (5 mCi/mmol; Amersham Corp.) per 40 ml of medium and incubation for 16 h at 37°C with strong shaking. The plasmid DNA was isolated from the harvested cells and purified by standard methods involving either cesium chloride gradient centrifugation or minipreparation procedures that included exclusion chromatography on Sephadex G-50 columns. In either case, the DNA was free of RNA and salt. The specific activity was between 1×10^6 and 5×10^7 cpm per μg of DNA.

Preparation of affinity beads. Antidigoxin Sepharose beads prepared by coupling affinity-purified antidigoxin antibodies to CNBr-activated Sepharose (Sigma) according to standard protocols. The antibodies were obtained from the Dig 26-115 hybridoma cell line (18), which was a gift from J. Abrams, DNAX Corp., Palo Alto, Calif. The antibodies were purified from tissue culture supernatants by centrifugation at 5,000 × *g* for 15 min, precipitation with ammonium sulfate at 0.55 saturation, and subsequent affinity chromatography on an ouabain-bovine serum albumin Sepharose column. About 1 mg of antidigoxin antibody was coupled to a 0.2-ml packed gel of CNBr-activated Sepharose. In some experiments, glutaraldehyde-activated polyacrylamide beads (P6; Bio-

TABLE 1. Transfection assay for recombination[a]

Line	DNA substrate(s)	No. Kan^r	Kan^r/Amp^r	Comments
1	Δ70X/*Xho*I + M13mp10*neo*1.3	118	0.20	Complete reaction
2	Δ70X/*Xho*I	0	0	Recipient only
3	Δ70X/*Xho*I	0	0	Recipient and no extract
4	M13mp10*neo*1.3	0	0	Donor only
5	Δ70X/*Xho*I + M13mp10*neo*1.3	0	0	Separately incubated and pooled for transfection
6	Δ70X/*Xho*I + M13mp10*neo*1.3	2	<0.01	No incubation
7	Δ70X/*Xho*I + M13mp10*neo*1.3	6	0.01	No extract
8	Δ70X/*Xho*I + M13mp10*neo*1.3	3	0.01	Heat-inactivated extract
9	Δ70X/*Xho*I + M13mp10*neo*1.3	36	0.06	−ATP, −creatine phosphate
10	Δ70X/*Xho*I + M13mp10*neo*1.3	15	0.03	−4 dNTPs
11	Δ70X/*Xho*I + M13mp10*neo*1.3	0	0	+50 mM EDTA
12	Δ248/*Nar*I + M13mp10*neo*1.3	96	0.18	Complete reaction
13	Δ248 + M13mp10*neo*1.3	87	ND[b]	Uncleaved recipient
14	Δ390/*Not*I + pIC20R*neo*1.0	84	ND	Complete reaction
15	Δ390 + pIC20R*neo*1.0	67	ND	Uncleaved recipient

[a] The transfection assay was performed by using fraction I as described in Material and Methods. Where the plasmid designation is followed by a restriction enzyme abbreviation, the plasmid was cleaved within the deletion site. The numbers of Kan^r colonies and the ratio of Kan^r to Amp^r colonies per microgram of protein were normalized according to the number of colonies containing the *lac* operator-promoter plasmid. Eight different types of control experiments were performed. Omission of ATP and creatine phosphate or, more strikingly, the four dNTPs resulted in a significant decrease in activity.

[b] ND, Not determined. The donor or recipient DNA was uncut, and therefore the number of Amp^r clones was so high as to make the ratio meaningless.

Rad) were used instead. Antidigoxigenin antibodies supplied by Boehringer Mannheim were also used, with little difference in outcome. Streptavidin agarose (1.2 mg of streptavidin per ml of packed gel) was purchased from Sigma. The quality of the affinity beads has been checked regularly in binding experiments with either biotinylated ^{3}H-pSV2*neo* or digoxigeninylated ^{3}H-pSV2*neo*. With use of the amounts and conditions described below, between 70 and 80% of the labeled DNAs is bound to the beads.

Assay for recombination. A mixture of 0.01 to 0.1 μg of recipient and donor DNAs was incubated with fractions I to V as described for the transfection assay. The reaction was terminated after 30 min by the addition of EDTA to 25 mM (Fig. 2). The terminated reaction mixture was incubated with 20 μg of proteinase K and SDS to 0.5% for 1 h at 37°C, after which the DNA was extracted twice with phenol-chloroform (1:1 if biotinylated recipients were used and 1:4 if digoxigeninylated recipients were used) and once with chloroform. The essentially protein free DNA solution obtained after ethanol precipitation and solution in TE (10 μM Tris hydrochloride [pH 7.4], 1 μM EDTA) was incubated with either 10 μl of the streptavidin agarose (diluted 1:4 with PBS) or 5 μl of the antidigoxin Sepharose with gentle rotation at room temperature for 30 to 60 min. The beads were separated from the reaction mixture by passage through a small amount of siliconized glass wool in 1-ml pipette tips and extensive washing with about 100 volumes of buffer W containing 0.3 M NaCl, 0.01% SDS, and TE. The beads were pulled out of the pipette tip and counted in a scintillation counter. The radioactivity bound to the washed beads is expressed as the percentage of the total input radioactivity (in counts per minute) per microgram of extract protein. When the recipient DNA was omitted or blank beads were used, radioactivity was barely detectable (<100 cpm). The wash solution containing unbound radioactive DNA and the DNA bound to the beads were counted separately in a scintillation counter.

Analysis of bead-bound DNA by PCR. After binding of the purified DNA to the affinity beads (see above), the beads were washed twice in the reaction tube with 10 volumes of wash buffer W and three times with TE. The beads were dialyzed by placing them separately on 0.025-μm-pore-size VSWP filters (Millipore Corp.) floating on the surface of 50 ml of TE. After 1 h, the beads were used for PCR analysis (41, 42). The reaction mixtures contained 10 mM Tris hydrochloride (pH 8.1), 1 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 150 μM each dNTP, 1.0 μM each oligonucleotide primer, 0.5 U of *Taq*I polymerase (Perkin Elmer Cetus), and the bead-bound DNA. After 35 cycles in a Perkin Elmer Cetus thermocycle apparatus (2 min at 95°C, 1 min at 56°C, and 1.5 min at 72°C), the products were analyzed by electrophoresis in a 0.7% agarose gel, staining with ethidium bromide, and UV illumination.

