MOLECULAR AND CELLULAR BIOLOGY, Sept. 1987, p. 3124–3130
0270-7306/87/093124-07$02.00/0

Vol. 7, No. 9

Characterization of an ATP-Dependent DNA Strand Transferase from Human Cells

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Received 2 March 1987/Accepted 15 June 1987

We have characterized an enzymatic activity from human cell nuclei which is capable of catalyzing strand exchange between homologous DNA sequences. The strand exchange activity was Mg^{2+} dependent and required ATP hydrolysis. In addition, it was capable of promoting reannealing of homologous DNA sequences and could form nucleoprotein networks in a fashion reminiscent of purified bacterial RecA protein. Using an in vitro recombination assay, we also showed that the strand exchange activity was biologically important. The factor(s) responsible for the activity has been partially purified.

A large series of experiments conducted over the last several years have conclusively demonstrated that somatic mammalian cells have all the enzymatic activities required to catalyze recombination between two homologous plasmid or viral molecules or between plasmid and homologous chromosomal sequences (see, for example, references 7, 8, 16, 28, 29, 33, and 35). Though much information about several aspects of this reaction is available, the precise nature of the enzymes that are needed for the initiation and completion of the reaction remains unelucidated.

Though several enzymatic activities can be postulated to be required for recombination, one enzymatic activity that is uniquely required for recombination is the DNA strand exchange or strand transfer activity. Such an activity has been purified from *Escherichia coli* (22, 26, 27) and the fungus *Ustilago maydis* (12–14). These two proteins have several common properties, including their dependence on and requirement for ATP for completion of the DNA transfer reaction. Much information about the properties of the bacterial RecA protein is available, and this protein is the paradigm for other strand transfer enzymes (for a review, see reference 24).

Bacterial RecA protein is capable of promoting a pairing reaction between single-stranded DNA (ssDNA) and homologous double-stranded DNA (dsDNA) in vitro by (i) polymerization of RecA on ssDNA, (ii) formation of presynaptic networks with dsDNA, which is independent of homology, (iii) search for homology, followed by the formation of a duplex between homologous sequences, and (iv) release of RecA accompanied by ATP hydrolysis.

With the long-term goal of a complete biochemical understanding of the recombination process in somatic mammalian cells, we have developed a cell-free system which is capable of catalyzing homologous recombination (17). Two deletion derivatives of the plasmid pSV2neo (31) were incubated with nuclear proteins isolated from human bladder carcinoma cell line EJ, and the DNA was then used to transform *recA E. coli*. Recombination was detected by the reconstruction of an intact *neo* gene. In these experiments we were able to show that recombination catalyzed by the extract occurred at high efficiency, double-strand breaks in the region of homology of one or both plasmid substrates increased recombination, and the reaction was homology dependent and required Mg^{2+}, ATP hydrolysis, and the presence of all four deoxynucleoside triphosphates (17, 30).

To understand the biochemical aspects of the homologous recombination reactions, we have begun to fractionate the human cell nuclear extracts by a number of biochemical methods. We used a number of assays to measure DNA strand transfer activity in our extracts and fractions derived from them. We now provide evidence for a high level of purification of a factor(s) which is capable of DNA strand exchange in an ATP-dependent manner. While this work was in progress, Hsieh et al. (10) reported partial purification of an enzymatic activity from a human B cell line which is ATP-independent and capable of partial strand exchange between homologous DNA molecules.

MATERIALS AND METHODS

Preparation of cell extracts. Human EJ cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. For each experiment approximately 2×10^8 to 6×10^8 cells were harvested, and the nuclear extract was prepared as described previously (17, 30). Cells were scraped from the petri dishes by using a rubber policeman and collected in 0.89% NaCl at 4°C. The cells were centrifuged at $300 \times g$ for 10 min, and the pellet was suspended in 80 ml of buffer 1 (20 mM Tris, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM dithiothreitol [DTT], and 200 mM sucrose). The pelleted cells were suspended in 24 ml of buffer 2A (20 mM Tris, pH 7.5, 5 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM DTT, and 1 mM phenylmethylsulfonyl fluoride [PMSF]). The cells were left on ice for 20 min and disrupted by homogenizing with a Dounce homogenizer (B-pestle, 60 strokes). Cell lysis and release of nuclei were monitored by phase-contrast microscopy, and the nuclei were pelleted by centrifugation at $1,200 \times g$. The supernatant was removed with a Pasteur pipette, and the nuclear pellet was suspended in 24 ml of buffer 2B (20 mM Tris, pH 7.5, 5 mM KCl, 500 mM NaCl, 10 mM EDTA, 0.5 mM DTT, and 1 mM PMSF). This solution was separated into four equal fractions of 6 ml each and sonicated on ice with a Fisher sonic dismembrator (model 300) equipped with a microtip at a relative output of 0.6. The solution was sonicated four times for 10 s each with 1 min between each sonication. The mixtures were pooled and centrifuged at $100,000 \times g$ for 1 h, and the supernatant was separated into two fractions of 12 ml each and loaded onto two 5-ml DEAE-Sepharose columns (Sigma CL6B) equili-

