RNA-DNA hybridization promoted by *E.coli* RecA protein

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ABSTRACT

RecA protein of *E.coli* plays a central regulatory role that is induced by damage to DNA and results in the inactivation of LexA repressor. *In vitro*, RecA protein binds preferentially to single-stranded DNA to form a nucleoprotein filament that can recognize homology in naked duplex DNA and promote extensive strand exchange. Although RecA protein shows little tendency at neutral pH to bind to RNA, we found that it nonetheless catalyzed at 37°C the hybridization of complementary RNA and single-stranded DNA sequences. Hybrids made by RecA protein at 37°C appeared indistinguishable from ones prepared by thermal annealing. RNA-DNA hybridization by RecA protein at neutral pH required, as does RecA-promoted homologous pairing, optimal conditions for the formation of RecA nucleoprotein filaments. The cosedimentation of RNA with those filaments further paralleled observations made on the formation of networks of nucleoprotein filaments with double-stranded DNA, an instrumental intermediate in homologous pairing *in vitro*. These similarities with the pairing reaction support the view that RecA protein acts specifically in the hybridization reaction.

INTRODUCTION

The induction of RecA protein *in vivo* triggers a set of cellular responses, commonly referred to as the SOS response (1). This pleiotropic response is complex and only partially understood. Induction of the SOS response is triggered by various lesions to duplex DNA which activate the cleavage of the LexA repressor by RecA protein (2, 3). In addition to its role in inactivating Lex A repressor, which results in the derepression of some 20 genes of repair and recombination, RecA protein *in vitro* catalyzes a number of DNA pairing reactions (4, 5). For simplicity, we will refer to the pairing of single-stranded DNA with double-stranded DNA as *homologous pairing* , while *renaturation* will be reserved for the reannealing of two complementary single-strands, and *hybridization* for the annealing of complementary RNA with single-stranded DNA.

The prototypic recombination reaction of RecA protein is the pairing of single-stranded DNA with duplex DNA, and subsequent strand exchange that produces a new heteroduplex molecule and a displaced strand from the original duplex molecule. The principal instrument of this reaction is the RecA nucleoprotein filament. The filament displays right-handedness with a helical repeat unit of 1 RecA monomer for every 3.5 or 3.6 nucleotides (4–7), in which the DNA bases manifest an axial spacing that is 1.5 times that of B-form duplex DNA (8). The phosphodiester backbone lies with its bases likely perpendicular to the helical axis (9, 10), in the deep groove of the filament. The RecA nucleoprotein filament provides a helical scaffold for strand exchange (7, 11).

RecA protein will also promote the renaturation of complementary single strands, provided that one of the two complementary strands or some part of both is not coated by RecA protein (12, 13, 14, 15). The inhibition of renaturation of complementary strands of DNA when both are fully coated by RecA protein, and the interaction of single-stranded RecA nucleoprotein filaments with naked duplex DNA that underlies homologous pairing show that recognition of complementarity is mediated by one molecule to which RecA protein is bound and one molecule that is free of protein. Accordingly, previous evidence that RecA protein reacts less well with RNA than DNA (16) suggested that it might promote the hybridization of RNA with DNA via a reaction of the DNA nucleoprotein filament with naked RNA. Investigation of the latter reaction is the subject of this report. A discussion of reaction kinetics and of the effects of competing nucleic acids is presented in the accompanying paper.

MATERIALS AND METHODS

Materials

RecA protein was purified essentially as described (17). SSB protein was purchased from U.S. Biochemical Corporation. The monoclonal antibody against RecA protein was kindly prepared by Dr. John Flory (18). T3 and T7 RNA polymerases were purchased from Promega and Boehringer Mannheim. Dithiothreitol (DTT) was from Promega and diethylpyrocarbonate from Sigma. RNasin^r Ribonuclease inhibitor was obtained from Promega, RNase-free DNaseI and Proteinase K from Boehringer Mannheim, sodium dodecyl sulfate (SDS) from Sigma. 'Molecular Biology Certified' grade agarose was purchased from Bio-Rad, Inc. Restriction enzymes BclI, HindIII and SalI were purchased from Boehringer Mannheim, EcoRV and AatII from

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New England BioLabs, restriction enzymes EcoR1, Kpn1 and Sac1 from International Biotechnologies, Inc. The Klenow fragment from DNA Polymerase I was from Boehringer Mannheim, T4 DNA ligase from International Biotechnologies, Inc. The DH5αF′ cell strain was purchased from Stratagene. ATP was purchased from Sigma, ATPγS, from Boehringer Mannheim. The four unlabeled ribonucleoside triphosphates used for the *in vitro* transcriptions were obtained from Promega corporation, as components of a transcription kit. Their concentrations were checked by U.V. spectrophotometry.

