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A stimulatory RNA associated with RecBCD enzyme

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ABSTRACT

RecBCD enzyme acts in the major pathway of homologous recombination of linear DNA in *Escherichia coli*. The enzyme unwinds DNA and is an ATP-dependent double-strand and single-strand exonuclease and a single-strand endonuclease; it acts at Chi recombination hotspots (5′-GCTGGTGG-3′) to produce a recombinogenic single-stranded DNA 3′-end. We found that a small RNA with a unique sequence of ~24 nt was tightly bound to RecBCD enzyme and co-purified with it. When added to native enzyme this RNA, but not four others, increased DNA unwinding and Chi nicking activities of the enzyme. In seven similarly active enzyme preparations the molar ratio of RNA molecules to RecBCD enzyme molecules ranged from 0.2 to <0.008. These results suggest that, although this unique RNA is not an essential enzyme subunit, it has a biological role in stimulating RecBCD enzyme activity.

INTRODUCTION

RecBCD enzyme (exonuclease V, EC 3.1.11.5) of *Escherichia coli* is required for the major pathway of genetic exchange following P1 transduction and Hfr conjugation, recovery from DNA damage, maintenance of cell viability and degradation of foreign DNA (reviewed in 1,2). The RecBCD enzymatic activities supporting these biological functions include ATP-dependent DNA unwinding and double-stranded (ds) DNA and single-stranded (ss) DNA exonuclease. The enzyme makes a single-strand endonucleolytic cut in DNA containing the 8 bp sequence Chi (5′-GCTGGTGG-3′; 3,4), which stimulates recombination in *E.coli* and bacteriophage λ (reviewed in 5). Single base pair mutations in the Chi sequence co-ordinately reduce or eliminate Chi-dependent cleavage and Chi stimulation of recombination (6,7). The RecBCD enzyme subunit that recognizes Chi is unknown.

RecBCD enzyme has multiple activities on linear dsDNA that help promote homologous recombination. Purified RecBCD enzyme contains three polypeptides with molecular masses of 134, 129 and 67 kDa, the products of the *recB*, *recC* and *recD* genes respectively (2,8). RecBCD enzyme binds to the ends of linear dsDNA and initiates unwinding in the presence of ATP (9,10). The DNA degradative activities of RecBCD enzyme, critical for production of a recombinogenic single-strand 3′-end, are regulated by several factors, including Chi sites and the concentration of Mg^{2+} and ATP (11–14). Reactions produce, with continued unwinding, a recombinogenic ssDNA 3′-end at Chi, a critical event in RecBCD enzyme-promoted recombination. Mutants lacking this activity lack Chi-stimulated recombination in genetic crosses and many are recombination deficient (3,15,16).

During our studies on the subunit composition of RecBCD enzyme we found a small unique RNA molecule that co-purifies with RecBCD enzyme. Here we describe the characteristics of the RNA and its biological activity.

MATERIALS AND METHODS

Enzymes

RecBCD enzyme was purified from *E.coli* strain AFT380 (3) or V182 and assayed as described (3,17,18). Enzyme from fraction IV (19) was further purified by heparin–agarose chromatography (fraction V) and glycerol gradient centrifugation (fraction VI). An additional preparation of RecBCD enzyme was purified from a derivative of *E.coli* strain CSH50 containing plasmids pB520 and pB800 (strain V2350; 20). The enzyme was purified by chromatography on heparin–agarose (4) followed by chromatography on DEAE–Sepharose (21) or on hydroxyapatite (22). The double-strand exonuclease specific activities, determined as described previously (17), were typically 2.2–3.3 × 10^5 U/mg protein. In our most active preparation there are 330 000 U/mg protein or ~5.6 × 10^9 enzyme molecules (9.3 fmol) per U dsDNA exonuclease activity. Typically, stocks of RecBCD enzyme were stored in buffer C [20 mM potassium phosphate, pH 6.8, 0.1 mM EDTA, 0.1 mM dithiothreitol, 100 µg/ml polyvinylpyrrolidonone K-60 (PVP)] containing 10 or 50% glycerol.

Two samples of RecBCD enzyme, provided by S.Kowalczykowski (University of California, Davis, CA), had specific activities of 5 × 10^4 (preparation 2) and 8.2 × 10^4 (preparation 3) U/mg protein and were in buffer B (20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50% glycerol). A sample of RecBCD enzyme provided by D.Julin (University of Maryland, College Park, MD) had a specific activity 1.1 × 10^5 U/mg protein and was in buffer D (10 mM potassium phosphate, pH 6.9, 0.1 mM EDTA, 0.1 mM dithiothreitol, 50% glycerol).