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brated with buffer 3 (50 mM Tris, pH 7.5, 10 mM DTT, 1 mM EDTA, 1 mM PMSF, 500 mM NaCl, and 10% glycerol). Each column was eluted with buffer 3, and 15 fractions of 1.5 ml each were collected. The protein concentration of the different fractions was measured by absorption at 280 nm, and the protein-rich fractions (OD_{280}, >0.7/ml) were pooled. Solid ammonium sulfate (0.313 g/ml) was added slowly while the fractions were mixed continuously. The protein was allowed to precipitate for 10 min at 4°C and centrifuged at 13,800 × *g* for 10 min. The pellet was suspended in 2 ml of buffer 4 (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, and 0.1 mM PMSF). This fraction, referred to as the nuclear extract, was dialyzed against six changes of buffer 4 (500 ml each) in a 3-h period, divided into 1.5-ml tubes, and quickly frozen in liquid N_2. The frozen extracts were stored at −80°C.

In vitro recombination assay. The parental plasmid pSV2neo provided by Southern (31) was used to generate two deletion mutants, pSV2neo DL and pSV2neo DR, as described previously (16). The mutant neo gene from DR was cloned into the M13mp11 phage vector, generating MHX phage (25). Double-stranded pSV2neo DL and the ssMHX were used as substrates for recombination. pSV2neo DL DNA (0.5 μg) linearized within the region of homology by *Sal*I digestion was mixed with 1 μg of ssMHX and incubated with the protein fraction as described previously (25). DNA from the reaction mixture was purified and used to transform *E. coli* DH1 (*recA*) as described by Mandel and Higa (19) and Maniatis et al. (20).

ATPase activity. The ATPase activity of the nuclear extract and of the different protein fractions was determined by a modification of the method described by Weinstock et al. (37). Nuclear extract (10 μl) or protein fractions (30 μl) were incubated with 2 μl of [γ^{32}P]ATP (specific activity, 3,000 Ci/mmol; 10 mCi/ml) in a reaction mixture containing 25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.5 mg of bovine serum albumin per ml, and 0.5 mM unlabeled ATP, in the presence or absence of 2 μg of ssDNA (M13mp11). The final volume was 40 μl. After incubation at 37°C for 45 min, excess EDTA and unlabeled ATP and ADP were added. The ATP was separated from the reaction products, ADP and P_i, by vertical thin-layer chromatography on polyethyleneimine-cellulose (J. T. Baker Chemical Co., Phillipsburg, N.J.) in 1 M formic acid containing 0.5 M LiCl. The ATP and ADP spots were visualized under UV light, the ATP spot was scraped off, and the radioactivity was measured in a Beckman scintillation counter. The ssDNA-dependent ATPase activity was determined by subtracting the activity obtained in the absence of ssDNA from the activity obtained in the presence of ssDNA. One unit of DNA-dependent ATPase activity is defined as the amount of protein that hydrolyzed 2 nmol of [^{32}P]ATP in 45 min.

Endogenous labeling of DNA. A *thy* derivative of *E. coli* JM103 was obtained by selection in aminopterin (23). The strain, LWT4, was maintained in medium containing thymine at a concentration of 20 μg/ml. M13 phage and replicative form (RF) DNA were obtained by established techniques (21). To label DNA, phages were grown on LWT4 for 6 h in LB containing thymine (20 μg/ml), uridine (50 μg/ml), and [^{3}H]thymidine (1 mCi/ml). Unlabeled thymidine was added 20 min before the cells were harvested for DNA.

Assays for formation of joint molecules. (i) D-loop assay. The D-loop assay was performed by a modification of the method of Beattie et al. (1) and Das Gupta et al. (6). The substrates used in the assay were uniformly labeled M13 RF DNA (double-stranded; specific activity, 9,600 cpm/μg) and homologous or heterologous ssDNA from M13 or φX174 phage. The reaction mixture contained 20 mM Tris, pH 7.5, 15 mM $MgCl_2$, 0.4 mM DTT, 6 mM phosphocreatine, creatine phosphokinase (10 U/ml), 1 mM ATP, 3.3 μM ssDNA, and 2 to 3 μm purified bacterial RecA protein (a generous gift from C. Radding) or up to 12 μl of the protein fractions. After incubation at 37°C for 10 min, 6.6 μm [^{3}H]dsDNA linearized with *Bam*HI or *Hpa*I was added, and the mixture was again incubated at 37°C for 15 min. The total volume was 20 μl. The reaction was stopped by the addition of 100 μl of cold 25 mM EDTA, followed by 2 ml of cold 10 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate). One hundred microliters of the resulting solution was spotted on nitrocellulose filters (Schleicher & Schuell Inc., Keene, N.H.) to determine the total amount of radioactivity, and the rest of the mixture was filtered through nitrocellulose filters and washed with 8 ml of 10 × SSC. The filters were dried and the radioactivity was measured in a Beckman scintillation counter. One unit of D-loop activity is defined as the amount of protein that converted 100 fmol of dsDNA into D-loop-containing molecules.