DNA preparations

Single-stranded DNA constructs

Three single-stranded DNA constructs were prepared, referred to hereafter as constructs C, D and I. Genes encoding tetracycline resistance (Tet^r) and chloramphenicol resistance (Cm^r) were subcloned into the phage M13 derivative, M13mp18. Both genes were derived from plasmid pBR328: The Tet^r gene was removed with a double restriction digest at the unique HindIII and AvaI restriction sites, yielding the gene on a fragment 1394 nucleotides long. A fragment containing the Cm^r gene was generated with a double restriction digest at the unique HindIII and AatII sites in pBR328, yielding a fragment 1741 nucleotides long. The ends of the Tet^r fragment were filled by the Klenow enzyme, while the AatII end of the Cm^r fragment was resected with the Klenow enzyme, and the HindIII end then filled. Both fragments were subcloned into the SmaI site of the polylinker region of M13mp18. Positive subclones yielded blue plaques on a lawn of DH5αF′ cells, in the presence of X-gal (5-bromo-4-chloro-3-indol-β-D-galactopyranoside) (19). Preparations of hybrid phage DNA were done on a small scale (20), and characterized.

Two single-stranded DNA clones with the Tet^r sequence were identified, each entailing one particular strand of the gene. One clone was identified with the non-transcribed strand ('plus' strand) of the Cm^r gene. Large scale preparations of the three single-stranded DNA constructs C, D and I were done as described (21).

Double-stranded DNA constructs

Double-stranded DNA constructs C and D were produced by subcloning the Tet^r gene, prepared as described above, into the unique SmaI site of the polylinker of Bluescript vector KS (+) (Strategene). In construct C, the Tet^r gene was oriented opposite to the Amp^r gene in the vector, whereas in construct D, Tet^r was oriented in the other direction.

The pBR328-derived fragment containing the Cm^r gene (22) was subcloned into the polylinker region of the Bluescript vector, at proximal sites to the T7 and T3 transcription initiation sequences. The Kpn1 site was proximal to the T3 initiation site, while the Sac1 site was proximal to the T7 initiation site. The Bluescript vector was prepared for subcloning by successive restriction digests with Kpn1 and at Sac1, followed by resection of the protruding 3′ ends with the Klenow enzyme. Blunt-end ligation with the Cm^r fragment yielded subclones with one orientation only. Since pBR328 contained an inverted duplication of the distal end of the Tet^r gene, positioned between the Bcl1 and the Cla1 restriction sites of the Cm^r fragment used in the subclonings (23), we removed that sequence from the construct by successive restriction digests with restriction enzymes Bcl1 and Sal1. The resulting construct we designated as I, and used subsequently for *in vitro* preparations of I:EcoR1 RNA. Large scale plasmid preparations of the three double-stranded constructs were followed, as described (20).

Preparation of linear DNA templates

Double-stranded DNA constructs C and D were each digested with restriction enzyme Sal1. Additional construct D was separately digested with EcoRV. In the three instances, each digest yielded two fragments, with the T7-dependent transcription units residing on the larger of the two fragments. The fourth template was generated by linearizing construct I at its unique EcoR1 site. The DNA templates were purified with phenol and chloroform extractions, precipitated in 95% ethanol and resuspended in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) for use in the transcription reactions.

RNA preparations

Unlabeled RNA and uniformly labeled high specific activity RNA were prepared by *in vitro* transcription protocols outlined by Promega. Transcripts C:Sal 1, D:Sal 1 and D:EcoRV were generated by T7 RNA polymerase, while transcript I:EcoR 1 was generated by T3 RNA polymerase (Fig. 1). The quality of the transcripts was monitored by agarose gel electrophoresis, and their concentration determined by U.V. spectrophotometry. The residue concentration of labeled RNA was determined by computing the number of pmols of incorporated labeled ribonucleotide in a total preparation of purified RNA substrate, and then multiplying that number by a factor of 4.

RNA and single-stranded DNA substrates which share sequence complementarity are represented by the same alphabetical letter. C:Sal1 RNA designates RNA generated from the double-stranded DNA template restricted at Sal I, and capable of forming a hybrid with single-stranded DNA-C.

Preparation of reaction buffers

Reaction buffers used in experiments involving ATPγS contained 33 mM Hepes buffer, pH 7.4, and 2 mM DTT. In ATPγS experiments, the nucleotide cofactor and Mg^{2+} were added separately. Those components were present together in the 2× concentrated reaction buffer used in the experiments with ATP and a regenerating system. The composition of the 2× concentrated buffer was: 66 mM Tris-HCl pH 7.5, 4 mM DTT, 3.0 mM ATP, 16.0 mM phosphocreatine (Sigma) and 2 mM $MgCl_2$. Creatine phosphokinase (Sigma) was added at a final concentration of 10 units/ml. The pH values for the buffers stocks were determined both at room temperature and at 37°C.

Description of assays

Immunoprecipitation of RecA protein-RNA complexes

A monoclonal antibody against RecA protein was used to compare the binding of RecA protein to RNA versus single-stranded DNA. The immunoprecipitation conditions were optimized with ^{35}S-labeled RecA protein, with respect to the molar ratio of antibody to RecA protein, and length of time of incubation with antibody.

An immunoprecipitation experiment involved incubation of RecA protein with ^{3}H-uniformly labeled RNA at 37°C, under the standard conditions, in the presence of ATPγS, for 10 minutes. The antibody was then added at 1/2 the micromolar concentration of RecA protein, and an aliquot from the reaction mixture was removed and used to measure total counts. Incubation of the remainder of the reaction was continued for another 10 minutes at 37°C, followed by centrifugation at 13000 g for 5 minutes at room temperature. An aliquot was removed from the supernatant, and the percentage of labeled RNA in immunoprecipitable complexes recorded.

