Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 9067–9071, October 1991
Biochemistry

The human homologous pairing protein HPP-1 is specifically stimulated by the cognate single-stranded binding protein hRP-A

(genetic recombination/recombination complex/DNA repair/DNA replication)

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Communicated by Howard A. Nash, July 8, 1991 (received for review April 13, 1991)

ABSTRACT **Homologous pairing and strand exchange of DNA are catalyzed by the human homologous pairing protein HPP-1 in a magnesium-dependent, ATP-independent reaction that requires homologous DNA substrates and stoichiometric quantities of HPP-1. Here we show that the addition of the purified human single-strand binding (SSB) protein hRP-A to the reaction mixture stimulates the rate of homologous pairing 70-fold and reduces the amount of HPP-1 required for the reaction at least 10-fold. The identification of hRP-A as a stimulatory factor of HPP-1-catalyzed reaction was facilitated by its recognition as a member of a high molecular weight complex of recombination components. Neither the *Escherichia coli* SSB protein, bacteriophage T4 gene *32* protein, nor the highly conserved *Saccharomyces cerevisiae* yRP-A SSB protein could substitute for hRP-A in this stimulation. Because only the cognate SSB was capable of stimulating HPP-1, these results suggest that eukaryotes depend on unique and specific interactions between DNA recombination components.**

The initiation of genetic recombination begins with pairing of chromosomes at sites of sequence homology and is followed by an exchange of DNA strands forming a crossover structure (Holliday junction) (1). Several proteins have been purified that catalyze the homologous pairing and strand exchange of model DNA substrates and produce partial or complete Holliday intermediates (2–7).

The RecA protein of *Escherichia coli* is the prototypical recombination homologous-pairing activity (8–10). Purified RecA protein has been shown to catalyze homologous pairing and strand exchange of DNA substrates that contain sequence homology, at least one DNA strand break, and a single-stranded region at the site of exchange (11). Stoichiometric quantities of RecA protein are required to coat the single-stranded region and form a nucleoprotein–filament complex prior to strand exchange (10). The amount of RecA protein needed for strand exchange can be reduced 10-fold if the *E. coli* single-strand binding (SSB) protein is included in the reaction mixture (13–15). Furthermore, it appears that nearly any single-stranded DNA (ssDNA)-binding protein can be substituted for SSB protein in the RecA-catalyzed strand-exchange reaction regardless of the species of origin (refs. 16–18; W.-D. Heyer and L.E., unpublished data). These and other results support the idea that relaxation of DNA secondary structure is the major role for SSB protein in the RecA-catalyzed reaction.

Several eukaryotic homologous-pairing proteins have been purified and characterized, including SEP-1 from *Saccharomyces cerevisiae* (5), Rec1 from *Ustilago maydis* (4), STase from *Drosophila* (6), and HPP-1 from human cells (7). Additionally, Heyer and Kolodner (18) have purified and characterized a factor from yeast (ySSB protein) based on its ability to stimulate the SEP-1 reaction. The ySSB protein binds to ssDNA and reduces the amount of SEP-1 required for the strand-exchange reaction at least 10-fold. However, neither *E. coli* SSB protein nor T4 gene *32* protein could be substituted for ySSB protein in the stimulation of SEP-1 (18). In a comparison of protein sequences, ySSB protein was found to have significant homology with one of the subunits of the hRP-A holoprotein complex (17, 19). hRP-A was originally identified as an essential factor for simian virus 40 origin and large tumor antigen-dependent replication reaction *in vitro* (hRP-A = RP-A = RF-A = hSSB) and consists of three peptide subunits with relative molecular masses of 70 kDa, 32 kDa (17), and 14 kDa (20–22). The ySSB protein is homologous to the 70-kDa subunit of hRP-A [designated hRP-A(70) (17, 19); 31% identity, 45% conserved amino acids]. The ssDNA-binding activity has been localized to the hRP-A(70) subunit (19, 23). Furthermore, a functional gene for ySSB (RPA-1) is essential for cell survival in yeast (19). A recent report has demonstrated that a replicative excision repair reaction derived from crude human cell extracts appears to require hRP-A *in vitro* (24).