(ii) S1 nuclease protection assay to measure heteroduplex formation. The reaction conditions for this assay are identical to those used for the D-loop assay. However, in this case the dsDNA was unlabeled and the ssDNA was uniformly labeled (M13; specific activity, 167,000 cpm/μg). The dsDNA that was used was either M13 RF digested with *Ava*I or MHX RF digested with *Pst*I. After incubation at 37°C for 1 h, 80 μl of S1 nuclease mix containing 250 mM NaCl, 62.5 mM sodium acetate (pH 4.6), 0.12% sodium dodecyl sulfate (SDS), 2.5 mM $ZnSO_4$, and 96 U (final concentration) of S1 nuclease per ml was added and incubated at 37°C for 30 min. After the addition of 5 μg of salmon sperm DNA, trichloroacetic acid (TCA)-precipitable counts were measured. The results were expressed as picomoles of ssDNA which became S1 nuclease-resistant.

Nucleoprotein network formation. This assay measures homology-independent coaggregation of ssDNA and dsDNA in complexes that sediment rapidly (4). The substrates used in this assay were M13 RF [^{3}H]DNA linearized with *Bam*HI and φX174 circular phage DNA. The reaction mixture contained 3 μM circular ssDNA, 33 mM Tris, pH 7.5, 1.3 mM ATP, 3 mM phosphocreatine, phosphocreatine kinase (10 U/ml), bovine serum albumin (88 μg/ml), 1.8 mM DTT, 3 mM $MgCl_2$, and 2 to 3 μM RecA or up to 12 μl of protein fraction. After incubation at 37°C for 10 min, 6 μM dsDNA and 10.3 mM $MgCl_2$ were added, and the mixture was further incubated for 15 min at 37°C. The total volume was 60 μl. After centrifugation for 2 min at 15,000 × *g*, two 28-μl portions from the supernatant were counted to determine the total radioactivity of the supernatant, and the precipitate was suspended in 100 μl of distilled water and counted. The results were expressed as percentage of [^{3}H]dsDNA present in the sedimented network.

Assay for exonuclease activity. pBR322 DNA linearized with *Eco*RI was end labeled at the 5′ end by treating the DNA with alkaline phosphatase followed by T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP and at the 3′ end by DNA polymerase large fragment in the presence of [α^{32}P]ATP by the method of Maniatis et al. (20). The reaction mixture contained 25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 2 mM ATP, 5′- or 3′-end-labeled DNA (approximately 5×10^4 cpm), and up to 12 μl of protein fraction. The total volume was 80 μl. After incubation at 37°C for 60 min, carrier DNA was added and TCA-precipitable radioactivity was measured.

Reannealing assay. Reannealing reactions were carried out by the methods of Weinstock et al. (36) and Bryant and Lehman (2). Briefly, 20 μl of final reaction mixture contained 25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 5% glycerol, 0.5 mM ATP, 6 mM creatine phosphate, creatine phosphokinase (10 U/ml), 60 ng of M13 phage [^{3}H]DNA (185 pmol; 167,000 cpm/μg) and 200 ng (615 pmol) of heat-denatured M13 RF linearized with *Ava*I. Up to 7 μl of enzyme fraction or 0.6 μg of RecA protein was added last, and the reaction mixture was incubated for 15 min at 37°C. Subsequently, 80 μl of S1 nuclease mix containing 250 mM NaCl, 62.5 mM sodium acetate, pH 4.6, 0.12% SDS, 2.5 mM $ZnSO_4$, and 96 U of S1 nuclease was added, and incubation was continued for a further 30 min. After addition of 5 μg of carrier DNA, the reaction mixtures were spotted onto Whatman 3MM filter paper and the DNA was precipitated by addition of 5% TCA. After being washed with ethanol, the filters were dried and counted.