RNA

A synthetic RNA, designated control RNA, with sequence 5′-AUCGAACGUACGUAUGGCCAAGAUCUUCUG-3′ (5′- and 3′-OH) was purchased from US Biochemical or Cruachem. Two additional synthetic RNAs with the sequence 5′-AGGAAGGUGGCGGAACCACCAGC-3′ or 5′-AGGAAGGUGGCGGAACC-3′ were purchased from Cruachem and used in tests of RNA

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activity. Leucine$_1$ tRNA was provided by W.M.Holmes (Virginia Commonwealth University and the Medical College of Virgina, Richmond, VA).

Preparation of RecBCD RNA or control RNA for labeling

Boiling method. RecBCD enzyme or the control RNA was diluted in TE. Samples were diluted below 5 mM KPO_4 to prevent inhibition of T4 polynucleotide kinase (23). Samples were boiled for 10 min and cooled on ice, treated with calf intestinal phosphatase (New England Biolabs) or shrimp alkaline phosphatase (US Biochemical), which were then inactivated by addition of EGTA to 25 mM (for the former) and incubation at 65°C for 20 min.

Proteinase K method. RecBCD enzyme was diluted in TE containing 0.5% SDS, treated with 20 µg/ml proteinase K (Boehringer Mannheim) for 90 min at 37°C and extracted with phenol and chloroform. RNA was recovered by precipitation with ethanol after addition of sodium acetate to 300 mM and glycogen (Boehringer Mannheim) to 10 µg/ml. Proteinase K-treated samples were treated with phosphatase and labeled as described below. The two methods gave similar yields of labeled RNA.

Labeling and quantitation of RNA

The 5′-end of RNA was labeled using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol; New England Nuclear) as described (24). The 3′-end of RNA was labeled using T4 RNA ligase (New England Biolabs) and [5′-^{32}P]pCp (3000 Ci/mmol; New England Nuclear) according to D'Alessio (25). Unincorporated nucleotides were removed by electrophoresis through a 20% polyacrylamide–7 M urea gel in Tris–borate buffer (24). Labeled products were visualized by autoradiography and the excised bands quantitated by Cerenkov radiation in a liquid scintillation counter. After overnight elution in the cold the labeled material was recovered by precipitation with ethanol as described above. Elution was usually >70% and precipitation was ~75%. The extent of labeling RecBCD RNA using T4 polynucleotide kinase was calculated from the mol of RecBCD enzyme used, the specific activity of [γ-^{32}P]ATP and the amount of radioactive material in the excised gel fragment.

The amount of unlabeled RNA used in the experiment shown in Figure 3A was estimated by labeling a portion of the RNA and quantitation as above. The amount of RNA in Figure 3B was estimated within a factor of about three from an autoradiograph of the electrophoretic analysis of labeled RNA.

RNA sequencing

RNA was labeled at the 3′- or 5′-end, purified by gel electrophoresis and sequenced using nucleotide-specific RNases (25). RNA was partially digested with RNases T1 (cuts at G), U2 (cuts at A), CL3 [cuts at C, weakly at 5,6 dihydrouridine (D)], and Phy M (cuts at A and U), and *Bacillus cereus* RNase (cuts at C and U) according to the manufacturer's directions (US Biochemical). A ladder of oligoribonucleotides was prepared by RNase ONE digestion (Promega) or by alkaline hydrolysis in 50 mM sodium carbonate, pH 9.5, at 90°C for 5–30 min (25). Reaction products were separated by electrophoresis through a 20% polyacrylamide–7 M urea gel containing Tris–borate buffer and visualized by autoradiography. The 5′-terminal nucleotide, liberated by digestion with P1 nuclease (25), was identified by thin layer chromatography (26) in four different solvent systems.

RecBCD enzyme reaction conditions and DNA substrates

Plasmid pBR322 χ^+F χ^+H (11) or χ^+F DNA was digested with *Nde*I (New England Biolabs) and labeled at the 3′-ends with [α-^{32}P]dTTP (800 Ci/mmol; New England Nuclear) by incubation with Sequenase (US Biochemical). Unincorporated nucleotides were removed from the substrate by passage through an SR200 minicolumn (Pharmacia Biotech). RecBCD enzyme Chi cutting and DNA unwinding activities were measured as described (27). Reaction products were separated by electrophoresis through 1% agarose gels in Tris–acetate buffer (24). Gels were dried under vacuum onto Whatman DE51 paper and visualized by autoradiography.

RESULTS

A small RNA co-purifies with RecBCD enzyme

To test the hypothesis that RecBCD enzyme contains an RNA component, we searched for material in RecBCD enzyme that could be labeled with polynucleotide kinase and subsequently digested with RNase. RecBCD enzyme (fraction IV) was boiled to denature the polypeptides, treated with alkaline phosphatase and labeled. Samples were analyzed by electrophoresis through denaturing polyacrylamide gels, followed by autoradiography. A major label-accepting species was observed on a 20% polyacrylamide–urea gel (Fig. 1A, lanes 1, 4 and 7); larger species were not observed on 4 or 8% polyacrylamide–urea gels (data not shown). The appearance of the labeled species was dependent on RecBCD enzyme (Fig. 1A, lane 3). The labeled species was sensitive to treatment with RNase (Fig. 1A, lanes 2, 6 and 9) but not to DNase I (Fig. 1A, lanes 5 and 8) or proteinase K (data not shown). The size of the RNA was estimated to be <30 nt by comparison with an RNA marker (data not shown). The RNA was not detectably labeled if either the boiling or the phosphatase step was omitted (data not shown). These results are consistent with RecBCD enzyme containing a small RNA component bearing a 5′-terminal phosphate sequestered within the enzyme molecule.