RESULTS

Our initial assays for the repair of gaps and deletions in the recipient DNA *neo* sequence relied on the restoration of *neo* function, specifically the ability to confer kanamycin resistance after transfection into *E. coli*. The gapped DNAs (i.e., plasmids Δ70X, Δ248, and Δ390 linearized at the deletion site) were incubated with either of the two donor DNAs (M13mp10*neo*1.3 or pIC20R*neo*1.0 as circular double-stranded DNAs) and the nuclear extract (fraction I). After termination of the reaction, the DNA was extracted, purified, and transfected into a *recA* mutant strain of *E. coli* as described in Materials and Methods. The results with different combinations of recipient and donor DNA and various conditions are summarized in Table 1.

In the complete reaction (line 1), about 20% of the Amp^r transformants were also Kan^r. Incubation of the recipient or donor DNA alone (lines 2 to 4) failed to yield Kan^r transformants. Moreover, incubation of the recipient and donor DNAs separately and transfection with the pooled DNAs did not produce Kan^r transformants (line 5). This finding indicates that restoration of the *neo* gene requires the simultaneous presence of the recipient and donor DNAs during the incubation with fraction I. Elimination of the incubation (line 6) or the extract (line 7) or the use of heat-inactivated extract (line 8) caused a reduction in the yield of Kan^r colonies to less than 5% of that of the complete system. Removal of ATP and creatine phosphate (line 9) or of the four dNTPs (line 10) reduced the yield of Kan^r transformants to about 30

and 10%, respectively, of that of the complete system. The addition of EDTA (line 11) completely blocked the reaction, indicating a role for a divalent cation. Surprisingly, the uncleaved recipient substrates (lines 13 and 15) were almost as efficiently repaired as those which had ends within the deletion (lines 12 and 14). These data indicate that the extract contains an activity which is able to repair the deleted *neo* gene when supplied with a duplex DNA containing the deleted sequences. With deletions ranging from 70 to 390 bp, repair is nearly as efficient as with the corresponding gapped structures.

An in vitro recombination assay based on DNA transfer. Although useful, the bacterial transfection assay has several limitations. First, the time needed to detect bacterial transformation by the recombined gene is too slow for routine analysis or for enzyme purification. Second, the products of the recombination are not examined directly, and there is considerable uncertainty about the contribution made by *E. coli* to the recombination process, even if a number of controls have been done.

In almost every intermolecular recombination reaction, DNA strands are transferred from one DNA duplex to the other. Accordingly, we have developed an alternate assay which measures the amount of transfer of radioactive labeled DNA from the donor to the recipient molecule. To measure the amount of DNA strand transfer, a reliable, specific, and rapid separation of the two reacting DNAs is essential. This can be achieved by modifying one of the two DNA partners in the recombination in such a way that it can be separated from the other. For this purpose, the recipient DNA molecules were labeled with a low level of nucleotides bearing either biotin or digoxigenin substituents. Such labeled molecules are readily bound to streptavidin agarose or to antidigoxin monoclonal antibodies linked to Sepharose beads. Under the same conditions, unlabeled DNA remains unbound. Thus, after binding and extensive washing, the biotinylated or digoxigeninylated DNAs can be separated from the [^{3}H]DNA and their ^{3}H labeling can be determined (see Materials and Methods). Products and intermediates containing the recipient DNA are recovered from the beads, and their structures are analyzed.

Figure 2 depicts how the selective binding of the biotinylated or digoxigeninylated recipient DNA to streptavidin Sepharose or antidigoxin Sepharose beads is used to assay the recombination reaction. To stop the reaction, the mixture is treated with EDTA, SDS, and proteinase K, and the extracted DNA is bound to the streptavidin or antidigoxin beads. Any of the ^{3}H label transferred to the recipient DNA is also bound to the beads. A critical requirement for this assay is that the amount of bound labeled donor DNA be lower than the experimental value. With use of the procedure and washing conditions described in Materials and Methods, the amount of donor DNA bound to the antidigoxin beads was very low (Table 2, lines 4 to 12, 15, 16, and 29 to 31). The binding of labeled donor DNA with streptavidin agarose was somewhat higher but still usable.

Various fractions obtained by cation-exchange chromatography of fraction I were used to compare the transfection assay for repair of the *neo* sequence with the DNA transfer measurements (Fig. 3). Clearly, in the fractions obtained by stepwise elutions with increasing NaCl concentrations, the bulk of activity measured in both assays eluted with 500 mM salt. The 300, 500, and 700 mM salt fractions were also assayed in the DNA transfer reaction with ^{3}H-labeled λ DNA as the donor, and it is apparent that the 500 mM salt fraction did not promote DNA transfer from a nonhomologous donor.

Table 2 presents the results of a series of experiments with Δ390/*Not*I(dig) and Δ70X/*Xho*I(dig) recipient DNAs, respectively. These experiments included the use of different enzyme fractions (see Materials and Methods), a variety of donor DNAs, and variations in the conditions of the reaction. Lines 1, 13, 20, and 27 show the extent of ^{3}H transfer from the donor DNA to the gapped digoxigeninylated recipient DNA catalyzed by the complete system with fractions I, II, and V, respectively. When the enzyme fraction was omitted (lines 11 and 30) or heat-inactivated extract was used (lines 12 and 31), the ^{3}H transfer was very low. Lines 18 and 26 indicate that the uncleaved recipient with the 390-bp deletion acquired nearly as much ^{3}H label as did the cleaved recipient. Additionally, if the donor DNA lacked any homology with the recipient (lines 15 and 29), there was little or no transfer of ^{3}H label. Donor DNA that lacked *neo* sequences but whose vector backbone was homologous to the vector sequence in the recipient DNA failed to transfer ^{3}H label with fraction I (line 4) and did so poorly with fraction V (line 23). Similarly, donor DNAs whose *neo* sequences had deletions that overlap those of the recipient DNA (lines 7 to 10) were ineffective in transferring DNA to the recipient. Donors that shared only the *neo* sequences with the gapped recipient (lines 14 and 28) were about 10 to 15% as effective as those with complete homology. Note the lack of ^{3}H transfer when the recipient lacked the digoxigenin substituents (line 16). Linear donors with intact *neo* sequences and with ends homologous to sequences in the recipients (lines 2, 3, 21, and 22) were only somewhat less effective than the circular forms in transferring their ^{3}H label. For example, the donor plasmid linearized at the *Eco*RI site, which is outside the *neo* gene (Fig. 1), was about as effective as the circular donor (lines 2 and 21). Interestingly, linearization of the pSV2*neo* donor just at the site corresponding to the gap in the recipient (lines 3 and 22) caused a 50% decrease in the DNA transfer. Linearization of the recipient far from the deletion in the *neo* gene (line 19) lowered the DNA transfer to about 40% that of the circular recipient DNA. No significant difference was found between the Δ390 (lines 1 to 26) and Δ70X (lines 27 to 35) recipients. However, detailed comparative studies on different DNA substrates and different forms of these substrates are under way. When the donor DNA lacked *neo* but shared backbone sequences (lines 5, 6, 24, and 25), the strand transfer reaction was distinctly impaired. With the most purified fraction (fraction V), omission of ATP reduced the DNA transfer activity to about a third (line 33), and omission of the four dNTPs (lines 32 and 34) reduced the transfer even more. Inclusion of EDTA in the reaction (line 17) as well as omission of Mg^{2+} (line 35) markedly lowered the transfer of ^{3}H label to the recipient.