During the purification of HPP-1, we found a substantial fraction of the homologous-pairing and strand-exchange activity associated in a high molecular weight form. Because a limiting amount of HPP-1 was present in this high molecular weight complex, we surmised that either HPP-1 was more active in an oligomeric form or that a heteropeptide stimulatory component was present. Here we demonstrate that hRP-A is a stimulatory factor for HPP-1-catalyzed strand exchange and that it is present in the high molecular weight complex. The introduction of stoichiometric quantities of hRP-A into the strand-exchange reaction mixture reduces the requirement for HPP-1 at least 10-fold to near catalytic amounts. In addition, we demonstrate that only the cognate SSB protein will stimulate the eukaryotic strand-exchange reaction.

MATERIALS AND METHODS

Chemicals and Enzymes. Ultrapure Tris (acid and base), $MgCl_2$, NaCl, EDTA, and analytical-grade KCl were purchased from Amresco (Solon, OH). Molecular-biology-grade dithiothreitol was purchased from Sigma. Bovine serum albumin fraction V (BSA) was purchased from Pharmacia. Restriction enzymes were purchased from New England Biolabs. *E. coli* SSB was a gift from L. Harris and J. Griffith (University of North Carolina, Chapel Hill, NC). T4 gene *32* protein (T4g32p) was purchased from United States Biochemical and found to be free of single-stranded and double-

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Abbreviations: SSB, single-strand binding; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA.

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stranded nuclease and homologous-pairing activity (unpublished results). Yeast yRP-A and yRP-A(70^{*35}) were a gift from R. Kolodner, E. Alani, and W.-D. Heyer (Harvard Medical School, Boston). The yRP-A(70) was purified as a 35-kDa proteolytic fragment of the 70-kDa full-length peptide and is designated yRP-A(70^{*35}) (18). Human HPP-1 was purified from HPB-ALL immature T-lymphocyte cells as described (7). Human-derived hRP-A was purified from HeLa S3 cells as described (25). The 70-kDa subunit hRP-A(70) was partially purified from an *E. coli* overexpression construct by a modification of the published procedure (20). Briefly, the bacteria were lysed by freezing and thawing, and the lysate was made 2 M in NaCl and loaded onto a hydroxyapatite (Calbiochem) column. The 70-kDa subunit eluted with 30 mM Hepes (pH 7.5), 0.25% inositol (buffer C) plus 70 mM KPO_4 (pH 7.5), and 100 mM KCl. This material was dialyzed against 50 mM Tris (pH 8), 200 mM NaCl, and 2 mM EDTA (buffer D) and loaded onto a ssDNA-agarose column (Bethesda Research Laboratories). The column was washed with buffer D and eluted with buffer C plus 2 M NaSCN, 0.25 mM EDTA, and 0.01% Nonidet P-40. This material was concentrated on a mini-hydroxyapatite column prior to use. Purified HPP-1 has a trace $3' \rightarrow 5'$ exonuclease, as described by Moore and Fishel (7). The hRP-A holoprotein complex has no detectable nuclease and the partially purified hRP-A(70) preparation has a $3' \rightarrow 5'$ exonuclease contaminant (≈1 pmol of nucleotides released after incubation for 30 min at 37°C).

SDS/PAGE and Western Blot Analysis. For Western blot analysis, 8–25% gradient gels were run on a PhastGel system and semidry transfer was performed on a PhastGel system (Pharmacia) according to manufacturer's recommendations. Nitrocellulose filters were blocked in 20 mM Tris (pH 8), 150 mM NaCl, and 2% bovine serum albumin (Sigma) (TBS) and washed with TBS plus 0.3% Tween 20 (TBST). The screening antibody was diluted in TBST and incubated with the filter, washed with TBST, and incubated with a conjugated anti-rabbit alkaline phosphatase second antibody (Promega). The Western blot was developed according to manufacturer's recommendations. Polyclonal rabbit antibodies to a peptide fragment (SP381) of HPP-1 were used in primary antibody screens at a 1:1000 dilution for detection of HPP-1 and will be described in detail elsewhere (S.P.M. and R.F., unpublished data). Polyclonal rabbit antibodies to whole hRP-A were used in primary antibody screens at a 1:5000 dilution for detection of hRP-A (26). For general protein composition analysis, PhastGel electrophoresis (Pharmacia) using 8–25% gradient SDS gels was performed.