Fractionation procedures. The nuclear extract obtained as described previously (17, 30) was dialyzed against buffer A (50 mM Tris, pH 7.5, 1 mM EDTA, 1 mM DTT, 10% glycerol, 0.1 mM PMSF), to which 200 mM NaCl was added, and loaded on a 5-ml phosphocellulose column (Sigma Chemical Co., St. Louis, Mo.) equilibrated with the same buffer. Five protein fractions were eluted stepwise (flow rate, 1 ml/min) with 1.5 ml of buffer A containing 0.2, 0.3, 0.4, 0.5, and 2 M NaCl. After dialysis against 660 volumes of buffer A for 1 h, the fractions eluted at 300 through 500 mM NaCl were pooled and loaded on a 2-ml ssDNA-cellulose column (Sigma) equilibrated with buffer A. The proteins were eluted with 7 ml of a linear gradient of NaCl (0 to 1 M) in buffer A. After dialysis against 2,000 volumes of buffer A for 1 h at 4°C, the active fraction was loaded on a Mono Q HR 55 ion-exchange high-pressure liquid chromatography (HPLC) column (Pharmacia, Piscataway, N.J.) and the proteins were eluted with 15 ml of a linear gradient of NaCl (0 to 1 M) in buffer containing 32 mM Tris, pH 7.6, 2 mM EDTA, 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 10 mM mercaptoethanol, and 10% glycerol. The HPLC fractions were dialyzed against 660 volumes of buffer A, frozen in liquid nitrogen, and kept at −80°C. All procedures with the exception of the HPLC run were conducted at 4°C.

RESULTS

Experimental strategy. Nuclear extracts from human bladder carcinoma cells were fractionated, and individual fractions were assayed for DNA-dependent ATPase activity. Active fractions were tested for strand exchange activity by the biological assay (in vitro recombination) and the D-loop assay. Fractions enriched for these activities were tested for other features that may be characteristic of human DNA strand transfer activity.

General characterization of the recombinationally active fraction from EJ nuclear extracts. We have previously shown that nuclear extracts from human EJ cells are capable of catalyzing recombination between two homologous double-stranded molecules (17) or between a double-stranded and single-stranded substrate (25). The enzymatic activities that are required to catalyze this reaction and their sizes were not known. In an effort to obtain this type of information, total EJ nuclear extracts were loaded onto a 5-ml 30 to 50% glycerol gradient and subjected to centrifugation at 189,000 × *g* at 0°C for 46 h. Ten equal fractions were collected and assayed for DNA-dependent ATPase activity, recombinational activity, and protein profile (Fig. 1). We observed maximal ATPase and biological activities in fractions 7 and 8. The size of the proteins in these fractions corresponded to 70 to 100 kilodaltons (KDa). A second peak of activity at 250 to 500 kDa was also observed. However, this second peak was not detectable in all of the experiments we conducted. We conclude that a certain fraction corresponding to a size range of 70 to 100 kDa contains the protein(s) necessary for catalyzing homologous recombination between the deletion mutants. The inconsistent presence of the second peak is not surprising, because it was shown that bacterial RecA can be detected in rapidly sedimenting forms depending on the protein concentration, salt concentration, and presence or absence of ATP (22).

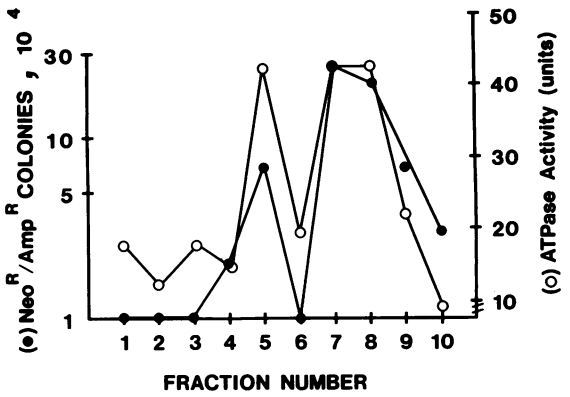

FIG. 1. Fractionation of nuclear extracts on glycerol gradients. Nuclear extract (2 mg) prepared as described in Materials and Methods was layered onto a 30 to 50% glycerol gradient (5 ml) and subjected to centrifugation as described in the text. Ten equal fractions were isolated and assayed for biological activity and ATPase activity as described in Materials and Methods. For each biological assay and ATPase assay, 30 μl of the fraction was used. Symbols: ●, in vitro recombination activity; ○, DNA-dependent ATPase activity.

Purification of a protein fraction which promotes strand invasion. In an attempt at fractionation, EJ nuclear extracts were loaded on a phosphocellulose column equilibrated with buffer A containing 200 mM NaCl and eluted as described in Materials and Methods. The fractions were dialyzed extensively against buffer A and assayed for recombination activity and ATPase activity. Fraction 3, eluted with 400 mM NaCl, was the only fraction which catalyzed homologous recombination between the substrates (Fig. 2). This fraction also contained maximal DNA-dependent ATPase activity. Examination of the plasmid DNA from the Neo^r colonies obtained from one of these experiments revealed that the majority of the plasmids (27 of 28) contained only wild-type molecules, with one case of a mixture between wild-type and DL molecules. Similar results were obtained when total nuclear extracts were used (25).

When assayed for joint molecule formation in the D-loop assay, the phosphocellulose fractions induced significant binding of radiolabeled DNA to the nitrocellulose filters in the presence of homologous or nonhomologous ssDNA, presumably due to the presence of nucleases. When tested directly, the total nuclear extract as well as phosphocellulose fractions 2 to 4 contained double-stranded 3′ and 5′ exonuclease activity.