Partial purification of the recombination activity. Using the DNA transfer assay, we undertook the purification of the nuclear extract. The various steps in the purification procedure are detailed in Materials and Methods. A more extensive description of the biochemical characteristics and of the purified fractions will be presented elsewhere. The 500 mM salt fraction obtained by chromatography of the nuclear extract on a BioRex70 column contained the bulk of the activity for repairing the gap in the *neo* sequence and for DNA transfer (Fig. 3). This resulted in a purification of about ninefold compared with the nuclear extract. Further fractionation was performed by chromatography of fraction II on a DEAE column, using step elution with 60, 300, and 600

TABLE 2. DNA transfer assay for recombination

Recipient	Line	Donor	Fraction	Comment	% cpm/μg of protein
Δ390/*Not*I (dig)	1	pSV2*neo*	I	Complete system	1.3
	2	pSV2*neo*/*Eco*RI	I	Linear donor	1.0
	3	pSV2*neo*/*Hin*dIII	I	Linear donor[a]	0.7
	4	pSV2*gpt*	I	Donor lacks *neo*	0.0
	5	pSV2*gpt*/*Eco*RI	I	Donor lacks *neo*, linear	0.1
	6	pSV2*gpt*/*Hin*dIII	I	Donor lacks *neo*, linear[a]	0.1
	7	Δ248	I	Δ mutant as donor	0.2
	8	Δ248/*Eco*RI	I	Δ mutant as donor, linear	0.2
	9	Δ248/*Nar*I	I	Δ mutant as donor, linear[b]	0.2
	10	Δ70X/*Xho*I	I	Δ mutant as donor, linear[b]	0.1
	11	pSV2*neo*		No extract	0.3
	12	pSV2*neo*	I	Extract heat inactivated	0.1
	13	pSV2*neo*	II	Complete system	8.8
	14	M13mp10*neo*1.3	II	15% homology with recipient	1.0
	15	λ	II	No homology with recipient	0.5
	16	M13mp10*neo*1.3	II	Recipient lacks digoxigenin	0.2
	17	pSV2*neo*	II	+5 mM EDTA	1.5
	18	pSV2*neo*	II	Uncleaved recipient	8.1
	19	pSV2*neo*	II	Recipient cut at *Eco*RI	2.9
	20	pSV2*neo*	V	Complete system	32.7
	21	pSV2*neo*/*Eco*RI	V	Linear donor	33.6
	22	pSV2*neo*/*Hin*dIII	V	Linear donor[a]	18.5
	23	pSV2*gpt*	V	Donor lacks *neo*	13.4
	24	pSV2*gpt*/*Eco*RI	V	Donor lacks *neo*, linear	5.8
	25	pSV2*gpt*/*Hin*dIII	V	Donor lacks *neo*, linear[a]	5.2
	26	pSV2*neo*	V	Uncleaved recipient	24.2
Δ70X/*Xho*I (dig)	27	pSV2*neo*	V	Complete system	34.8
	28	M13mp10*neo*1.3	V	15% homology with recipient	4.8
	29	M13mp10	V	No homology with recipient	0.4
	30	pSV2*neo*		No extract	0.2
	31	pSV2*neo*	V	Extract heat inactivated	0.7
	32	pSV2*neo*	V	−dNTP	7.5
	33	pSV2*neo*	V	−ATP, −PCK, −CP[c]	21.2
	34	pSV2*neo*	V	−dNTP, −ATP, −PCK, −CP	4.6
	35	pSV2*neo*	V	+10 mM EDTA, $-MgCl_2$[d]	0.9

[a] Donor cleaved at the site of deletion in the recipient.
[b] Deletion mutant cleaved at the site of deletion and used as donor.
[c] PCK, Phosphocreatine kinase; CP, creatine phosphate.
[d] Extract dialyzed against a $MgCl_2$-deficient buffer.

mM salt. The bulk of the activity appeared in the 600 mM peak fraction (fraction III). This fraction was enriched about 15-fold compared with the nuclear extract and was almost free of nucleic acids, judging from the ratio of A_{260} to A_{280} (55). After ammonium sulfate precipitation (55%) of fraction III, the recovered protein was dissolved in E200 (fraction IV); it had about the same specific activity as fraction III but was more concentrated. Fraction IV was then filtered through a Superose 6 FPLC column and eluted with E200 buffer. Figure 4 shows the elution profile of the protein and activities measured by the DNA transfer and transfection assays. Both assays revealed a major and a minor peak of activity; the major peak fraction occurred in the high-molecular-mass range at about 600 kDa, and the minor peak appeared at about 60 kDa. The discordance of the two major peaks may reflect the fact that the DNA transfer assay scores all reactions that result in ^3H-strand transfer to the recipient DNA, whereas the transfection assay measures those products that can confer kanamycin resistance after transfection into *E. coli*. The peak fraction in the 600-kDa range showed about 20 to 25 protein bands on a silver-stained SDS-polyacrylamide gel (data not shown). Our present indications are that a multiprotein complex is responsible for the activities being measured. Considering the multiplicity of reactions that are necessary for the recombination that we observe, one might not be surprised by this finding. The nature of the complex and its substituents are currently under study and will be reported elsewhere.