Homologous-Pairing and Strand-Exchange Assay. The homologous-pairing assay was performed essentially as described (7). The 10-μl reaction mixture included 20 mM Tris (pH 8), 40 mM KCl, 3 mM $MgCl_2$, 0.8 mM dithiothreitol, 80 μg of bovine serum albumin per ml, 8% glycerol, *Ssp* I-digested φX174 replicative form double-stranded DNA (dsDNA), and φX174 viral ssDNA. DNA concentrations are expressed in mol of nucleotides and are described in the figure legends. Quantitation was performed with an LKB scanning laser densitometer. The % product = relative joint molecules/relative dsDNA + joint molecules. The relative amount of ssDNA was neglected in this calculation, since the product linear ssDNA migrates at the same position as the substrate ssDNA in this system and, therefore, the ssDNA band cannot be distinguished as substrate or product.

RESULTS

HPP-1 and a Recombination-Stimulatory Activity Cofractionate in a High Molecular Weight Form. In a previous communication, we described the purification of the human homologous-pairing and strand-exchange protein HPP-1 (7). A majority of purification was obtained following chromatography on a Z-DNA affinity column (7). HPP-1 was then purified to near homogeneity as a 130-kDa protein using two subsequent chromatography steps. We have performed gel filtration chromatography of the Z-DNA chromatography eluate (Fig. 1). The results suggest that, at this stage of purification, the homologous-pairing activity resides in a high molecular weight