Agarose gel electrophoresis

Deproteinized reaction samples were electrophoresed on 1% agarose gels in TBE buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA), typically for 2 hours at 120 V and 100 mA. The gels were then dried and subjected to autoradiography. Native unhybridized RNA and RNA in hybrid form were readily distinguishable, as the migration of hybridized RNA was retarded (24). Quantitation was done by direct scanning of the dried gel with a Betascope 603 blot analyzer (Betagen, Waltham, MA).

RESULTS

Hybridization of RNA transcripts with single-stranded DNA by RecA protein at 37°C

The system

To study this reaction, we prepared the plasmid and phage vectors shown in Fig.1. The plasmid vectors served for the production of specific RNA transcripts *in vitro* from T7 or T3 promoters; and the phage vectors served for the production of a unique strand of DNA that was complementary to the transcript, but which lacked a complementary DNA strand.

RNA and DNA were hybridized under standard conditions in which single-stranded DNA was first incubated with RecA protein in the presence of a nucleotide cofactor and 1mM Mg^{2+}, followed by the addition of labeled RNA and more Mg^{2+}, bringing the latter to 12mM. With the concentration of RNA in the nanomolar range and that of DNA in the micromolar range, DNA was in large excess. The nucleotide cofactor in most cases was the non-hydrolyzable analog, ATPγS. In a few experiments, as indicated, ATP was used. All observed parameters have been the same for both cofactors. Hybridization was allowed to proceed for 20 minutes, unless otherwise specified. The reaction was terminated by a combined treatment with proteinase K and SDS, at final concentrations of 200 μg/ml and 0.4%, respectively. In control reactions where RecA protein was omitted, dialysis buffer (buffer R composition: 50 mM Tris-HCl pH 7.5, 5 mM DTT, 0.3 mM EDTA and 10% glycerol) was substituted. Hybridization was detected by electrophoresis of the deproteinized samples in 1% agarose gels. Hybridized and unhybridized RNA species were visualized by autoradiography of the dried gels, and measured quantitatively by direct gel scanning (see Methods).

Requirements

In the complete reaction, hybridization was indicated by the slower migration of RNA (Fig. 2). No reaction product was observed in the absence of complementary single-stranded DNA substrate, or in the presence of noncomplementary DNA. A trace signal was detectable, if Mg^{2+} ions were omitted, and more obvious but still minor hybrid bands were seen in the absence of RecA protein or a nucleotide cofactor. The yield of hybrid in the reaction promoted by RecA protein was greater than that of the protein-independent hybridization by a factor which varied from 10 to greater than 50. The hybrid band observed as a result of protein-independent hybridization was found to vary between 0% and 8% of total labeled RNA, and to be a function of a number of parameters (see Discussion).

An experiment with three sets of complementary single-stranded DNA and RNA substrates confirmed that hybridization was contingent on sequence complementarity between the RNA and the single-stranded DNA substrates (25). We further observed that for transcripts ranging from 200 to 800 nucleotides in length, there appeared to be no difference in the efficiency of hybrid formation as a function of RNA length (25).

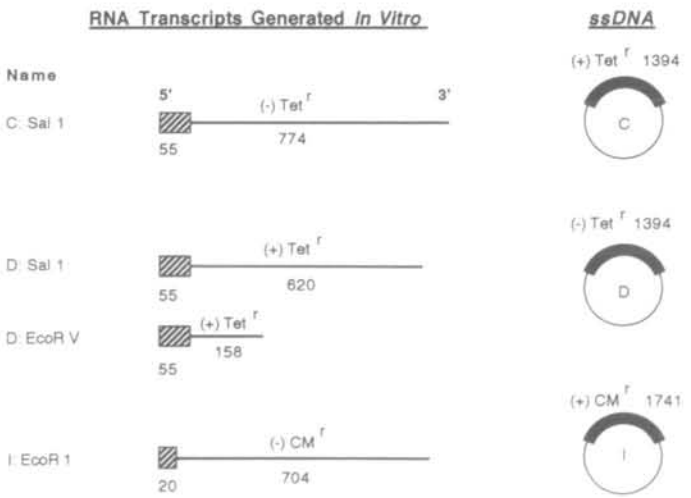


Figure 1. Single-stranded DNA and RNA substrates used in the model reaction. Fragments from pBR328, containing the genes encoding for tetracycline resistance (Tet^r) and for chloramphenicol resistance (Cm^r), were subcloned into M13mp18, to generate single-stranded DNA constructs. Subclones were obtained with both strands of the Tet^r gene, and with the (+) or non-transcribed strand of the Cm^r gene. The double-stranded DNA constructs were produced by inserting either antibiotic resistance gene into the Bluescript vector KS(+). The RNA transcripts were generated *in vitro*, after linearizing the double-stranded DNA constructs at a designated restriction site. The striped box at the 5′ end of the transcript corresponds to sequence complementary to polylinker sequence from the vector only, and therefore, noncomplementary to sequence from the antibiotic resistance gene.