For further purification, pooled phosphocellulose fractions 2 to 4 were chromatographed on an ssDNA-cellulose column equilibrated with buffer A and eluted as described in

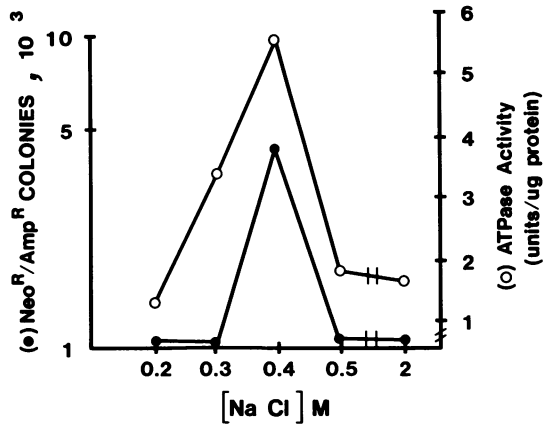

FIG. 2. Fractionation of nuclear extracts on phosphocellulose column. Nuclear extract (2 mg) was loaded onto a phosphocellulose column as described in Materials and Methods and eluted with a step gradient of NaCl. Individual fractions were dialyzed extensively, and 30 μl of each fraction was used for the recombination assay and ATPase assay. Fractions were assayed for in vitro recombination activity (●) and DNA-dependent ATPase activity (○).

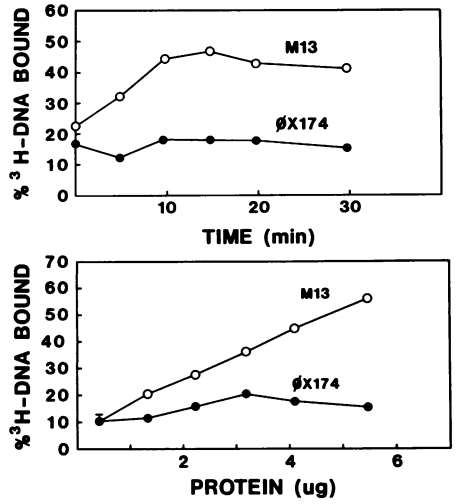

FIG. 4. (Top) Strand exchange activity is homology dependent. D-loop assays were conducted with dsM13 DNA and homologous M13 ssDNA (○) or heterologous ϕX174 ssDNA (●). (Bottom) Relationship of protein concentration and strand exchange activity. Symbols are the same as for the top panel.

Materials and Methods. After extensive dialysis against buffer A, the fractions were assayed for DNA-dependent ATPase activity and strand exchange activity. The DNA-dependent ATPase activity eluted at 160 to 200 mM NaCl (fraction 6, Fig. 3).

Properties of fraction 6. We conducted a series of experiments to examine the properties of fraction 6. Fraction 6 exhibited DNA strand exchange activity (D-loop) as well as DNA-dependent ATPase activity (Fig. 3). The strand exchange activity was homology dependent (Fig. 4). When heterologous (ϕX174) DNA was used as a substrate, we did not detect any strand exchange activity. The extent of strand exchange was proportional to the amount of protein added, and the strand exchange reaction was completed in 10 min (Fig. 4).

Since bacterial RecA and fungal Rec1 proteins require the presence of ATP and its hydrolysis to complete the strand exchange reaction, we examined whether fraction 6 had similar requirements. We removed specific components from the reaction mixture for strand exchange activity and tested the effects on the reaction (Fig. 5). The reaction required Mg^{2+}. When ATP was removed or a nonhydrolyzable analog of ATP, γS-ATP was used, the reaction did not proceed. When the ssDNA was omitted from the reaction, we did not observe any filter binding, indicating that fraction 6 did not contain substantial nuclease activity.

Evidence for formation of heteroduplexes. We tested whether fraction 6 could catalyze the formation of true heteroduplexes by the S1 nuclease protection assay (Table 1). When completely homologous substrates were used, we observed that a maximum of 50% of the labeled ssDNA entered into the S1-resistant fraction. When a dsDNA with terminal nonhomologies (of nearly 1 kilobase [kb] on each side) was used, we noted that a significant portion of the ssDNA entered a duplex structure. These experiments clearly demonstrate that fraction 6 is capable of catalyzing true DNA strand exchange in vitro.