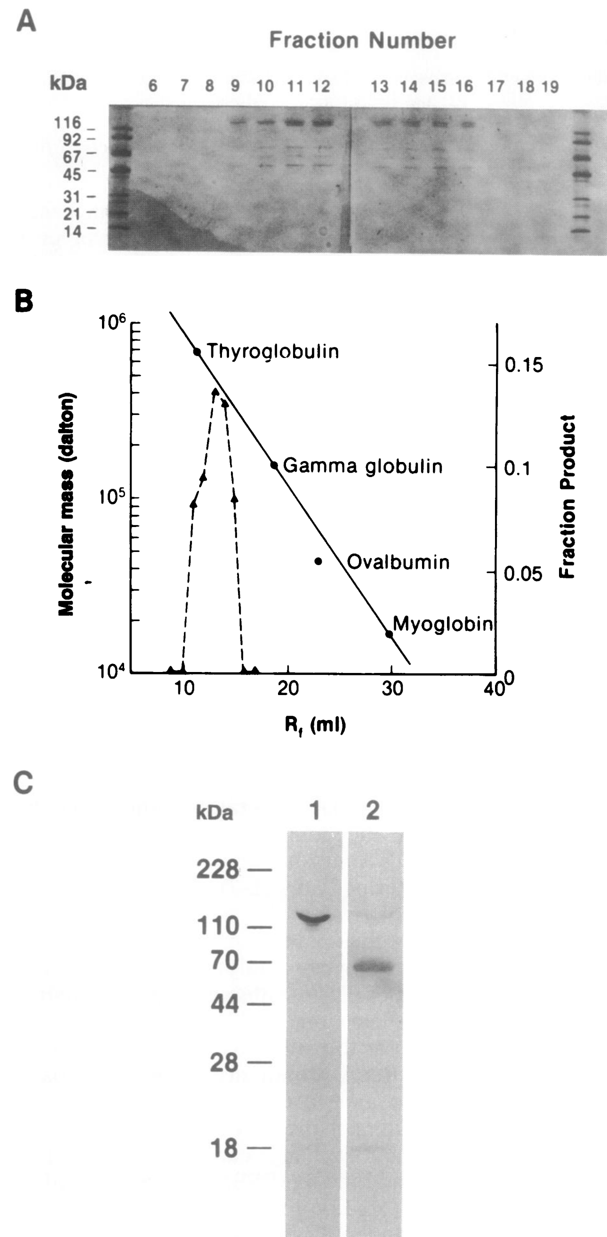

FIG. 1. Gel filtration and electrophoretic analysis of homologous-pairing activity. Gel filtration of the Z-DNA chromatography 0.3 M step eluate (76) was performed on a 1 cm × 30 cm Superose 6A column (Pharmacia) equilibrated with buffer B plus 50 mM KCl (7). (*A*) Protein content was analyzed by an 8–25% PhastGel system (Pharmacia) stained with silver according to the manufacturer's recommendations. Because 1-ml fractions were collected, the fraction numbers correspond exactly with R_f (ml) shown in *B*. (*B*) Homologous-pairing activity of gel filtration fractions was assayed according to ref. 7. Molecular mass markers correspond to thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa). (*C*) Western blot analysis of proteins present in the peak homologous-pairing activity fraction. The peak fraction corresponds to fraction 13 (*A*) and Rf13 (*B*), although it is from a separate identical experiment. Lane 1 was screened with anti-HPP-1 (SP381) and lane 2 was screened with anti-hRP-A as primary antibodies.

form of at least 500 kDa (Fig. 1B). Five proteins appear to be present in the peak fractions, having relative molecular masses of 150 kDa, 130 kDa, 70 kDa, 66 kDa, and 53 kDa (Fig. 1A). Western blot analysis using polyclonal antibodies to HPP-1 and hRP-A demonstrate that HPP-1 (130 kDa) and hRP-A(70) proteins are present in the peak fraction (Fig. 1C). By comparing the amount of silver-stained HPP-1 and hRP-A with corresponding purified HPP-1 or hRP-A standards, we estimate that there is approximately one molecule of HPP-1 for five molecules of hRP-A in the peak gel filtration fraction (data not shown). The 53-kDa protein appears to be a proteolytic product of the hRP-A(70) subunit, as has been reported by Wold et al. (20), and its presence is variable. The function of the 66-kDa peptide is unknown; however, there is a potent ATP-dependent blunt-end ligation activity apparently associated with the presence of the 150-kDa protein (27). Coincidental coelution appears unlikely since HPP-1 and hRP-A chromatograph as proteins of 160 kDa or less in their purified form (ref. 20; S.P.M. and R.F., unpublished data). Taken together, these data further suggest that HPP-1, hRP-A, and several additional proteins may be specifically associated in a high molecular weight complex.

The peak gel filtration fraction appears to promote the formation of ≈10 times the amount of strand-exchange product found with an equivalent weight of purified HPP-1 (data not shown). Because HPP-1 is about one-fifth the weight fraction of the proteins in the peak fractions we estimate that the complex form is ≈50-fold more active for the homologous-pairing and strand-exchange reaction than purified HPP-1. This observation suggested either that HPP-1 is more active in an oligomeric form or that a stimulatory factor was present in the high molecular weight complex. The recent demonstration that the *S. cerevisiae* SEP-1-dependent homologous-pairing stimulatory activity (ySSB) was homologous to the hRP-A(70) subunit suggested that a potential stimulatory activity in the high molecular weight fraction might be hRP-A. It is of interest to note that we have been unable to detect the hRP-A(32) subunit in the complex form, although the hRP-A(70) and hRP-A(14) are present. Since the polyclonal antibody used as a probe for hRP-A has been shown to react strongly with the hRP-A(32) subunit (26), this result may reflect the *in vivo* absence of hRP-A(32) in the recombination complex or incidental loss during protein fractionation.