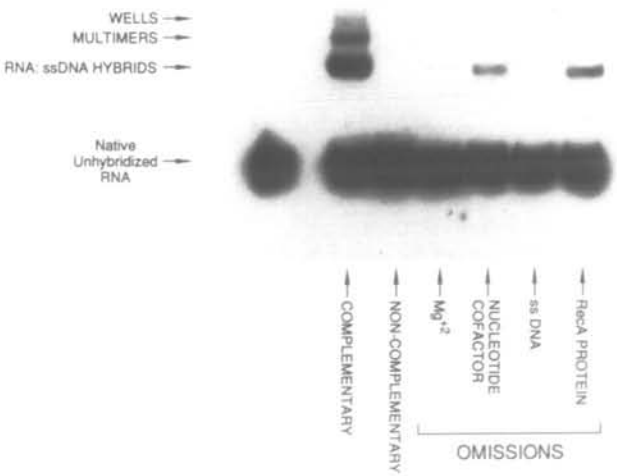


Figure 2. Requirements for the hybridization reaction. The standard hybridization protocol, described in Methods, was modified by the substitution of ATP and a regenerating system for ATPγS. Single-stranded DNA-D and ^{32}P-labeled D:EcoRV RNA substrates (Fig. 1) were at final concentrations of 10 μM and 1 nM, respectively. For the noncomplementary reaction, single-stranded DNA-I was substituted for single-stranded DNA-D at the same final concentration. The ratio of RecA/single-stranded DNA nucleotides was 1/2, and the reactions were done in Hepes buffer at pH 7.4. Where RecA protein was omitted, protein dialysis buffer was substituted at an equivalent volume. Deproteinization and autoradiography were described in Methods.

Stoichiometry

In an experiment done under standard conditions with ATPγS, the ratio of RecA monomers to nucleotide residues of single-stranded DNA was varied from 1/1 to 1/25, while the concentration of single-stranded DNA was kept constant at 10 μM. A maximal yield of hybrid around 50% was observed with ratios of 1/1, 1/2 and 1/4, whereas decreasing yields were associated with ratios of 1/10 or less (Fig. 3). Similar yields were observed when the titration experiments were done with ATP as nucleotide cofactor, in the presence of a regenerating system (data not shown). A ratio of 1/4 suffices for a threshold level of saturation of single-stranded DNA by RecA protein (6, 7), while ratios of 1/2 and 1/1 entail excess RecA protein. Optimal hybridization conditions were, therefore, ones which supported the formation of saturated RecA nucleoprotein filaments. By contrast, RecA-mediated renaturation of complementary strands of DNA is optimal at a RecA/nucleotide ratio of 1/30 (13). As mediated by RecA protein, RNA-DNA hybridization and DNA renaturation were further distinguishable: Whereas excess RecA protein inhibits renaturation (13, 14), it did not inhibit hybridization (Fig. 3, and see Discussion).

As may be noted in the experiments described above, although the DNA in these hybridization reactions was in excess by three orders of magnitude, RNA was not completely incorporated into hybrids. This factor is investigated in the following paper.

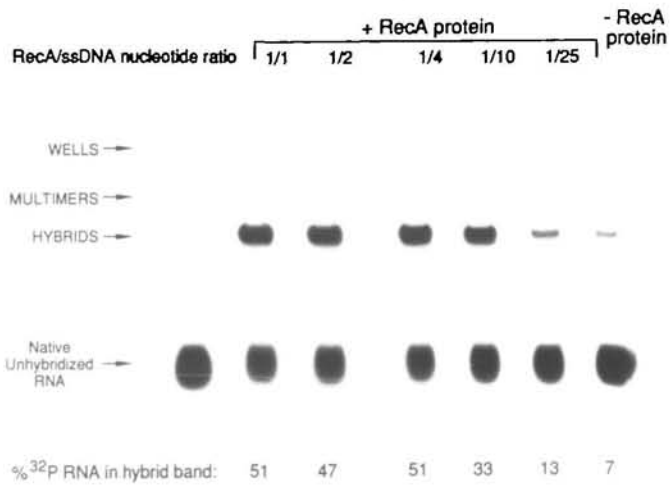


Figure 3. Requirement for stoichiometric amounts of RecA protein. Reactions followed the standard protocol, with ATPγS as cofactor. Single-stranded DNA-I and ^{32}P-labeled I:EcoR1 RNA were at final concentrations of 10 μM and 1 nM, respectively. The ratio of RecA protein to single-stranded DNA was changed by adjusting the final concentration of RecA protein. The percentage of labeled RNA in hybrid form is given for each lane, as determined by direct scanning of the gel.

Characterization of the product
Comigration with annealed hybrids

The products of RecA-mediated hybridization were compared by agarose gel electrophoresis, with hybrids prepared by thermal annealing (Fig. 4). The comparison was undertaken with two pairs of substrates: Single-stranded DNA-D plus ^{32}P-labeled D:EcoRV RNA and single-stranded DNA-I plus ^{32}P-labeled I:EcoR1 RNA. Hybrids in lanes A were prepared with RecA protein, following the standard hybridization protocol with ATPγS, while the hybrids in lanes B were prepared by a thermal annealing protocol, as described (see legend, Fig. 4; Ref.20). With both sets of substrates, bands corresponding to the hybrid species prepared thermally and enzymologically co-migrated. Curiously, the unhybridized RNA did not migrate identically for the enzymological and the thermal reaction mixtures. Since we have observed elsewhere differences in electrophoretic mobility of RNA samples that change upon heating, we attribute the differences in the present case to secondary structure or intermolecular interactions between RNA transcripts.