Verification of gap and deletion repair by PCR. Both the transfection and DNA transfer assays with various enzyme fractions indicate that gaps and deletions in the *neo* sequence are repaired by an intact *neo* gene in the donor DNAs. This conclusion has been verified by using PCR to examine the products made with fractions I, II, and V. PCR was carried out by using the streptavidin or antidigoxin Sepharose beads to which the recipient DNAs containing either biotin or digoxigenin substituents, respectively, were bound. Such DNAs are readily amplified in our standard PCR protocol (see Materials and Methods). Figure 5A shows the organization of the Δ70X and Δ248 recipient DNAs and the sites of the gaps and deletions in the *neo* gene. The structure of the M13mp10*neo*1.3 donor DNA, which includes the *neo* sequences missing from the two recipient DNAs, is also shown. The short arrows indicate the positions and the 5′-to-3′ directionality of the oligonucleotides used to prime the PCR. Note that one of the primers (II) anneals to the sequence missing from the two recipient DNAs; thus, under the conditions of the PCR, only the repaired product, pSV2*neo*, yields a 720-bp amplified fragment.

The electrophoretic patterns shown in Fig. 5B, lanes A to

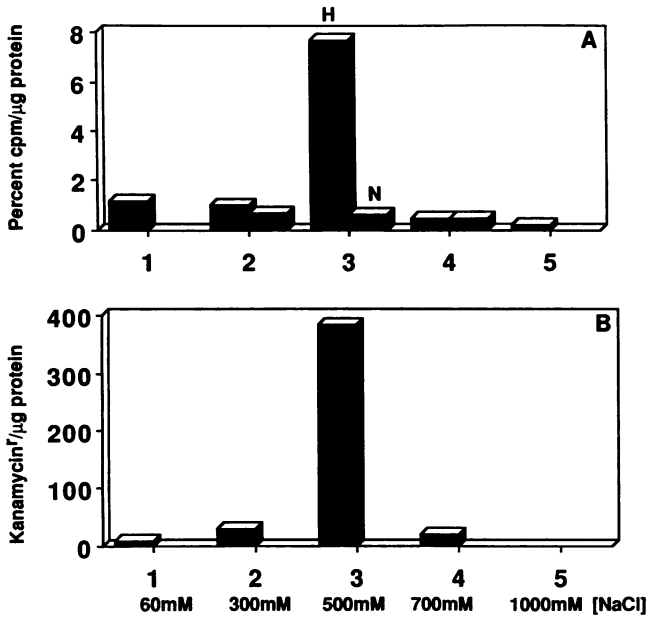

FIG. 3. Transfection and DNA transfer assays of cation-exchange column chromatographic fractions. In the DNA transfer assay (A), the recipient DNA was digoxigeninylated Δ70X, cleaved at the *Xho*I site, and the donor was ^{3}H-pSV2*neo* (H). The two DNAs were incubated with each of the five fractions eluted in steps from a BioRex70 column with increasing amounts of NaCl. In some experiments the donor was the nonhomologous λ DNA (N). The transfection assay (B) was performed as described in the text, using Δ70X/*Xho*I and M13mp10*neo*1.3 as DNA substrates.

V, were obtained after 35 cycles of PCR with the products made in a variety of recombination reactions. The expected 720-bp fragment was produced when authentic pSV2*neo* was used as the template (lane D). The same band was formed with the product of the reaction made in the complete system with either *Nar*I-cleaved Δ248 (Δ248/*Nar*I) DNA (lane E) or Δ70X/*Xho*I DNA (lanes L, P, and V) as the recipient and M13mp10*neo*1.3 as the donor. That band was not detected if λ phage DNA (lane K) or M13mp10 double-stranded DNA (lane H) was used as the donor, if inactivated extract was used (lane O), or if the *neo* donor was omitted (lane Q). Also, if the Δ248/*Nar*I recipient and the M13mp10*neo*1.3 donor DNAs were incubated separately and their pooled contents were carried through the whole assay procedure, no 720-bp PCR fragment was formed (lane F). The 720-bp fragment diagnostic of the intact *neo* sequence was not produced when 100 ng each of Δ248/*Nar*I and M13mp10*neo*1.3 DNAs or 100 ng of only one of the DNAs was applied directly to PCR without prior incubation in the recombination reaction (lanes A to C). Also, when PCR was performed with the products from incubations with donors that lack the *neo* sequence, such as pSV2*gpt* (line I), or without the recipient DNA (lane M), no 720-bp fragment was formed. The finding of the 720-bp PCR fragment among the products of the reaction with the uncleaved deletion recipients, Δ70X (lanes N, S, and U) and Δ248 (lanes G and T), affirms the finding indicated earlier (Table 2) that deletions are repaired during incubation with the donor. These data show clearly that the *neo* sequences of at least some of the recipient DNAs bound to the Sepharose beads are fully repaired on both strands during the recombination reaction in vitro. All enzyme preparations, fractions I to V, were demonstrated to catalyze this reaction.

The oligonucleotide primers described above were selected to determine whether the sequences lacking from the recipient DNA *neo* gene are restored during the incubation. PCR analysis with different sets of oligonucleotide primers revealed that there were also other recombination products formed in the reaction. Figure 6 shows the locations of the alternate PCR primers, their binding locations on several recombinant structures that could be formed during the

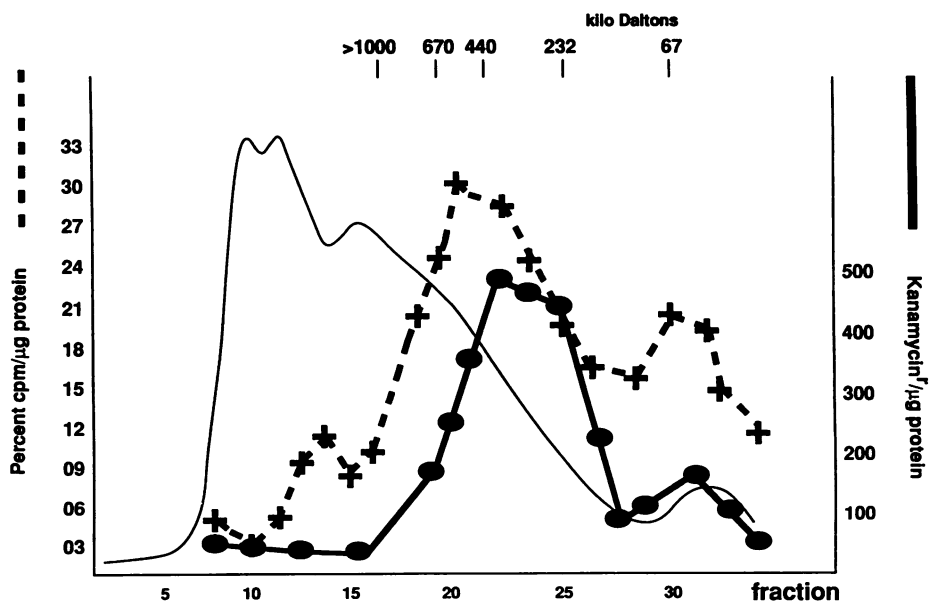

FIG. 4. Gel filtration column chromatography. Shown is an elution profile of fraction IV, loaded on a Superose 6 FPLC column, that had been equilibrated with E200. Elution of proteins was measured by the A_{280}. Fractions were assayed by the transfection and the DNA transfer assays.