The labeling reaction was used on other fractions of enzymatically pure RecBCD enzyme (fractions IV, V and VI) from one purification and yielded RNase-sensitive ^{32}P-labeled molecules with a similar mobility and yield in each case (Fig. 1B and data not shown). This result shows that the RNA co-purified with enzymatic activity during column chromatography and during sedimentation through a glycerol gradient. In addition, RecBCD enzyme provided by S.Kowalczykowski (preparation 2) also contained a small ^{32}P-accepting RNA of indistinguishable mobility (Fig. 1A, compare lanes 4 and 7). We shall refer to the RNA associated with RecBCD enzyme as RecBCD RNA.

Ten to twenty percent of the RecBCD enzyme molecules contain RNA

To determine the molar ratio of RNA to RecBCD polypeptides in the enzyme preparation, we compared the observed number of RNA molecules labeled with the number of RecBCD enzyme molecules present in the labeling reaction. We estimate that only 10–20% of the purified RecBCD enzyme molecules contain a label-accepting component.

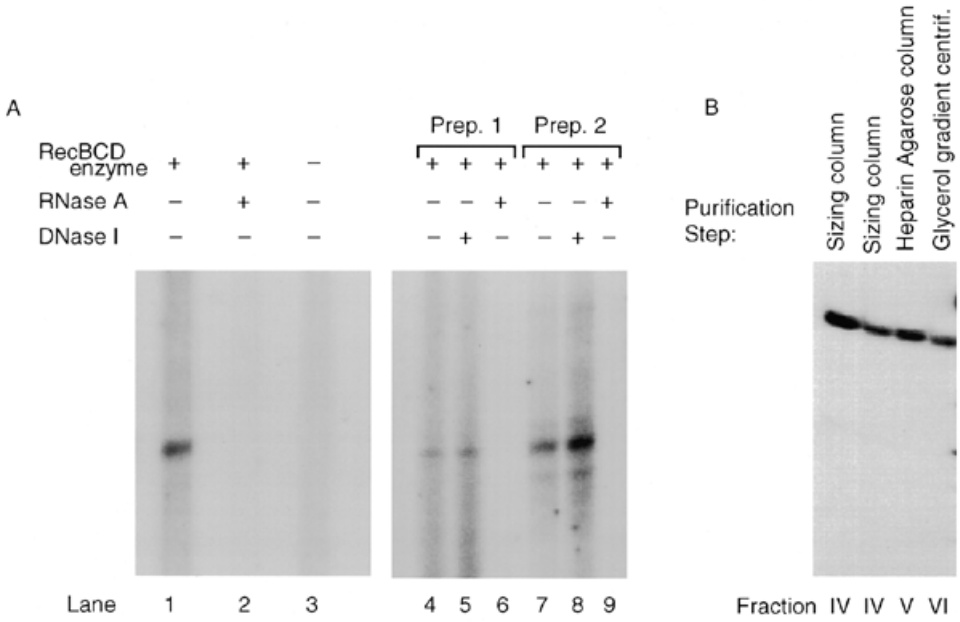

Figure 1. Purified RecBCD enzyme contains RNA. (**A**) RecBCD enzyme in buffer C (500 fmol, fraction IV, 48 U/µl, 1.7 mg/ml; lanes 1 and 2) or buffer C alone (lane 3) was processed and labeled using [γ-^{32}P]ATP and T4 polynucleotide kinase as described in Materials and Methods (boiling method). Half of the RecBCD enzyme labeling reaction was loaded onto a 20% polyacrylamide–7 M urea gel directly (lane 1) or after (lane 2) treatment with 4.4 fmol RNase A (Sigma) for 20 min at 37°C. Labeled products were visualized by autoradiography. Two additional preparations of RecBCD enzyme were analyzed; preparation 1 in buffer C (420 fmol, fraction V, 21 U/µl, 0.1 mg/ml; lanes 4–6) and preparation 2 in buffer B (1.2 pmol, 34 U/µl, 0.7 mg/ml; provided by S.Kowalczykowski; lanes 7–9). One third of each labeling reaction was loaded directly (lanes 4 and 7) or after treatment with 0.001 U DNase I (Pharmacia; lanes 5 and 8) or 4.4 fmol RNase A (Sigma; lanes 6 and 9) for 30 min at 37°C. (**B**) RecBCD enzyme (100 fmol) from fractions IV (in duplicate, 30 U/µl, 0.46 mg/ml), V (20 U/µl, 0.11 mg/ml) and VI (100 U/µl, 0.69 mg/ml) of a single purification was prepared by the proteinase K method and labeled at the 5′-end.