Sensitivity to RNase H

RNase H is a ribonuclease that specifically recognizes and degrades the RNA component of an RNA-DNA hybrid, but does not recognize single-stranded or double-stranded RNA or DNA (26). Hybrids were prepared by RecA protein under standard conditions with ATPγS, as described above, and purified in preparation for treatment with RNase H (see legend to Fig. 5). One half of the preparation was treated with RNase H, the other half with RNase H digestion buffer. The reactions were performed as described, and the outcome monitored by gel electrophoresis. The labeled RNA species in retarded bands, including those designated as multimers, were selectively degraded by RNase H, whereas unhybridized RNA was not. The bands designated as multimers were the likely result of RNA hybridization to concatemeric forms of the single-stranded DNA substrate: Higher molecular weight forms of the single-stranded DNA substrates were minor species observed by agarose gel electrophoresis, for all preparations of the phage single-stranded DNA (25).

Specificity and Path of the Reaction
Specific Requirements for Mg^{2+}

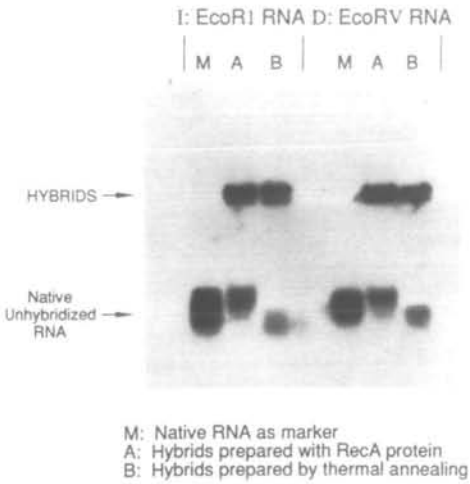
Homologous pairing of single-stranded DNA with duplex DNA by RecA protein requires the prior polymerization of RecA protein on single-stranded DNA to form the helical RecA nucleoprotein filament (7, 27). In the absence of *E.coli* SSB, this polymerization is favored by low concentrations of Mg^{2+}, which reduce secondary structure in single-stranded DNA (7, 28). However, synapsis requires a higher concentration of Mg^{2+} than that which is optimal for the presynaptic step (11, 29, 30). We, therefore, investigated the effect of Mg^{2+} ion concentration on the preincubation and the hybridization steps of the hybridization reaction.

The concentration of Mg^{2+} was held constant at 1 mM during the preincubation step, but varied during hybridization. Hybrid product was not formed when the Mg^{2+} concentration was at 1 mM during the hybridization step, but the yield increased as Mg^{2+} was raised from 3 mM to 12mM (Fig. 6, left-hand panel). Concentrations of 3 mM or 12 mM Mg^{2+} during preincubation, on the other hand, severely inhibited the reaction, even though the final Mg^{2+} concentration during hybridization was at 12 mM (Fig. 6, right-hand panel).

Cosedimentation of RNA with RecA nucleoprotein filaments is sequence independent

Sedimentable networks of duplex DNA with RecA nucleoprotein filaments are intermediates in homologous pairing of DNA molecules (29, 31). We sought to determine if the RNA substrate would cosediment with RecA nucleoprotein filaments in the pellet fraction, upon centrifugation of a hybridization mixture (Fig. 7).

Standard hybridization protocols with 12 mM Mg^{2+} and ATPγS were followed for 20 minutes, whereupon reaction



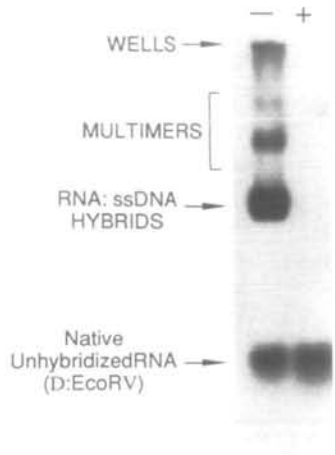


Figure 4. Comigration of hybrids made by RecA protein with those made by thermal annealing. Hybrids in lanes A were prepared with RecA protein, following the standard hybridization protocol with ATPγS, as described in Methods. The hybridization reactions were done for 20 minutes. The hybrids in lanes B were prepared by a thermal annealing protocol, as described (20). RNA was incubated with its complementary single-stranded DNA, in formamide buffer. The single-stranded DNA in either preparation was in approximately 200 fold nucleotide excess over its complementary RNA. The composition of the annealing buffer was 80% formamide, 40 mM Pipes pH 6.7, 0.4 M NaCl and 1 mM EDTA. The samples were heated at 80°C for 15 minutes, and then placed in a waterbath at 50°C for 3 hours. Each of the samples was subsequently desalted with 2 gel filtration sephadex G50 mini-columns, and run next to the hybrid preparations made with RecA protein.