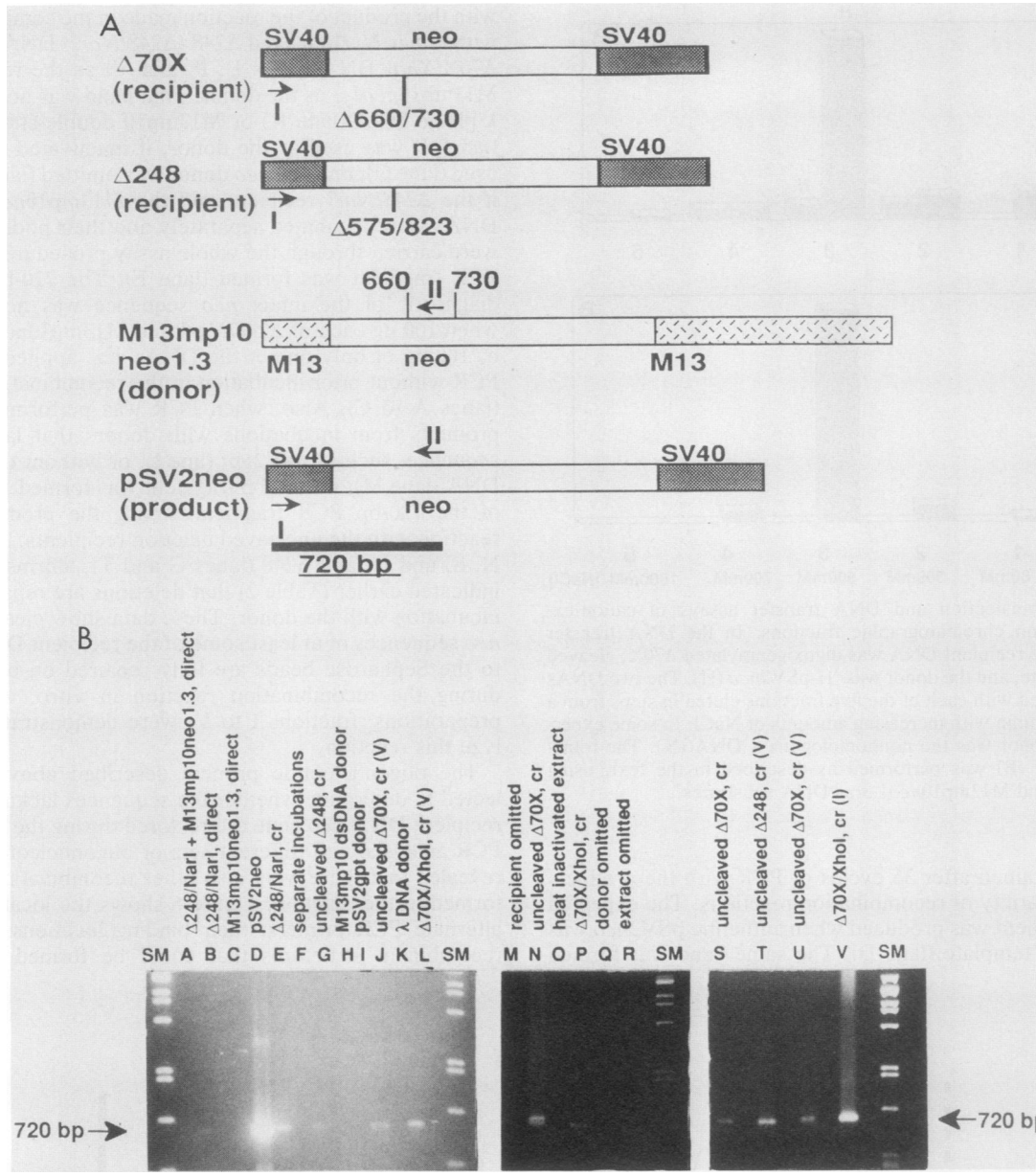

FIG. 5. PCR analysis of bead-bound DNAs. (A) Locations of primer pairs used for the PCR analysis on the bead-bound DNA molecules generated in the recombination experiments. Further details are provided in the text. (B) Gel electrophoretic patterns of ethidium bromide-stained PCR-amplified fragments. Unless otherwise noted, fraction II was used in the recombination reactions. cr, Complete reaction; ds DNA, double-stranded DNA. Lane A contained 100 ng each of Δ248/*Nar*I and M13mp10*neo*1.3, lane B contained 100 ng of Δ248/*Nar*I, and lane C contained 100 ng of M13mp10*neo*1.3. These DNAs were subjected to PCR without incubation in the recombination reaction. In all of the following experiments, the biotinylated recipient molecules were bound to the streptavidin beads after the incubation. Lanes: D, pSV2*neo*; E, Δ248/*Nar*I plus M13mp10*neo*1.3; F, as lane E, but DNAs were separately incubated and pooled before binding to the beads; G, Δ248 plus M13mp10*neo*1.3; H, Δ248/*Nar*I plus M13mp10; I, Δ248/*Nar*I plus pSV2*gpt*; J, Δ70X plus M13mp10*neo*1.3; K, Δ248/*Nar*I plus λ; L, Δ70X/*Xho*I plus M13mp10*neo*1.3 with fraction V; M, M13mp10*neo*1.3 only; N, Δ70X plus M13mp10*neo*1.3; O, Δ248/*Nar*I plus M13mp10*neo*1.3, incubated in heat-inactivated extract; P, Δ70X/*Xho*I plus M13mp10*neo*1.3; Q, Δ248/*Nar*I only; R, Δ248/*Nar*I plus M13mp10*neo*1.3, incubated without extract; S, Δ70X plus M13mp10*neo*1.3; T, Δ248/*Nar*I plus M13mp10*neo*1.3, incubated in fraction V; U, Δ70X + M13mp10*neo*1.3, incubated in fraction V; V, Δ70X/*Xho*I plus M13mp10*neo*1.3, incubated in fraction I; SM, size marker λ/*Pst*I.