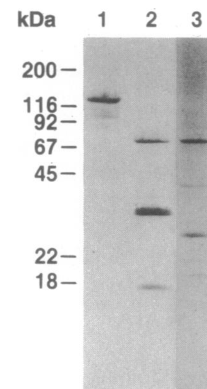

FIG. 2. Purified human recombination proteins. Electrophoretic analysis was performed on a PhastGel system (Pharmacia) with 8–25% gradient acrylamide gels in the SDS buffers recommended by the manufacturers. Lane 1, human HPP-1 purified from the HPB-ALL cell line (7); lane 2, human hRP-A purified from the HeLa cell line (25); lane 3, human hRP-A(70) purified from an *E. coli*-cloned overproducer (L.E. and T.K., unpublished). Relative molecular mass markers are shown on the left.

Homologous Pairing Is Stimulated by hRP-A. We tested the effect of purified hRP-A on the homologous-pairing reaction catalyzed by the human HPP-1. The assay for homologous pairing and strand exchange measures the conversion of single-strand circular and duplex linear DNA substrates to intermediate paired (joint molecules) and eventually duplex circular (form II) DNA products (7, 28, 29). HPP-1 and hRP-A were purified to near homogeneity as described (Fig. 2, lanes 1 and 2) (7, 20). In addition, the hRP-A(70) subunit was partially purified (Fig. 2, lane 3) from a bacterial construct overexpressing the protein (see *Materials and Methods*) and shown to bind ssDNA (L.E. and T.K., unpublished results).