In a series of seven 5′-end-labeling reactions with polynucleotide kinase we varied the amount of RecBCD enzyme from 50 to 1400 fmol (fraction IV). The 30 nt control RNA (Materials and Methods) of known concentration was labeled to 73–130% of the theoretical yield, whether labeled alone or in the presence of boiled RecBCD enzyme to test for inhibitory material (data not shown). RecBCD RNA was labeled to 9–15% of the theoretical yield. Since the extent of labeling did not vary significantly over the range of RecBCD enzyme tested, the conditions of labeling were apparently not the limiting factor. Whether RecBCD enzyme, prior to labeling, was denatured by boiling or by treatment with proteinase K, extraction with phenol and precipitation with ethanol, the extent of labeling was 9–17%. These results imply that there is one RNA molecule per 5–10 RecBCD enzyme molecules.

As a second measure of the molar ratio of RNA to RecBCD enzyme molecules, we determined the extent of labeling of the 3′-end of the RNA molecule using T4 RNA ligase and [5′-^{32}P]pCp. The extent of labeling of the 3′-end of the control RNA in four separate labeling reactions was 18–35% and that of RecBCD RNA was 1.8–3%. These are ~20% of the extent of labeling of the 5′-end (see above). The extents of labeling of either end of RecBCD RNA was, therefore, ~10-fold lower than that of the control RNA. These results indicate that there is about one RecBCD RNA molecule per 5–10 RecBCD enzyme molecules or that both ends are equally resistant to labeling; RNA resistant to labeling at both ends would, of course, be undetectable. The 3′-ends of 5′-end-labeled RecBCD RNA and control RNA molecules appeared to be good substrates for RNA ligase, because both were efficiently converted into products that behaved as circles (data not shown). We therefore conclude that RNA molecules labeled at the 5′-end can also be labeled at the 3′-end.

The extent of labeling per unit of dsDNA exonuclease was similar for fractions IV, V and VI of RecBCD enzyme (Fig. 1B). This demonstrates that the ratio of RNA-containing RecBCD enzymes to total RecBCD enzyme molecules remained approximately the same, but low, during enzyme purification (Fig. 1B).

Nucleotide sequence of the RNA

RNA isolated from RecBCD enzyme migrated during electrophoresis as a single species. To determine if the RNA had a unique sequence or was a population of similarly sized, but otherwise unrelated, molecules, the RNA was labeled at the 3′- or 5′-end, purified and then cleaved with nucleotide-specific RNases or snake venom phosphodiesterase. The 5′-terminal nucleotide (G) was identified by thin layer chromatography (data not shown). The following sequence was determined by partial RNase digestion of RNA from two separate RecBCD enzyme preparations (see Fig. 2A for one example): 5′-GNGAAGGUGGCGGAADDNNNNNN-3′, where N indicates an uncertain or undetermined nucleotide. One nucleotide may be present between the 5′-terminal G and the G that begins the sequence determined by partial RNase digestion. The last 2 nt in the sequenced region were weakly cleaved by RNase CL3 but not by the other RNases, suggesting the presence of 5,6-dihydrouridine (D; 28).

The 3′-end of the RNA molecule was resistant to cleavage by RNase ONE (Fig. 2B) and all of the RNases used for sequencing. Similarly, alkaline hydrolysis of the RNA revealed a ladder of