Nucleoprotein network formation. The first step in the strand exchange process by bacterial RecA consists of the

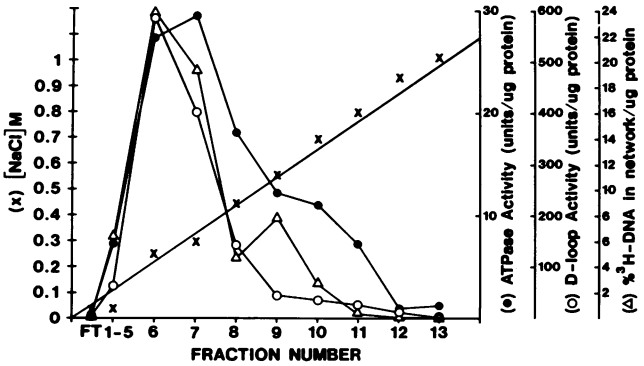

FIG. 3. Fractionation of active fraction from the phosphocellulose column on the ssDNA-cellulose column. Fractions 2 to 4 from the phosphocellulose column were loaded onto a DNA-cellulose column and eluted with a linear gradient of NaCl (0 to 1 M). Individual fractions were assayed for DNA-dependent ATPase activity (●), D-loop activity (○), and ability to form nucleoprotein networks (Δ). ×, NaCl concentration.

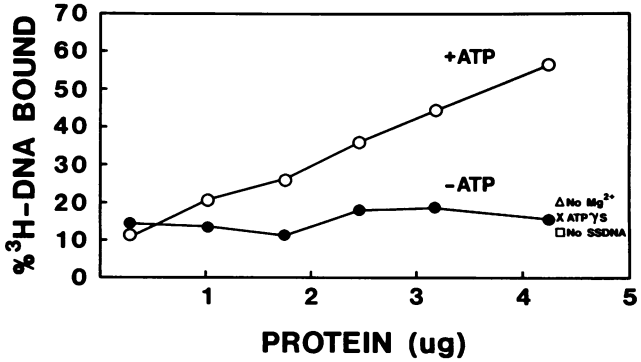

FIG. 5. Requirements of the strand exchange reaction. D-loop assays were conducted in a complete reaction mixture (+ATP), in the absence of Mg^{2+}, ATP, or ssDNA, or in the presence of γS-ATP.

TABLE 1. Formation of S1-resistant joint molecules

Sample[a]	S1-resistant DNA (pmol)
No fraction 6	
ssM13 DNA + dsM13 DNA + S1 nuclease	1.4
ssM13 DNA + S1 nuclease	1.35
ssM13 DNA	150
With fraction 6	
Rxn mix with dsM13 DNA	66.3
Rxn mix with dsMHX[b] DNA (with heterologous ends)	9
Rxn mix without dsDNA	1.35

[a] The amounts of the components in the reaction mixtures were as follows: ssM13 DNA, 50 ng; dsM13 DNA, 100 ng; dsMHX DNA, 100 ng; fraction 6, 5.4 μg. Rxn, Reaction.

[b] For details about the construction of the MHX phage, see text and Rauth et al. (25).

polymerization of RecA on ssDNA in a presynaptic phase. The presynaptic complexes can bind homologous or nonhomologous duplex DNA, causing the formation of rapidly sedimenting networks which contain ssDNA, RecA, and dsDNA (4). These large nucleoprotein networks, which presumably could rearrange themselves to search for homology, are believed to be synaptic intermediates in the strand exchange process (34). We examined whether fraction 6 from human extracts was capable of catalyzing such a reaction. We used two nonhomologous DNA substrates, radiolabeled linearized M13 RF DNA and φX174 circular ssDNA for this experiment. DNA-cellulose fractions were preincubated with the ssDNA, followed by the addition of labeled dsDNA. After incubation, the sample was centrifuged, and the label retained in the supernatant and the precipitate was measured. We observed that this activity was maximal in fraction 6 (Fig. 3). At low protein concentrations, the activity was dose dependent and Mg^{2+} dependent and required the presence and hydrolysis of ATP. Moreover, for protein concentrations up to 3.2 μg per reaction mixture, there was no formation of rapidly sedimenting complexes with the dsDNA in the absence of ssDNA. However, at higher protein concentrations (>4.3 μg per reaction mixture), the active fraction induced the formation of rapidly sedimenting complexes with only dsDNA. This process seemed to be ATP independent and was only partially inhibited by the absence of Mg^{2+} (results not shown).

Reannealing activity. Since a major function of the strand exchange protein would be to catalyze the pairing of homologous single-stranded molecules, we tested whether fraction 6 had any such reannealing activity. All of the fractions from the DNA-cellulose column were tested for this activity as described in Materials and Methods. The reannealing activity comigrated with the D-loop activity. The properties of this activity are summarized in Table 2. This reaction was completely homology dependent, partially dependent on Mg^{2+}, and ATP independent. These features are reminiscent of RecA, which exhibits similar properties (36).