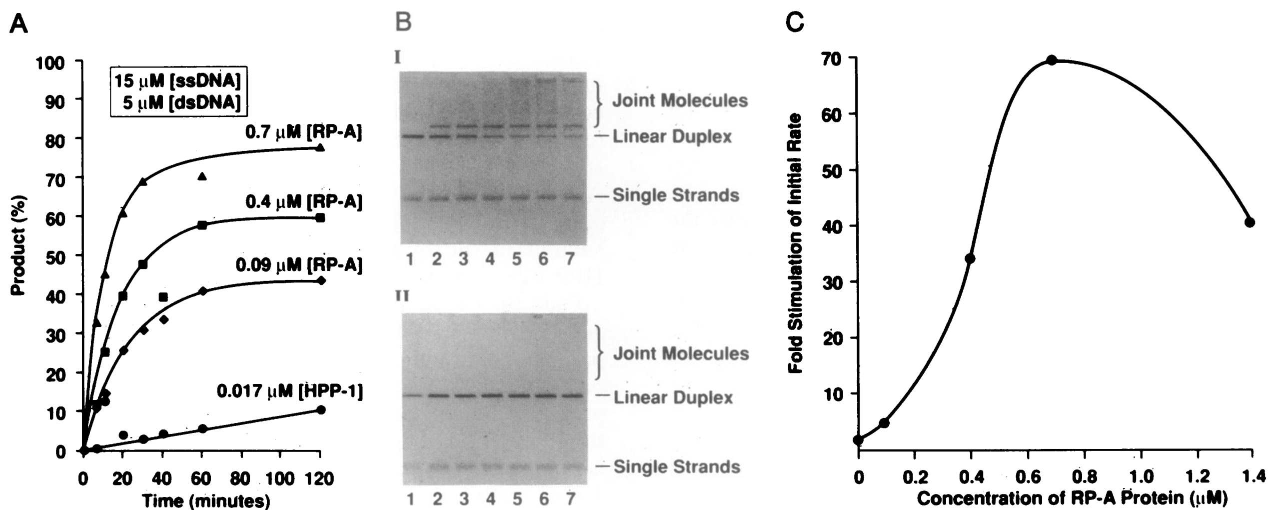

FIG. 3. Stimulation of HPP-1-catalyzed homologous pairing by human hRP-A holoprotein. Reaction conditions and DNA concentrations were as described in ref. 7 and included 20 mM Tris (pH 8), 40 mM KCl, 3 mM $MgCl_2$, 0.8 mM dithiothreitol, 80 μg of bovine serum albumin per ml, 8% glycerol, 5 μM *Ssp* I-digested ϕX174 replicative form DNA, and 15 μM ϕX174 viral ssDNA. (*A*) Quantitated hRP-A stimulation. HPP-1 (0.017 μM) was present in all reaction mixtures and resulted in a background conversion of 10% joint molecule forms after 120 min. Incremental amounts of hRP-A were added to the reaction mixture and the kinetic rate of formation of joint molecules is shown. (*B*) Primary data from hRP-A-stimulated kinetic experiments shown in *A*. Time points were taken at 0 min (lane 1), 5 min (lane 2), 10 min (lane 3), 20 min (lane 4), 40 min (lane 5), 60 min (lane 6), and 120 min (lane 7). Gel I contained 0.017 μM HPP-1 and 0.7 μM hRP-A. Gel II contained 0.017 μM HPP-1 only. (*C*) Plot of the hRP-A-dependent stimulation of the initial rate versus the amount of added hRP-A. Fold stimulation of the initial rate was calculated by determining the percentage of joint molecules formed after a 5-min reaction and dividing it by the percentage of joint molecules formed in the unstimulated reaction (0.017 μM HPP-1 only) after 5 min. For the hRP-A-stimulated reactions, the proteins were mixed prior to their addition to the DNA/buffer solution.

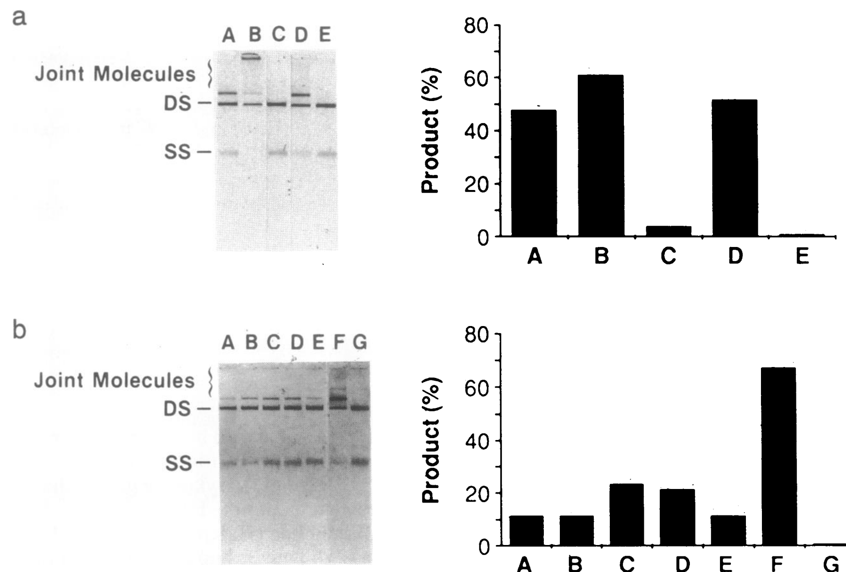

FIG. 4. Effect of heterologous SSB proteins on the HPP-1-catalyzed strand-exchange reaction. Conditions for the duplex linear versus single-strand circular strand-exchange reaction were identical to those described in ref. 7 and the legend to Fig. 3 except for the experiments in *b*, where the DNA concentrations were 4.05 μM for the double-strand (DS) substrate and 4.8 μM for the single-strand (SS) substrate, and the reaction was stopped and examined after 40 min. (*a*) Effect of human SSB protein. Lane A, 0.015 μM HPP-1 and 0.7 μM hRP-A; lane B, 0.015 μM HPP-1 and 0.7 μM hRPA(70); lane C, 0.015 μM HPP-1 only; lane D, 0.2 μM HPP-1 only; lane E, no protein. The material that remained adjacent to the well in lane B was examined by electron microscopy and found to contain >70% joint molecules. (*b*) Effect of heterologous SSB proteins. Lane A, 0.02 μM HPP-1 only; lane B, 0.02 μM HPP-1 and 0.2 μM yRP-A; lane C, 0.02 μM HPP-1 and 0.2 μM yRP-A(70*35) [note: yRP-A(70*35) was purified as a 35-kDa proteolytic product (17) of the 70-kDa full-length protein]; lane D, 0.02 μM HPP-1 and 5 μM T4 gene *32* protein; lane E, 0.02 μM HPP-1 and 5 μM *E. coli* SSB protein; lane F, 0.2 μM HPP-1 only; lane G, no protein. In addition to the amount of added heterologous SSB protein shown in *b*, a complete titration of heterologous SSB proteins at six different concentrations was performed. At peak stimulation concentrations, the amount of joint molecule product for T4 gene *32* (5 μM) was potentially 4-fold over the unstimulated control and decreased to baseline at 10 μM; for yRP-A(70*35) (0.3 μM), product formation was 2.5-fold over unstimulated control and no product over the unstimulated control was observed for either yRP-A or *E. coli* SSB. For the experiment shown, 0.13 μM hRP-A was used as a control stimulatory protein and promoted the formation of 37% of the joint molecule product (the calculated amount of product formed by this ssDNA/protein ratio, based on the experiments shown in Fig. 3, is 40%). All measurements were performed in the kinetically linear portion of the homologous-pairing reaction.