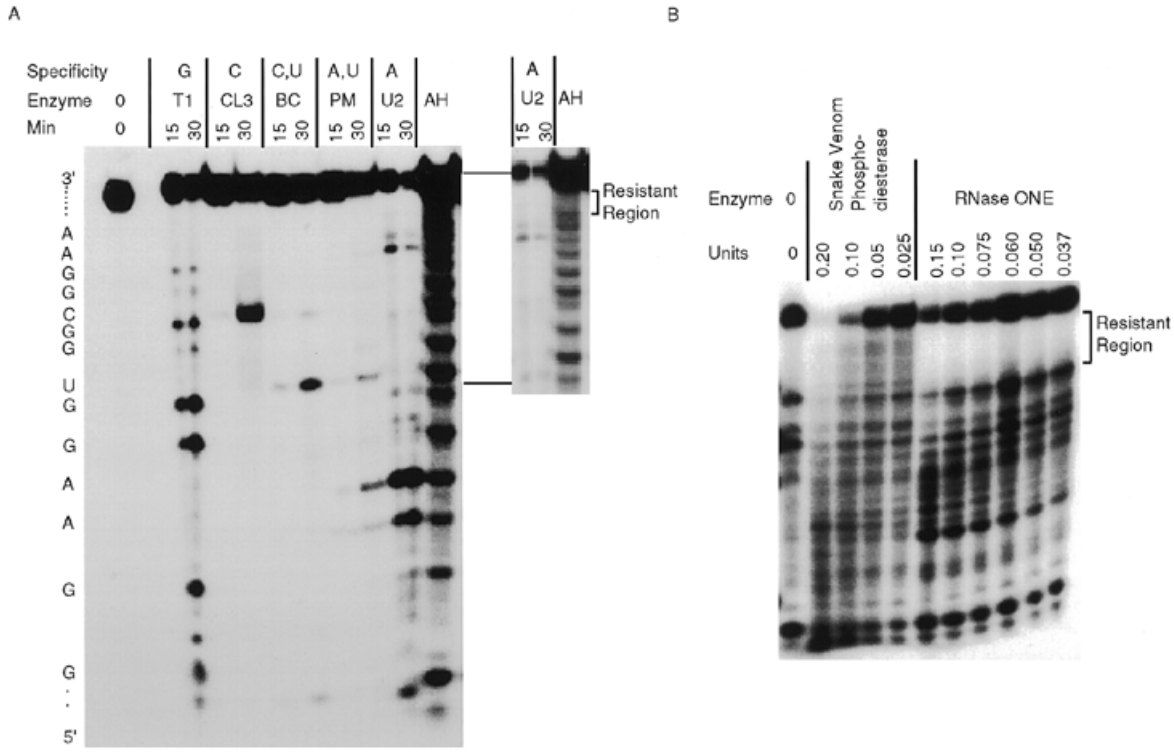

Figure 2. Nucleotide sequence of 5′-end-labeled RecBCD RNA. (**A**) RNA (~1500 d.p.m.) was digested with the indicated nucleotide-specific RNases (Materials and Methods) or hydrolyzed in alkali (AH) (~5000 d.p.m.; 25) and resolved in a 20% polyacrylamide–7 M urea gel. The nucleotide sequence derived from this autoradiograph (and others) is shown on the left of the figure. A lighter rendition of the lanes containing the products of alkaline hydrolysis and U2 cleavage shows the region resistant to alkaline hydrolysis. (**B**) RNA (~400 d.p.m./lane) was digested with snake venom phosphodiesterase (Sigma) or RNase ONE (Promega) for 30 min at 37°C.

~17 nt and a region of nucleotides resistant to hydrolysis (see Fig. 2A for one example). These results demonstrate that 5–8 ribonucleotides at the 3′-end of the molecule are modified in such a way, perhaps by 2′-OMe, as to make them resistant to hydrolysis by alkali, RNases or DNase (25; Fig. 1). The 3′-region was sensitive to digestion by snake venom phosphodiesterase (which digests nucleic acids from a 3′-OH end), demonstrating that nucleotides are present (Fig. 2B). The alkali- and RNase-resistant region of RecBCD RNA was also observed in molecules labeled at the 3′-end (data not shown).

While we were unable to determine the entire RNA sequence, sequencing of RNA from two RecBCD enzyme preparations revealed the same sequence in the RNase-sensitive region. The same sequence was found for the 5′- and the 3′-end-labeled RNAs. Based on the limited background of RNA visualized on autoradiographs, we estimate that ≥90% of the gel-purified RNA was a single species (data not shown). Thus, the majority of the RNA in RecBCD enzyme appears to be a single species with a unique sequence.

Addition of RecBCD RNA to native RecBCD enzyme increases enzymatic activity

The preceding observations show that ~10–20% of the RecBCD enzyme molecules contain a small RNA with a unique sequence. We reasoned that addition of exogenous RecBCD RNA to native enzyme might increase the activity of RecBCD enzyme. The DNA unwinding and Chi-dependent strand cleavage (Chi cutting) activities were measured using a linearized derivative of pBR322 (pBR322 $\chi^+F\chi^+H$; 11) that contains two Chi sites (Fig. 3A). Under the conditions of reaction used here RecBCD enzyme enters the DNA at either end and produces two radioactive DNA fragments from Chi cutting and two (co-migrating) products of DNA unwinding (3). A 6 h incubation of native RecBCD enzyme (9.3 fmol) with a 60-fold molar excess of RecBCD RNA increased the Chi cutting and DNA unwinding activities ~8-fold [Fig. 3, compare products of native enzyme titration in lanes 12–16 with the products in lane 3 (similar to 0.06 U activity) and those in lane 7 (similar to 0.5 U activity)]. Enhancement of activity was also seen by a reduction in the amount of unreacted DNA substrate remaining after the reaction (Fig. 3A, compare double-stranded substrate in lanes 4, 6 and 8). Enhancement of RecBCD activity by RecBCD RNA was specific: the control RNA, 30 nt long but with no sequence similarity to the RecBCD RNA, failed to stimulate enzyme activity (Fig. 3A, compare lanes 5 and 6 with lanes 3 and 4). Neither of the purified RNAs alone had any detectable activity (Fig. 3A, lanes 9–11). In six additional experiments using 30- to 100-fold molar excesses of RecBCD RNA to native enzyme Chi cutting and DNA unwinding activities were stimulated 2- to 5-fold (data not shown). In these experiments the control RNA and two additional synthetic RNAs with sequences related to that of the RecBCD RNA (Materials and Methods) failed to stimulate Chi cutting or DNA unwinding activities (data not shown). Thus, RecBCD RNA, but not three other RNAs, stimulated two enzymatic activities of RecBCD enzyme 2- to 8-fold.