Biological activity of fractions from the DNA-cellulose column. We examined whether individual fractions obtained from the DNA-cellulose column (fractions 1 to 13, Fig. 3) were capable of catalyzing recombination between homologous substrates in the biological assay. Linearized pSV2neo DL was mixed with MHX DNA and incubated with the fractions, and the DNA was used to transform *recA E. coli* (Table 3). We observed that maximal biological activity was

TABLE 2. Reannealing activity of fraction 6[a]

Substrates		Reaction conditions	S1-resistant DNA (pmol)
Labeled DNA	Unlabeled DNA		
ssM13	Denatured M13 RF	Complete	14.4
ssM13	None	Complete	3.6
ssM13	Denatured φX174 RF	Complete	3.4
ssM13	Denatured M13 RF	No ATP	17.0
ssM13	Denatured M13 RF	No Mg^{2+}	11.4
ssM13	Denatured M13 RF	No enzyme	5.5

[a] The reaction was performed as described in Materials and Methods. The amounts of the components used in the reaction mixture were as follows: [^{3}H]ssM13 DNA, 60 ng; dsM13 or φX174 DNA, 200 ng; fraction 6, 3.15 μg.

exhibited by fraction 5, whereas fractions 4 and 6 had a low level of activity. These results could be interpreted to mean that the biological activity and the strand exchange activity are independent. Alternatively, it is possible that the strand exchange activity alone is not sufficient for biological activity and that one or more proteins present in fractions 4 and 5 are required for the manifestation of biological activity. If this were the case, fraction 5 would have overlapping peaks of activities from fractions 4 and 6, explaining its high level of biological activity. To test this feature, we incubated the DNA substrates with fraction 6 for 1 h and then added fraction 4 followed by an additional period of incubation. The DNA from this reaction was used for bacterial transformation. As shown in Table 3, fractions 4 and 6 acted in a synergistic fashion to yield maximal biological activity.

Fractionation by HPLC. In an attempt at further purification of the strand exchange protein, we fractionated fraction 6 on an ion-exchange column (Mono Q; Pharmacia) by HPLC. Each of the individual fractions was tested for ATPase and D-loop activity (Table 4). We observed that two fractions, 1 and 3, contained DNA-dependent ATPase activity, while only fraction 1 contained the D-loop activity. These results indicate that in the fraction 6 which was loaded onto the HPLC column, there were two ATPase activities, only one of which was associated with the strand exchange reaction. Strand exchange catalyzed by fraction 1 was homology dependent and required Mg^{2+} as well as ATP. When γS-ATP was used, the strand exchange reaction did not proceed. When this fraction was directly assayed for 5′ and 3′ exonuclease activity, we did not detect any such activity (results not shown). The levels of protein recovered

TABLE 3. Biological activity of fractions from the DNA-cellulose column[a]

Fraction[b]	Neo^r/Amp^r colonies	Frequency (10^4 colonies)
4	3/3,720	8.1
5	30/2,370	127
6	1/1,460	6.8
7	1/3,100	3.2
4 + 6	72/1,270	567

[a] pSV2neo DL DNA (0.5 μg) digested with *Sal*I was mixed with 1 μg of ssMHX DNA. The mixture was incubated with 20 μg of protein from the fractions indicated, except fraction 4, for which we used 2 μg of protein. After incubation at 37°C for 1 h under the conditions described in Materials and Methods, the DNA was purified by phenol extraction and ethanol precipitation and used to transform *recA E. coli* strain DH1. A fraction of the transformed bacteria were plated on ampicillin-containing plates, and the rest were plated on kanamycin-containing plates.

[b] Fractions 4 through 7 are sequential fractions obtained from the salt gradient.

TABLE 4. Strand transfer activity in fractions from ion-exchange HPLC

Fraction	Protein (μg)	Activity (U/μg of protein)	
		DNA-dependent ATPase[a]	D-loop[b]
1	11.8	440	18,500
2	11.8	ND	ND
3	29.1	148	ND
4	0.78	ND	ND
5	37.7	ND	ND
6	53.4	ND	ND
7	184.7	ND	ND

[a] ND, Not detectable (limit of detection, 0.0075 U/μg of protein).
[b] ND, Not detectable (limit of detection, 3 U/μg of protein).

and the different activities in the various fractions are shown in Table 5.

DISCUSSION

Work in our laboratory (17, 25, 30) and others (5, 9) has shown that nuclear extracts from human and mouse cells are capable of catalyzing recombination between homologous DNA molecules. Symington et al. (32) and Hotta et al. (9) have obtained similar results with yeast extracts. Keene and Ljundquist (11) presented evidence for a D-loop-forming activity in human cells. Though the exact mechanistic steps leading to a recombinant product are not understood, a characteristic of the recombination process is the exchange of information between homologous molecules. Thus, the key enzymatic activity is that which catalyzes this strand exchange reaction.