incubations, and the fragment sizes that would be produced from a particular set of primers and the structures shown. These are summarized in Table 3 along with the fragments actually detected after PCR. Thus, as noted earlier, PCR with primers I and II yielded a 720-bp fragment diagnostic of the recipient DNA's fully repaired *neo* gene. Primers I and III permitted the amplification of an 1,875-bp fragment from the repaired recipient and a 1,627-bp fragment from the unrepaired Δ248 *neo* gene. Using oligonucleotide primer pair IV-V, V-VI, or II-V, each of which permits amplification of specific regions of the M13mp10*neo* DNA, allowed the structure of the donor molecule *neo* gene to be monitored. In

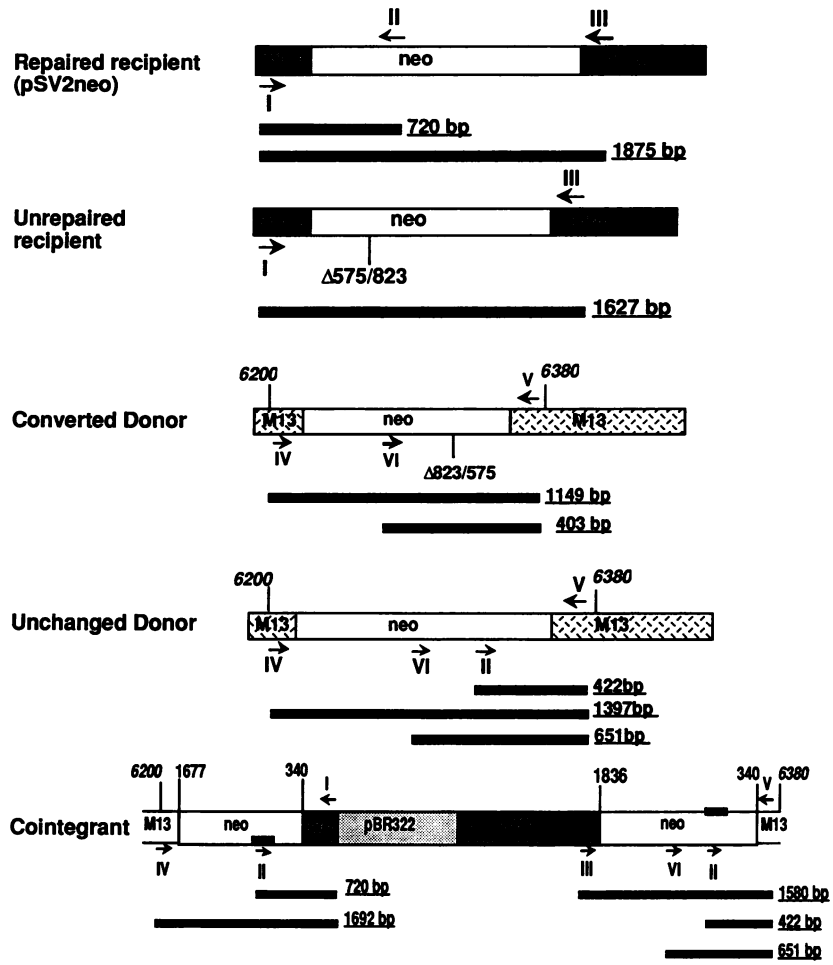

FIG. 6. PCR analysis of different recombination products. Bead-bound recombination products were used as described in Materials and Methods. The base pair numbering in roman type refers to numbers of pSV2*neo* (45); numbers in italics are for M13mp10. SV40, Simian virus 40.

this case, the donor molecules were labeled with biotin, and the PCR analysis was carried out with material fixed to the streptavidin agarose beads. The unaltered donor *neo* sequence would be expected to yield characteristic fragments: with primers IV and V, 1,397 bp; with primers V and VI, 651 bp; and with primers II and V, 403 bp. If the donor *neo* gene acquires the deletion originally present in the recipient DNA *neo* sequence, primers IV and V will amplify an 1,149-bp fragment and primers V and VI will amplify a 403-bp fragment. Each of these fragments was detected among the

TABLE 3. Recombination products in the repair of Δ248 detected by PCR[a]

Oligonucleotide primer pair	Expected size of fragment (bp)						Observed fragment (bp)
	Repaired recipient	Unrepaired recipient	Converted donor	Unchanged donor	Repaired integrant	Unrepaired integrant	
I + II	720				720		720
I + III	1,875	1,627					1,880 + 1,620
III + IV							None
III + V					1,580	1,332	1,580 + 1,330
I + V							None
I + IV					1,692	1,444	1,690 + 1,440[b]
II + V				422	422		420
IV + V			1,149	1,397			1,390 + 1,150[c]
V + VI			403	651	651	403	650 + 400[c]

[a] The locations to which various primer pairs anneal and the sizes of the PCR fragments that could be produced are shown in Fig. 6.
[b] Detected as a weak band only; generated from an unrepaired *neo* sequence within the cointegrant molecule.
[c] Clearly less abundant when the Δ248 DNA was cleaved at the deletion site.

PCR products primed with the indicated oligonucleotides. These fragments were most abundant in cases in which the uncleaved recipient DNA had been applied. This result shows that the partially purified fraction can promote a gene conversion which transfers the 248-bp deletion from the mutant *neo* sequence to the previously intact *neo* sequence of the donor.

Perhaps the most surprising finding is evidence that cointegrant molecules containing sequences from both the donor and recipient DNAs are formed (Fig. 6). The diagnostic fragments favoring such a cointegrant structure are the fragments produced in the PCR with primer pairs I-IV and III-V. Thus, primer I and III pair with sequences unique to the recipient DNA, and primers IV and V anneal only to sequences present in the donor DNA. The bottom of Figure 6 indicates the fragment sizes expected by the PCR amplification from a cointegrant DNA.

Table 3 summarizes the fragment sizes formed with each of these primer pairs. The possible origin of such molecules is addressed in Discussion; briefly, they are the presumptive products that arise by crossovers within the paired structures involved in the repair of gaps and deletions (52). Of the possible cointegrants, the one in which both *neo* genes are intact was the most prevalent. These findings indicate that our enzyme fractions catalyze a variety of recombination reactions and therefore provide a good starting source for identifying the responsible enzymatic activities.