Figure 5. Hybrids made by RecA protein are sensitive to RNase H treatment. Hybrids between single-stranded DNA-D and ^{32}P-labeled D:EcoRV RNA were prepared with RecA protein, following the standard hybridization protocol with ATPγS, as described in Methods. The reaction was terminated by a combined treatment with proteinase K and SDS. The reaction products were purified with phenol-chloroform extractions, followed by precipitation in 95% ethanol at −70°C for 30 minutes. The resuspended pellet was dissolved in DEPC-treated water. One half of the products were treated with 1.9 units of RNase H, the other half with RNase H digestion buffer only. RNase H treatment was in 40 mM Tris-HCl, pH 8.0, 5 mM $MgCl_2$, and 1 mM DTT, for 5 minutes at 32°C. The reaction was quenched with 10 mM EDTA, pH 8.0 and 200 μg/ml of proteinase K, for 15 minutes at 37°C. The RNase H-treated samples were then placed on ice and loaded onto a 1% agarose gel for electrophoresis.

mixtures were subjected to centrifugation at room temperature and at 13000 g, for 5 minutes. Three aliquots were sequentially removed, and labeled supernatants 1 and 2, and pellet. Each was treated with proteinase K and SDS, prior to gel electrophoresis. When RecA protein was present, between 80 and 85% of the labeled RNA was found in the pellet for both complementary and noncomplementary pairs of substrates. In contrast, when RecA protein was omitted in a reaction with complementary substrates, labeled RNA was distributed evenly across all fractions. Preferential localization of RNA in the pellet fraction was, therefore, dependent on the presence of RecA protein, and independent of sequence complementarity. Experiments done in parallel showed that nucleoprotein filaments formed by RecA protein on ^{3}H-labeled single-stranded DNA, with ATPγS and 12 mM Mg^{2+} were sedimentable (data not shown). RNA, therefore, does not cause the aggregation of RecA nucleoprotein filaments, but rather cosediments with them or their aggregates, under optimal hybridization conditions. This result is further indication of a reaction mechanism which involves interactions between RecA nucleoprotein filaments and naked RNA (see Discussion).

Binding of RecA protein to RNA

Early literature on the enzymology of RecA protein showed ribohomopolymers to be poor cofactors for RecA protein-dependent ATPase activity (16, 32) and binding (33). Ogawa and colleagues found that phage RNAs were also poor cofactors for the RecA-dependent ATPase activity (16).

We compared the ability of RecA protein to form immunoprecipitable complexes with single-stranded DNA versus RNA, at neutral pH (Table 1). Either polynucleotide was incubated with RecA protein at 1 mM or 12 mM Mg^{2+}, with ATPγS as nucleotide cofactor. The final concentration of Mg^{2+} did not appear to affect in any obvious way the interaction of the monoclonal antibody with RecA protein, as the immunoprecipitation signals for RecA-single-stranded DNA complexes, in the absence of SSB protein, were comparable at both Mg^{2+} concentrations.

In the absence of SSB protein, at both 1 mM and 12 mM Mg^{2+}, all single-stranded DNA was found in immunoprecipitable complexes, and whereas 60% to 70% of the RNA was found in complexes at 1 mM Mg^{2+}, only 7% was in complexes at 12 mM Mg^{2+}. The formation of immunoprecipitable complexes of RecA protein with RNA at 1 mM Mg^{2+} depended on the presence of a nucleotide cofactor, as the omission of ATPγS reduced the signal from 60−70% to 10%. The apparent Mg^{2+} ion-dependence of the binding affinity of RecA protein for RNA was supported by other experiments: A gradual drop in the levels of immunoprecipitable complexes between RecA protein and RNA was observed, as the Mg^{2+} concentration was raised in increments from 1 mM to 12 mM (25). We also detected a sharp drop in immunoprecipitable complexes when the Mg^{2+} ion concentration in a reaction mixture was raised directly from 1 mM to 12 mM (25). The lesser binding by RecA protein to RNA than single-stranded DNA, was also seen when SSB protein was present (Table 1). The binding of SSB protein to RNA has been reported by others (34).

We also examined the ability of RNA to serve as a cofactor for ATPase activity, in a direct comparison with single-stranded DNA. At pH 7.4, RecA protein manifested single-stranded DNA-