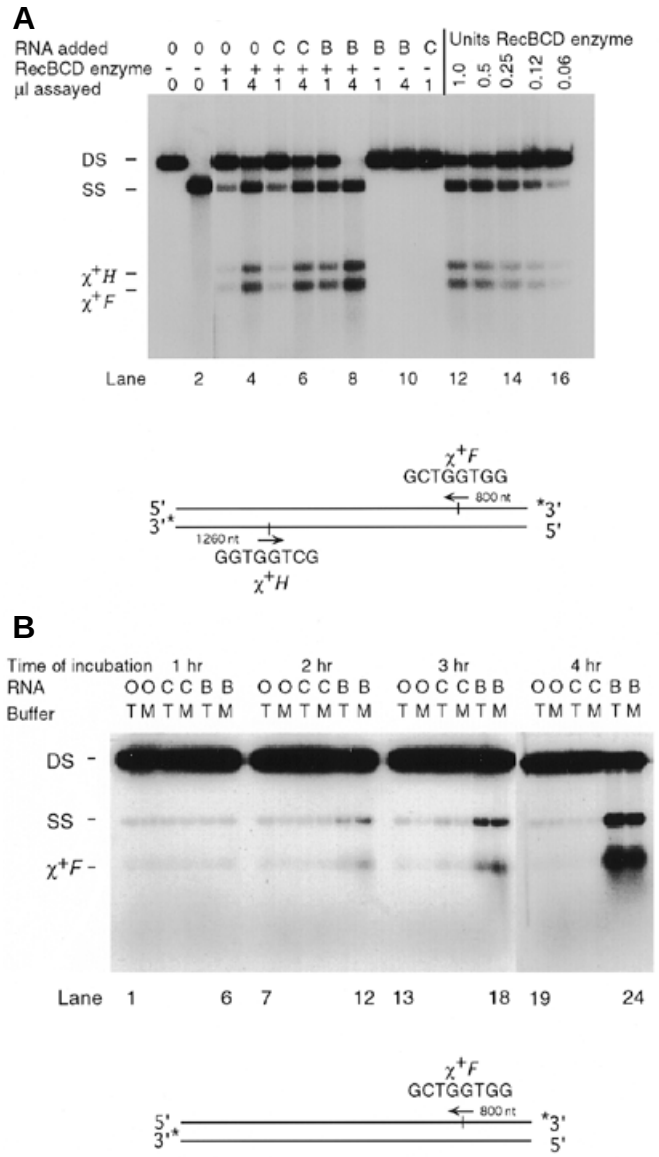

Figure 3. Addition of RecBCD RNA increases RecBCD enzyme unwinding and Chi cutting activities. (**A**) RecBCD enzyme (9.3 fmol, fraction V, 20 U/µl, 0.11 mg/ml) in buffer C was mixed with 560 fmol RecBCD RNA (B) or control RNA (C) or TE (O) in a total volume of 10 µl and incubated for 6 h at room temperature. Samples (1 or 4 µl) of the mixture, the RNA alone or the indicated amounts of RecBCD enzyme were assayed for Chi cutting and DNA unwinding (2 min reaction; Materials and Methods) using the 3′-^{32}P-labeled substrate shown. The duplex substrate (DS), unwound full-length substrate (SS) and single-stranded fragments from cleavage at χ^+H (χ^+H) and cut at χ^+F (χ^+F) were identified using appropriate markers (data not shown). (**B**) Stimulation of RecBCD enzyme requires prior incubation of RecBCD enzyme and RecBCD RNA. RecBCD enzyme (28 fmol, fraction V, 20 U/µl, 0.1 mg/ml) in buffer C was mixed with ~1 pmol RecBCD RNA (B) or control RNA (C) or TE (O) without (T) or with (M) 3 mM $MgCl_2$ and 5 mM ATP in a total volume of 15 µl and incubated for up to 4 h at room temperature. At the times indicated a 1 µl sample of the mixture was diluted 10-fold and assayed as described above for Chi cutting and DNA unwinding (2 min reaction) except that the DNA substrate was pBR322 χ^+F.