DISCUSSION

Considerable progress has been made in recent years to detect and characterize homologous recombination events in mammalian cells (for reviews, see 24 and 50). Much of what we know derives from the studies of the recombination products that are formed in vivo from specially designed transfected DNAs (5, 19, 20, 24, 27, 28, 50). Only recently, however, have some of the models that have been proposed to explain the mechanisms of reciprocal and nonreciprocal recombinations been explored in vitro (25). One approach has been to focus on the presumptive initiating event in recombination. Thus, eucaryotic proteins, somehow analogous to procaryotic *recA*, which promote strand displacement in duplex DNA by homologous single strands (8, 43) have been described (10, 12, 14, 17, 31, 34).

But the more complex challenge has been to devise assays that measure the overall process, that is, the formation of relatively rare recombination products. One successful solution is to rely on bacterial transformation to assay the in vitro products for recombination that result in the formation of selectable markers, for example, drug resistance genes (for a review, see reference 25). Although recombination-deficient bacteria (e.g., *recA*) are used as test organisms, it is difficult to rule out the possibility that events initiated in vitro are completed by bacterial enzymes in vivo. To avoid this complexity, some studies in yeast recombination have relied on Southern blotting techniques to monitor the formation of homologous recombinants in vitro (51). However, the sensitivity of this approach is limiting, especially for use with mammalian systems. Furthermore, both the transfection assay and blotting assays are time consuming and do not lend themselves to the needs of enzyme fractionation.

This report describes an in vitro system that catalyzes the repair of deletions or gaps in duplex DNA. To monitor these reactions, we used an assay that monitors the repair by measurements of DNA strand transfer from an intact, homologous DNA sequence (the donor) to the gapped or deleted DNA (the recipient). Such a transfer is a predicted outcome of the DSBR model proposed by Szostak et al. (52) and of other recombination models as well (27, 28, 35, 39). Simple gap repair using intact DNA as a donor leads to the transfer of a strand at least as long as the gap, but probably longer because of branch migration. If crossovers occur during the repair process, cointegrant molecules are formed and strand transfer is more extensive.

The DNA transfer assay relies on the rapid and efficient separation of an isotopically labeled intact donor DNA from a ligand (biotin or digoxigenin)-labeled recipient DNA containing the gap or deletion. The separation is achieved by using affinity beads to bind the ligand-labeled DNA and to remove other components in the reaction. DNA strand transfer is monitored by the amount of radioactivity associated with the affinity beads. DNA lacking biotin or digoxigenin substituents binds very poorly to the corresponding beads, whereas more than 80% of the DNA containing either ligand is retained by the corresponding affinity beads. Comparisons of the bacterial transfection results with those obtained by using the DNA transfer assay with enzyme fractions at various stages of purification give comparable estimates for the repair of the altered *neo* genes. This finding suggests that the two assays are measuring a common feature of the overall reaction or possibly common products of the in vitro reaction. The DNA transfer assay is direct, easy, rapid, and reproducible (±15%) and therefore is well suited for monitoring enzyme purification and for surveying the reaction, the requirements, and kinetics. We anticipate that both intermediates and products bearing the ligands will be retained by the affinity beads and therefore be accessible to further analysis. This general approach appears likely to be useful for analyzing similar types of recombination processes and possibly a wide variety of other intermolecular interactions. Furthermore, preliminary experiments suggest that relatively stable, protein-dependent complexes can be adsorbed to the affinity beads if the SDS, proteinase K, and phenol-chloroform extractions are eliminated from the workup of the reaction mixtures.

Both the transfection and DNA transfer assays reveal an absolute dependence for the simultaneous incubation of the recipient and donor with the enzyme. Surprisingly, deletions ranging from 70 to 390 bp were repaired almost as efficiently (70 to 90%) as the gapped molecules generated by double-strand cleavage within the same deletions. Several models (27, 28, 35, 46, 52, 54) suggest that gaps are repaired more efficiently than deleted DNA because of the putative role of ends in initiating the process (5, 6, 35–37, 39, 46). Support for such a model has been provided by several studies (14, 25, 26, 30, 44), but there is also evidence (23), besides our own, that deletions are repaired as well as their gapped counterparts.

Besides the necessity for the simultaneous presence of both the recipient and donor DNAs and the enzyme fraction, the most notable requirement for recombinational repair is that the donor molecule's homology must overlap the ends of the gaps or deletions. Cleavages of the donor DNA far from the homology have little effect on the reaction, but cleavages within or near the donor's homology region severely impair the repair. There appears to be a clear requirement for a divalent cation but only little for ATP. The possibility of no ATP requirement has to be studied further, but it should be emphasized that some eucaryotic strand exchange proteins function in the absence of ATP (10, 17, 31). By contrast to the dispersibility of ATP, omission of the four dNTPs lowers the DNA strand transfer activity to about

20% of the complete system. This probably indicates a need for DNA synthesis, but the residual activity may reflect a novel recombination mechanism for the repair.

The bead-bound DNAs are evidently substrates for PCR. Presumably, the biotinylated DNA strands are retained on the beads, even after some denaturation (32), and are available as a template for *Taq*I polymerase. The use of such affinity beads and appropriately modified DNAs or primers provides a way to preselect DNA molecules from a mixture for PCR analysis and thereby lower the likelihood of nonspecific PCR products.

PCR analysis of the affinity bead-bound products establishes that both the gapped and deleted *neo* genes in the recipient DNAs are fully repaired in the course of the recombination reaction. Thus, with primers I and II or I and III (Fig. 6), the PCR-generated fragments serve to identify the presence of fully repaired *neo* sequences (Table 3). The diagnostic fragments of about 720 and 1,875 bp indicate that the 248-bp gap or deletion is restored during the reaction. Similar results were obtained in parallel experiments with the Δ70 and Δ390 gapped or deleted recipient DNAs, using fractions I to V (Fig. 5 and data not shown). However, these PCR analyses do not indicate how much of the bead-bound recipient DNA has been fully repaired.

Controls in which the enzyme fraction was omitted or inactivated, the donor or recipient DNA was omitted, or the two DNAs were incubated separately and then mixed prior to binding to the beads failed to generate these or other PCR fragments. No diagnostic PCR bands were detected with donors such as λ or M13mp10 (double-stranded DNA), neither of which contains a *neo* sequence. Moreover, there was no 720-bp fragment produced when pSV2*gpt*, which is homologous to the vector sequence of the recipient DNA, was used as a donor (Fig. 5). PCR analysis carried out directly on mixtures of more than 100 ng each of the recipient and donor DNAs did not yield the specific PCR fragments (Fig. 5). From this result we surmise that although theoretically possible, PCR-generated artifactual fragments were not formed. Furthermore, the likelihood of generating those false PCR fragments would have been greatly reduced by removal of the donor DNAs which are not bound to the beads used to recover the recombination products.