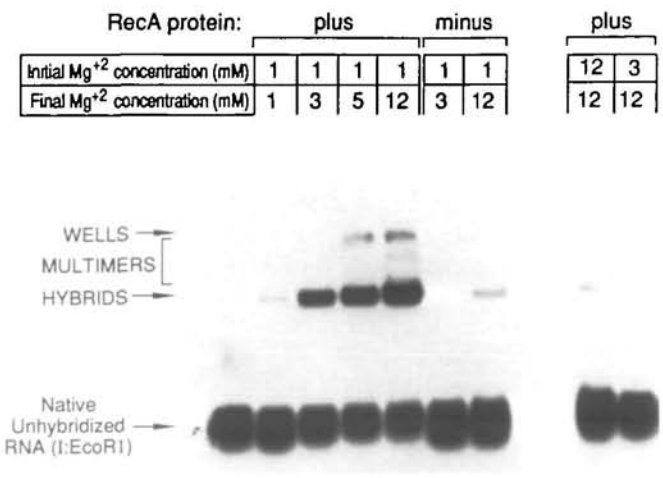


Figure 6. The requirements for specific concentrations of Mg^{2+} ions in RecA protein-promoted hybridization are similar to those for the DNA pairing activities catalyzed by RecA protein. Reactions were done according to the standard protocol in the presence of ATPγS (see Methods). The final concentrations of single-stranded DNA-I and ^{32}P-labeled I:EcoR1 RNA were 10 μM and 1 nM, respectively. The ratio of RecA/single-stranded DNA nucleotides was 1/4, and the reactions were done in Hepes buffer at pH 7.4. Left-hand panel: The concentration of Mg^{2+}, held constant during the preincubation of RecA protein with single-stranded DNA, was varied during the final incubation of the RecA nucleoprotein filaments with RNA. The left-most lane contained only labeled RNA as marker. Right-hand panel: The effect of higher concentrations of Mg^{2+} during the preincubation of single-stranded DNA with RecA protein.

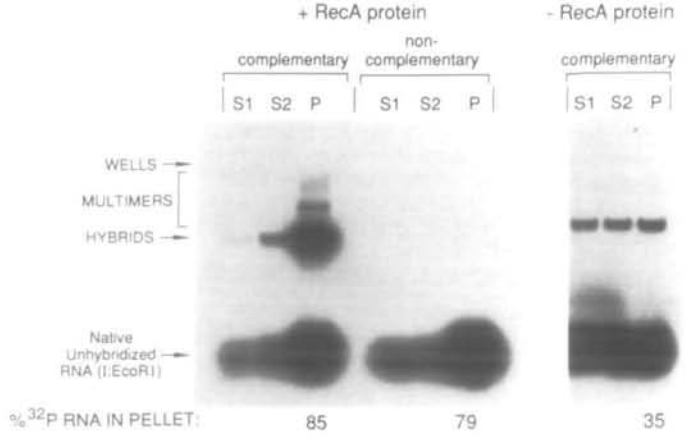


Figure 7. Cosedimentation of RNA with RecA nucleoprotein filaments at 12 mM Mg^{2+}, in the presence of ATPγS. The hybridization protocol was the standard protocol with ATPγS (see Methods). The hybridization reactions were allowed to proceed for 20 minutes, and then subjected to a spin in a benchtop microcentrifuge at 13 000 g, for 5 minutes. 3 aliquots were sequentially removed and labeled supernatant 1, supernatant 2 and pellet. Each fraction was then treated with proteinase K and SDS. Reactions were done with complementary and noncomplementary pairs of substrates in the presence of RecA protein, and a control reaction with complementary substrates was performed in the absence of RecA protein. The final concentrations of single-stranded DNA-I and D were 10 μM, and the final concentration of ^{32}P-labeled I:EcoR1 RNA, 1nM. The reactions were done in Hepes buffer at pH 7.4. Prior to loading on a 1% agarose gel, samples were counted directly by Cerenkov, and the % sedimentable labeled RNA in the pellet fraction determined for each of the reactions. The 1% agarose gel was run for 2 hours at 120 V and 100 mA, and the labeled RNA visualized by autoradiography.

Table 1. Immunoprecipitation of complexes of RecA protein with single-stranded DNA or RNA.

Polynucleotide	ATPγS	$[Mg^{2+}]$	SSB	% immunoprecip.
ssDNA	+	12	+	88
ssDNA	+	12	−	99
ssDNA	+	1	+	33
ssDNA	+	1	−	99
RNA	+	12	+	0
RNA	+	12	−	7
RNA	+	1	+	0
RNA	+	1	−	60/69**
RNA	−	1	−	10

The single-stranded DNA substrate was ^{3}H-labeled M13 circular single-stranded DNA; the ^{3}H-labeled RNA substrate, 238 nucleotides long, was prepared *in vitro* from the Bluescript cloning vector linearized at a unique PvuII restriction site. The preparation was completed as detailed in Methods, with the omission of the gel purification step. In all experiments, the concentration of RecA protein was 1.5 μM, and SSB protein 0.4 μM. In both cases, the proteins exceeded the concentration required for stoichiometric coating of single-stranded DNA, whose concentration was 3 μM. The concentration of labeled RNA substrate was in the range of 1 nM to 5 nM. ATPγS was at a final concentration of 3 mM. In experiments that included RecA protein and SSB protein, the polynucleotide was added last to the reaction mixture. (**) These represent the outcome of two individual experiments with the same preparation of RecA protein. Two other preparations of the protein yielded somewhat lower levels of immunoprecipitable RecA protein-RNA complexes, 46% and 48%, and another a somewhat higher yield, 80%.

dependent ATPase at both Mg^{2+} ion concentrations, while ATPase activity dependent on RNA was undetectable, at either Mg^{2+} concentration (25).