The homologous-pairing reaction catalyzed by HPP-1 is stimulated by the hRP-A holoprotein (Fig. 3). The presence of stoichiometric quantities of hRP-A results in the efficient production of joint molecules in the presence of 1/10th the amount of HPP-1 used in an unstimulated reaction. The cofactor requirements for hRP-A-stimulated homologous pairing are identical to the reaction catalyzed by purified HPP-1 (7) (data not shown). The rate of formation and the final amount of joint molecule product formed in the presence of subreactive amounts of HPP-1 are dependent upon the concentration of hRP-A holoprotein introduced into the reaction (Fig. 3*A*). These results suggest a lack of protein turnover during the reaction and mimic the kinetics of product formation in the presence of stoichiometric quantities of HPP-1. Thus, although the requirement for HPP-1 has been reduced to near-catalytic quantities, we can find no differences in the mechanism of the homologous-pairing reaction when hRP-A is present. The kinetics of joint molecule formation is linear for at least 30 min after addition of the reactants (Fig. 3*A*). A comparison of the stimulation produced after a 5-min reaction by various quantities of hRP-A in the presence of a fixed amount of HPP-1 is shown in Fig. 3*C*. The addition of RP-A holoprotein increases the initial rate of joint molecule formation up to 70-fold. Furthermore, when the concentration of hRP-A protein approaches the concentration of single-stranded nucleotides, the rate begins to decline, suggesting that high concentrations of hRP-A holoprotein may reduce the accessibility of HPP-1 to the DNA.

The hRP-A(70) subunit of the hRP-A holoprotein alone is sufficient for the stimulation of homologous pairing catalyzed by HPP-1 (Fig. 4*a*, lane B). This result is analogous to the ySSB protein stimulation of yeast SEP-1, in which a proteolytic fragment of the 70-kDa subunit of the yRP-A holoprotein complex was found to be sufficient for stimulation of the yeast homologous-pairing reaction. However, the amount of complete strand-exchange product (form II) appeared to be greater in the presence of the hRP-A holoprotein than in the presence of only the hRP-A(70) subunit (data not shown). Furthermore, a substantial portion of the reaction products fails to enter the gel system; although, by electron microscopy, they appear identical to normal joint molecule products (data not shown). This latter result appears similar to the SF-1 stimulation of SEP-1 observed by Norris and Kolodner (30). An understanding of the differences observed using the complete hRP-A holoprotein complex and the hRP-A(70) subunit in the homologous-pairing reaction catalyzed by HPP-1 requires further investigation.