We next tested the time requirements for stimulation of RecBCD enzyme by RecBCD RNA. RecBCD enzyme was incubated with an ~30-fold molar excess of RecBCD RNA and assayed at 1–4 h for DNA unwinding and Chi cutting activities (Fig. 3B). (In this experiment the DNA substrate contained only a single Chi site and only one Chi fragment was produced.) Both activities increased during incubation up to 4 h, but only in the presence of RecBCD RNA (lanes 5, 6, 11, 12, 17, 18, 23 and 24). Activity increased up to ~10-fold whether RecBCD enzyme and RecBCD RNA were incubated in buffer with EDTA (lanes marked T) or in a buffer with Mg^{2+} plus ATP (lanes marked M). Thus, stimulation required extended incubation of RecBCD enzyme with RNA but did not appear to be influenced by the presence of the enzyme cofactors in the incubation. In the absence of added RNA or in the presence of the control RNA little or no change in activity was observed, indicating that stimulation was not due to stabilization of the enzyme by RecBCD RNA and that stimulation was specific to RecBCD RNA.

Since the molar ratio of RNA molecules to RecBCD enzyme molecules at each step of purification was about the same (Fig. 1B), we expected that each fraction would be comparably stimulated by addition of RNA. Native enzyme from four steps in purification was incubated with purified RecBCD RNA; the Chi cutting and DNA unwinding activities of each fraction were increased to approximately the same extent (data not shown).

To demonstrate that the material responsible for stimulation of RecBCD enzyme was RNA, we treated an aliquot of the RecBCD enzyme-derived material with RNase A (proteinase K method; see Materials and Methods). Addition of untreated RNA increased RecBCD enzyme activity ~1.5-fold; RNase A treatment essentially eliminated the stimulatory activity of the factor extracted from RecBCD enzyme (data not shown), demonstrating that the stimulatory factor is, or contains, RNA.

To test the hypothesis that the RecBCD RNA is essential for enzyme activity, we tested the effect of RNase A treatment on RecBCD enzyme. RNase A bound RecBCD enzyme to the walls of test tubes unless high concentrations of BSA were present (data not shown). When BSA was added, RNase A had little effect on the RecBCD enzyme activities tested. This result might be due to only 10–20% of the RecBCD enzyme molecules having associated RNA (see above) or to the RNA being protected by the RecBCD enzyme protein subunits, making it inaccessible, under the conditions used, to RNase treatment.

Analysis of RecBCD RNA in enzyme prepared from cells containing over-expressing plasmids

If the RNA molecule that co-purifies with RecBCD enzyme is not essential for activity, some preparations of RecBCD enzyme might lack the RNA but nevertheless have enzymatic activity. Although RecBCD RNA was observed in four preparations studied earlier, we searched for it in two additional preparations of RecBCD enzyme from other laboratories. We observed an RNA molecule with a slightly greater mobility than that of RecBCD RNA (data not shown) in enzyme provided by S.Kowalczykowski (preparation 3), but no RNA or other nucleic acid could be detected in enzyme provided by D.Julin. These results suggest that RecBCD enzyme can be separated from the RNA under some conditions of purification. The specific activities of all six preparations tested were similar (see Materials and Methods), suggesting that the RNA is not essential for enzymatic activity. Since only ~10% of the enzyme molecules are associated with RecBCD RNA and since the RNA stimulates enzymatic activity ~5-fold, we would expect enzyme preparations with the RNA to have ~50% greater specific activity than those

without it. Limited precision in determining specific activity makes such a small difference difficult to detect.

We reasoned that if there was a limited number of RNA molecules available in *E.coli* to bind to RecBCD enzyme the molar ratio of RNA to RecBCD polypeptides in purified enzyme might depend on the number of RecBCD enzyme molecules per cell. We estimate that there are ~500 RecBCD enzyme molecules per cell of strain V182 containing pDWS2 (a pBR322 derivative carrying the *recBCD* genes; 3). The molar ratio of RNA to RecBCD enzyme from these cells was ~0.10 (see above). We prepared RecBCD enzyme from *E.coli* cells containing plasmids that express RecBCD protein to ~10% of total cellular protein (strain V2350; 20). The specific activity of the unfractionated extract (3.3×10^4 U/mg protein; data not shown) indicates that there were ~30 000 RecBCD enzymes/induced cell. RNA was assayed in RecBCD enzyme from four steps of purification as described previously. No labeled material of the size predicted for the RecBCD RNA was detected in unfractionated extracts or RecBCD enzyme-containing fractions from heparin–agarose, DEAE or hydroxyapatite columns (data not shown). Phosphorimager analysis allows us to detect molar ratios of RNA to RecBCD enzyme of 0.008 or more (data not shown). Thus, we cannot rule out a very low level of RNA associated with RecBCD enzyme from strain V2350. The observation that purified enzyme preparations have similar specific activities despite differences in RNA content suggests that the RNA is not an essential subunit, although it does stimulate enzymatic activity.