Evidence from several laboratories indicates that recombination of DNA introduced into somatic mammalian cells in culture could proceed by one of two pathways. Evidence from our laboratory and several others (for a review, see reference 15) indicates that the recombination process is conservative in the sense that there is no loss of information during the recombination process. Lin et al. (18) and others (for example, reference 3) have provided evidence for a nonconservative pathway in which a "recombinant" product is obtained by exonucleolytic digestion followed by annealing of the resulting single strands. The first reaction needs a true strand exchange activity similar to that of the RecA and Rec1 proteins, while the key component of the second type of reaction is an exonuclease. We believe that the activity we have purified is a true strand exchange activity. Several lines of evidence support this view. The reaction was completely homology dependent. No D-loop formation was detected with heterologous ssDNA (ϕX174). (ii) Incubation of the active fraction with dsDNA in the absence of ssDNA did not result in filter binding. (iii) There was no direct correlation between the nucleolytic activity of the fractions eluted from the ssDNA-cellulose column and D-loop formation. Several fractions exhibited nucleolytic activity higher than or equal to that of fraction 6, but only fraction 6 was active in the D-loop assay. (iv) HPLC fraction 1, which exhibited D-loop activity, did not have detectable 3′ or 5′ exonuclease activity. We were also able to demonstrate formation of a true heteroduplex by using unlabeled dsDNA and uniformly labeled circular ssDNA as substrates for the reaction. In this reaction heteroduplex formation was inferred by conversion of an S1 nuclease-sensitive DNA fraction to S1 nuclease resistance. When a dsDNA molecule which had up to 1 kb of nonhomologous sequence at either end was used as one of the substrates, we were still able to detect joint molecule formation (Table 3). The reduced level of heteroduplex formation with dsMHX DNA as a substrate was not unexpected, since similar results have been obtained with Rec1 protein (14). All of these lines of evidence clearly indicate that a true strand transfer reaction has taken place. Since as much as 44% of the ssDNA can enter an S1 nuclease-resistant fraction, it is possible to estimate that as much as 3 kb of DNA can enter into a duplex structure.

An additional line of evidence which supports the view that true strand exchange has occurred comes from the biological assays we conducted. Since each of the substrates carries a deletion in the *neo* gene, it is necessary for them to exchange information to generate a wild-type *neo* gene (17, 25). All of the fractions that were active in the D-loop assay were found to be necessary for the biological activity. Since exonucleolytic digestion alone was not capable of generating a wild-type *neo* gene, strand exchange must have indeed occurred. These results indicate that the protein(s) we are fractionating has biological significance.

Our results also indicate that in order to generate a wild-type *neo* gene, one or more activities that are distinct from the strand transfer activity are needed. This conclusion is based on experiments in which we mixed different fractions obtained from the DNA-cellulose chromatography and observed that a combination of fractions 6 and 4, neither of which exhibited significant biological activity, yielded a maximal number of Neo^r colonies. The nature of the additional factors that are required to mediate the biologically detectable recombination reaction is not known.

Fraction 6 as well as the active fraction obtained by HPLC required Mg^{2+} and ATP for their strand exchange function. We also observed that the presence of a nonhydrolyzable analog of ATP prevented the reaction. Both bacterial RecA and fungal Rec1 proteins have similar requirements, indicating that the mammalian strand exchange activity shares these important properties with its bacterial and fungal analogs. Hsieh et al. (10) have reported that partial purification of a protein from a human B-cell line which was capable of promoting strand exchange between homologous molecules. That fraction required Mg^{2+} for its activity but did not require an energy source. These observations raise the possibility that we are purifying a protein which is distinctly different from that reported by Hsieh et al. (10).

The activity we have fractionated has additional proper-

TABLE 5. Recovery of strand transfer activity at different stages of purification

Sample	Protein (μg)	Protein recovery (%)	DNA-dependent ATPase activity (U/μg of protein)	DNA-dependent ATPase recovery (%)	D-loop activity (U/μg of protein)	D-loop recovery (%)
Total extract	7,140	100	14	100	—[a]	—
Phosphocellulose column fractions 2–4	3,780	53	13	47	—	—
ssDNA-column fraction 6	475	7	28	13	560	100
HPLC fraction 1	12	0.2	440	5	18,500	82

[a] —, Not detectable due to nuclease activity.

ties characteristic of strand exchange enzymes. These include the ability (i) to reanneal homologous ssDNA molecules and (ii) to catalyze the formation of nucleoprotein networks which can be considered synaptic intermediates. Chow and Radding (4) have shown that bacterial RecA has the ability to catalyze the formation of nucleoprotein networks and considered that such networks may be the precursors for eventual strand exchange. Several studies (for example, reference 36) noted that RecA has the ability to reanneal homologous ssDNA molecules. The observation that a fraction from human nuclear extracts shares these properties indicates that eucaryotic and procaryotic enzymes which catalyze recombination may share important properties and act in a similar fashion.

ACKNOWLEDGMENTS

We appreciate the help of S. Ehrlich, S. Frey, and L. Wallace in many of the experiments. G. Smith, C. Radding, and O. Smithies provided valuable comments on the manuscript.

This work is supported by Public Health Service grants GM-33943 and GM-36565 to R.K. and CA-37145 to P.M. from the National Institutes of Health and by the March of Dimes Birth Defects Foundation (grant I-806 to R.K.). D.G. is a Leukemia Society Fellow.

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