PCR analysis was also used to examine the structure of the donor DNA *neo* sequence following the recombination reaction. In these experiments, the biotin ligand was introduced into the donor DNA so that it was bound to the streptavidin beads. Using the primer pair IV-V or V-VI (Fig. 6), each of which anneals to only the donor DNA, we observed PCR products that are indicative of *neo* genes with a 248-bp deletion as well as those predicted for the unaltered *neo* sequence (Table 3). Here too, the controls of the type mentioned above did not yield any fragments.

One interesting feature of the DSBR model is that crossovers between the nonexchanged strands of the double Holliday recombination intermediate lead to the formation of cointegrant molecules (52). Indeed, dimers have been found previously among plasmids recovered from bacteria transfected with the products of an in vitro recombination reaction (25). However, we obtained direct evidence for cointegrant molecules by PCR analysis of recipient or donor DNA bound to the affinity beads. PCR carried out with primers III and V (Fig. 6) generates a fragment of about 1,580 bp and a lesser amount of one of about 1,332 bp (Table 3). With the primer pair I-IV, the predominant PCR product was about 1,690 bp, with a smaller amount of a 1,440-bp fragment (Table 3). All in all, these results strongly suggest that the recombinational processes catalyzed by our preparations can affect repair of double-strand breaks (gaps), gene conversions resulting in the removal or introduction of deletions, and the formation of cointegrant molecules.

How can the origin of the various recombinant products be explained? The model of Szostak et al. (52) can account for the repair of gaps at double-strand breaks as well as the formation of cointegrant molecules with such substrates. But their scheme does not explain several of our findings: (i) the only slightly lower efficiency of repairing deletions, (ii) the gene conversion leading to introduction of a deletion from the uncleaved recipient into the donor molecule, and (iii) the formation of cointegrants from the deletion recipient and the donor DNAs. Figure 7 suggests a way in which all of the observed products of the deletion reaction can be formed. The key feature of the proposal is the formation of paired four-stranded synaptic structures in the region of their

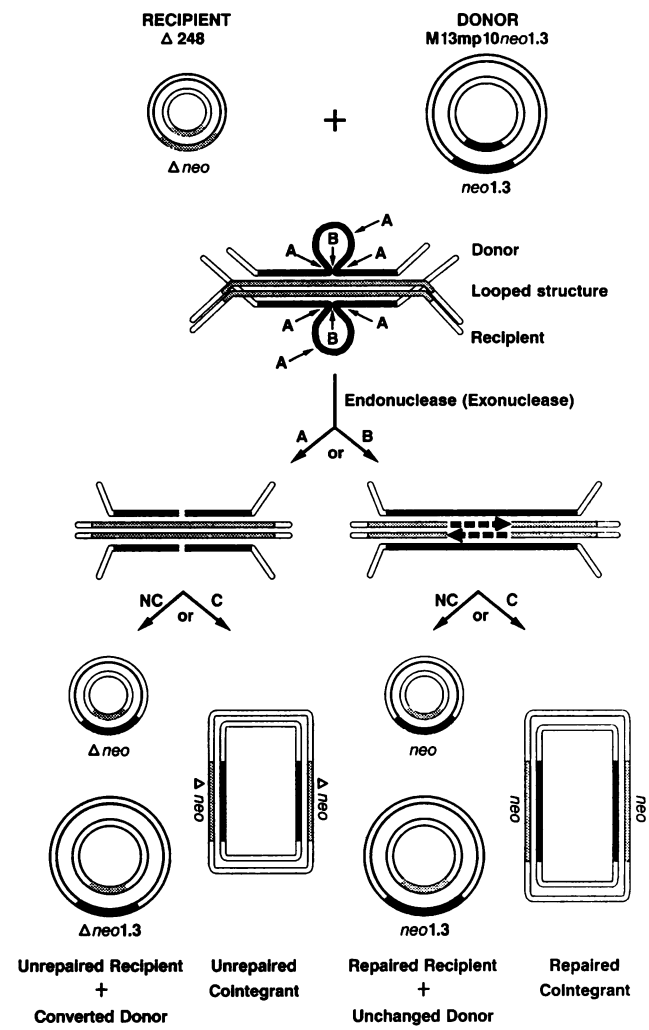

FIG. 7. Model for gap repair recombination with two circular DNA substrates. In this example, the recipient Δ248 and the donor M13mp10 *neo*1.3 DNAs were used. A and B stand for two alternative actions on the looped structure; in one, the endonucleolytic cleavage and possibly exonucleolytic degradation eliminates the single-strand loops, and in the other, the cleavages occur in the deleted DNA, allowing for repair of the gap. C and NC refer to the crossover and noncrossover outcomes, respectively.

shared homologies. Conversion of the donor could result from endonucleolytic (plus exonucleolytic) digestion of the single-strand loops, ligation of the nicked donor strands, and resolution of the paired structure. Alternatively, cleavage and digestion of the recipient strands opposite the loops by endonuclease and perhaps exonuclease action could lead to repair of the deletion, using the donor strands as a template. A double-strand crossover occurring in either of these putative recombination intermediates would lead to one of the two cointegrants. Further studies are needed to explore the validity of all or parts of this scheme.

Nuclear extracts from cultured human (HeLa and JM lymphocytes), monkey (COS1) and hamster (BHK-21) cells and fractions derived from them are equally proficient in using the donor DNA to repair various gapped or deleted forms of the *neo* gene. All experiments detailed here have been performed with both JM and COS1 cells. In addition, fractions I to III were obtained from HeLa and BHK-21 cells. Extracts from all four cell types have been used in the transfection assay and the DNA transfer assay, without any significant difference in their abilities to catalyze these reactions. Extracts from fetal calf thymus nuclei, and the corresponding fractions II to V, were also found to have the same activity as those from the cultured cells and should prove useful for large-scale purification. Cytoplasmic fractions alone are inactive, but whether they can complement the activity of nucleus-derived fractions needs further investigation. Judging from the position at which the active fractions eluted from the gel filtration column, we surmise that the recombination reactions are being catalyzed by a complex. Such a high-molecular-weight complex is likely to contain a duplex pairing activity, DNA polymerase, DNA ligase, and possibly endo- and exonuclease, topoisomerase, and helicase activities. But a biochemical characterization of this complex remains to be done.

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