DISCUSSION

A small unique RNA co-purifies with RecBCD enzyme

We have shown that a small RNA co-purifies with RecBCD enzyme (Fig. 1). The structure of RecBCD RNA was determined to be 5′-pGNGAAGGUGGCGGAADDNNNNNN-OH-3′, where N indicates an uncertain or undetermined nucleotide (Figs 1 and 2). The 3′-end of the RNA appeared to be composed of five to eight modified nucleotides that made this region resistant to alkaline hydrolysis or cleavage by RNases or DNase I (Figs 1 and 2). These nucleotides might bear 2′-OMe groups, which have this property (25).

We searched the complete *E.coli* DNA sequence (GenBank accession no. U00096) for homology to RecBCD RNA. Complete homology was identified to a part of each of the four copies of the gene encoding leucine$_1$ tRNA, but to no other sequences. The 5′-terminal nucleotide and 15 nt in the RNase-sensitive region of RecBCD RNA are identical to 16 of the 17 5′-terminal nucleotides in *E.coli* leucine$_1$ tRNA (5′-GCGAAGGUGGCG-GAADD-3′; 29,30). We were unable to determine if RecBCD RNA had a C in the 5′ penultimate position. If so, the homology to the 5′ fragment of leucine$_1$ tRNA cleaved in the dihydrouridine loop would be complete. It appears, however, that RecBCD RNA is not a simple cleavage product, because seven of eight nucleotides in leucine$_1$ tRNA corresponding to the RNase- and alkali-resistant region of RecBCD RNA would be expected to be sensitive to RNase and alkali. This suggests that if the 5′-end of RecBCD RNA is derived from leucine$_1$ tRNA this fragment is further modified on its 3′-end. The observation that this species of RNA, but apparently not others, binds to RecBCD enzyme suggests that this RNA has a biological role.

Addition of RecBCD RNA but not others stimulates RecBCD enzyme activity

The addition of RecBCD RNA, but not four other types of RNA, to native enzyme stimulated Chi cutting, DNA unwinding and dsDNA exonuclease activities ~2- to 10-fold (Fig. 3; additional results not shown). Leucine$_1$ tRNA before and after partial digestion with RNase A or T1 did not increase RecBCD enzyme activity (data not shown). This specificity further supports a biological role for the small unique RNA in RecBCD enzyme.

Further evidence for a biological role of the RNA associated with RecBCD enzyme might be obtained by analysis of the RecBCD enzyme-related phenotypes of a leucine$_1$ tRNA mutant. This experimental approach is difficult because leucine$_1$ tRNA is 4–7% of the total tRNA in *E.coli* (31) and its codon is the most frequent (32); cells lacking this tRNA are therefore likely inviable. The four copies of the gene encoding leucine$_1$ tRNA in the genome of *E.coli* (33) further complicate this approach.

Not all RecBCD enzyme preparations contain RNA

We estimate that there is one RNA molecule per 5–10 RecBCD enzyme molecules in each of five different enzyme preparations. In two other preparations we did not detect any RNA and estimate that their RNA content was <1 RNA molecule per 100 enzyme molecules.

If the amount of RecBCD RNA inside a cell is limited, the molar ratio of RNA to RecBCD enzyme molecules would be affected by the number of RecBCD enzymes in the cell. We estimate that there are ~500 RecBCD enzyme molecules/cell of strain V182 expressing the *recBCD* genes on a pBR322-based plasmid (3). The molar ratio of RNA to RecBCD enzyme purified from strain V182 is ~0.1 (see Results). This suggests that there are ~50 RNA molecules available to bind to RecBCD enzyme in each cell. In strain V2350, highly overproducing RecBCD enzyme, we estimate that there are 30 000 enzyme molecules/cell and would expect only 50 of these enzyme molecules (0.2%) to have an RNA molecule, below our level of detection. Failure to detect RNA in RecBCD enzyme from strain V2350 is consistent with there being a limited amount of RNA per cell. The estimated 50 RNA molecules/cell is, however, enough to saturate the 10 RecBCD enzyme molecules/wild-type *E.coli* cell. These considerations, plus the ability of the RNA to increase the activity of purified RecBCD enzyme, suggest a biological role for this RNA.

The stimulation of enzyme activity by a biological macromolecule has precedence: other enzymes are stimulated by, but do not require, a unique macromolecule for activity. For example, the RNA subunit of RNase P has cleavage activity *in vitro* that is stimulated ~20-fold by its protein subunit; both subunits are required in *E.coli* cells (34). A DNA target molecule is cleaved in two steps by the endonuclease encoded by the R2 element of *Bombyx mori*; conversion of a single-strand nick to staggered dsDNA cleavage is stimulated ~10-fold by RNA (35). The RNA associated with RecBCD enzyme similarly stimulates RecBCD enzyme but is not absolutely required for its activity.

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