Only the Cognate SSB Is Capable of Stimulating Homologous Pairing in Eukaryotes. HPP-1-catalyzed homologous pairing and strand exchange are not significantly stimulated (see legend to Fig. 4) by phage, bacterial, or yeast SSB proteins (Fig. 4*b*, lanes B–E). This observation indicates that the stimulation of homologous pairing by hRP-A holoprotein is not exclusively a result of its ability to bind ssDNA. Table 1 is a compilation of the stimulatory activity produced by

Table 1. Stimulation of homologous pairing

Protein	RecA*	SEP-1*	HPP-1
SSB			
E. coli	+	−	−
T4 gene *32*	+	−	−
RP-A			
Yeast	+	+	−
Human	+	−	+

*See refs. 15 and 17 (W.-D. Heyer and L.E., unpublished data).

several SSB proteins on the reaction catalyzed by three different homologous-pairing proteins (17). The inability of the yRP-A holoprotein to stimulate the HPP-1-catalyzed homologous pairing, in spite of its close similarity to hRP-A holoprotein (and likewise for yeast SEP-1), suggests two possibilities: (*i*) recombination in eukaryotes may require specific protein–protein interactions that do not cross taxonomic kingdoms or (*ii*) a DNA/RP-A/HPP-1 complex is formed that is organism specific. It has been proposed that a secondary role for SSB protein in the RecA-catalyzed reaction is to increase the lifetime of RecA–ssDNA complexes (31). Such a role for hRP-A in the HPP-1-catalyzed reaction could also be imagined; however, the protein–protein contacts are likely to be more remarkable, since hRP-A holoprotein uniquely stimulates the homologous-pairing reaction.

DISCUSSION

We have demonstrated that hRP-A holoprotein uniquely stimulates the homologous-pairing and strand-exchange reaction catalyzed by HPP-1. These experiments were performed with proteins that were independently purified to near homogeneity and demonstrate in mammalian cells a function for hRP-A outside of the replication reaction. Our studies further suggest that a reconstitution of the human homologous recombination reaction with purified protein components is possible. The use of Z-DNA column chromatography has facilitated the identification of human recombination proteins because they appear to bind and elute from the matrix in a complex form. Two apparent components of this complex have been recognized here as HPP-1 and hRP-A. Identification and purification of other protein components are necessary.

We have estimated that there are $\approx 10^3$ molecules of HPP-1 per human nucleus (7), which is not enough to promote homologous pairing in human cells, if it were required in stoichiometric quantities *in vivo* as it is *in vitro*. hRP-A is an abundant nuclear protein that could substantially reduce the requirement of HPP-1 in homologous recombination. Furthermore, the hRP-A(32) subunit is a phosphoprotein that is phosphorylated at G_1–S phase and dephosphorylated at S–G_2 phase of the cell cycle (32, 33). These observations may indicate that replication and recombination in eukaryotic cells are modulated by hRP-A function. The apparent absence of the hRP-A(32) subunit from the high molecular weight complex may indicate that recombination functions utilize an altered form of the hRP-A holoprotein.

The homologous-pairing reaction catalyzed by HPP-1 and hRP-A holoprotein appears similar to the reaction observed in less purified preparations of HPP-1 (27). Yet, there is no requirement for ATP in the reaction, as has been observed by several laboratories (12, 34), nor do the protein components turn over during the reaction (7). The addition of the hRP-A holoprotein to the human recombination reaction mixture makes it more likely that a catalytic coupling and turn-over activity can be identified, if it exists.

The specific stimulation of replication, excision repair, and homologous pairing by cognate SSBs appears to be a general trait of eukaryotic systems. The observed specificity could be mediated by unique protein–protein contacts or through specific structural changes in the DNA promoted by protein binding. Both possibilities can be addressed experimentally. Our results suggest that general recombination, recombination-dependent radiation repair, and perhaps the enhancement of gene targeting in human cells may require several levels of synchronized protein and DNA interactions. Studies are necessary to determine the molecular nature of any protein contacts between HPP-1 and hRP-A in human cells.

We thank Richard Kolodner for communicating unpublished results, providing yeast yRP-A and ySSB [yRP-A(70*35)] proteins, and suggesting that hRP-A might be the stimulatory component in the recombination complex. Myra Derbyshire and David Garfinkel provided critical comments, Candice Harris assisted in the purification of HPP-1, and Julie Ratliff prepared the manuscript. This research was sponsored in part by the National Cancer Institute, Department of Health and Human Services, under Contract N01-CO-74101 with ABL.

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