DISCUSSION

The experimental data presented here show that RecA protein catalyzes the formation of RNA-DNA hybrids. The reaction signal observed in the presence of RecA protein was greater by 10 to 50 fold over a spontaneous or protein-independent hybridization signal. The latter itself was contingent on a number of parameters: The concentration of Mg^{2+} ions (Fig. 6), the final nucleotide concentration of hybridizing single-stranded DNA and the presence of any competing polynucleotides (see accompanying paper). Unlike the RecA protein-dependent signal, the protein-independent signal did not increase over time.

The experiments presented here indicate an apparent similarity in the pathways for hybridization and DNA pairing. In both hybridization and homologous pairing, the two substrates are recognized differently by RecA protein at physiological pH. The stable nucleoprotein filament constitutes the active hybridization or pairing scaffold, while the second substrate, RNA or duplex DNA, remains largely uncoated and presumably is assimilated as a naked polynucleotide into the nucleoprotein filament. The hybridization and the binding data presented in this paper support that view.

Optimal hybridization was observed under conditions that were suitable for the formation of active RecA nucleoprotein filaments. These included the stoichiometric coating of single-stranded DNA with RecA protein (Fig. 3), specific requirements for Mg^{2+} ions during the preincubation and the hybridization steps of the reaction (Fig. 6) and a requirement for ATP (Fig. 1) or the non-hydrolyzable analog, ATPγS (Fig. 3). The sequence-independent cosedimentation of RNA with RecA nucleoprotein filaments, after

the centrifugation of a reaction mixture (Fig. 7), was reminiscent of the networks formed by short duplex DNA with RecA nucleoprotein filaments, detected by a similar assay (29; and see Discussion, accompanying paper).

The similarity in the pathways for hybridization and homologous pairing highlights some striking differences between the RNA-DNA hybridization and the renaturation of complementary single-strands of DNA. A central feature of RecA enzymology is that sequence alignment between two polynucleotides cannot be mediated through two nucleoprotein filaments (12). Because the substrates in the renaturation reaction are identical, and are, thus, recognized in the same manner by RecA protein, renaturation *in vitro* has only been documented under conditions, where either the complementary single-strands are only partially coated (13, 14, 15), or where saturated RecA-single-stranded DNA-ATPγS complexes are first purified by gel filtration chromatography, prior to incubation with protein-free deoxyribooligonucleotides (12). Concentrations of RecA protein in excess or in saturating amounts, therefore, inhibit renaturation (12, 13, 14). By contrast, when ATP and a regenerating system are present, RecA protein in excess of catalytic amounts displays no inhibitory effect on either hybridization (25) or DNA pairing.

The binding data support the proposition that, under the hybridization conditions, RNA is not bound by free RecA protein. We showed that interactions *in vitro* between RecA protein and RNA were weak, in a direct comparison with single-stranded DNA (Table 1), and found that it is a poor cofactor for ATP hydrolysis (16, 25). More recently, Tessman and colleagues identified a number of RecA mutant cell strains, which were constitutive for the SOS response. Both purified mutant RecA proteins demonstrated greater binding affinity *in vitro* for tRNA and rRNA than for single-stranded DNA, and were also shown to have the ability to use either RNA as cofactor in the cleavage of Lex A repressor protein (35, 36). Those studies argued, therefore, against a strong primary interaction *in vivo* between wild-type RecA protein and RNA.

The reaction pathway proposed for RecA protein-driven hybridization is further suggested by observations made with the *E.coli* single-stranded DNA binding protein (SSB). Our results suggested that excess SSB interfered with RecA-driven hybridization by binding to the RNA substrate, and that the inhibition could be relieved with the presence of noncomplementary RNA transcripts in the reaction mixture (25). This capacity of SSB protein to interfere with hybridization strengthens the case for a hybridization pathway based on interactions between a RecA nucleoprotein filament and naked RNA.

Is there a binding site specific for RNA on the RecA nucleoprotein filament? The existence of at least two polynucleotide binding sites for RecA protein has been inferred on the basis of enzymological and electron microscopic data (5). It has been proposed that polynucleotide sites on the RecA monomer could display different substrate specificities or affinities. The preferential binding of single-stranded DNA at one site, presumably, activates another polynucleotide binding site (5). Current data do not allow us, however, to discern if the RNA binding site might be a subset of the duplex DNA binding site or a physically distinct one.

The reaction product made by RecA protein at 37°C was, after deproteinization, indistinguishable from hybrids prepared by thermal annealing, judging from their common mobility by gel electrophoresis (Fig. 4). Hybrids made with RecA protein were, after deproteinization, also shown to be sensitive to treatment with RNase H (Fig. 5).

In spite of the large excess of single-stranded DNA, the hybridization reaction did not incorporate all RNA into stable hybrids. ATP hydrolysis, itself, did not appear to improve the yield of hybrids (Fig. 2), as ATPγS, a non-hydrolyzable analog, was readily substituted for ATP (Fig. 3), resulting in comparable yields. From the standpoint of chemical energy, RecA-promoted hybridization is clearly efficient: New hydrogen bonds are created without any ATP hydrolysis. The apparent incompleteness of the reaction is examined in the accompanying paper. That issue notwithstanding, RecA-promoted hybridization of RNA transcripts with DNA is rapid at 37°C and, as shown in the following paper, remarkably insensitive to inhibition by large excesses of DNA and other forms of RNA.

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