RNA and Protein Catalysis in Group II Intron Splicing and Mobility Reactions Using Purified Components†

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ABSTRACT: Group II introns encode proteins with reverse transcriptase activity. These proteins also promote RNA splicing (maturase activity) and then, with the excised intron, form a site-specific DNA endonuclease that promotes intron mobility by reverse splicing into DNA followed by target DNA-primed reverse transcription. Here, we used an *Escherichia coli* expression system for the *Lactococcus lactis* group II intron Ll.LtrB to show that the intron-encoded protein (LtrA) alone is sufficient for maturase activity, and that RNP particles containing only the LtrA protein and excised intron RNA have site-specific DNA endonuclease and target DNA-primed reverse transcriptase activity. Detailed analysis of the splicing reaction indicates that LtrA is an intron-specific splicing factor that binds to unspliced precursor RNA with a K_d of ≤0.12 pM at 30 °C. This binding occurs in a rapid bimolecular reaction, which is followed by a slower step, presumably an RNA conformational change, required for splicing to occur. Our results constitute the first biochemical analysis of protein-dependent splicing of a group II intron and demonstrate that a single intron-encoded protein can interact with the intron RNA to carry out a coordinated series of reactions leading to splicing and mobility.

Mobile group II introns, which have been found in bacteria and organelles, are site-specific retroelements (*1*−*4*). The introns insert at the same locations in intronless alleles ("homing") and also transpose at low frequency to ectopic sites that resemble the normal homing site. Studies with the yeast mtDNA aI1 and aI2 introns and the *Lactococcus lactis* Ll.LtrB intron showed that homing is mediated by a multifunctional intron-encoded reverse transcriptase (RT)[1] (*5*−*9*). This protein functions in RNA splicing (maturase activity) and then remains bound to the excised intron RNA to form an RNP complex that has site-specific DNA endonuclease activity. The endonuclease initiates mobility by making a double-strand break at the intron insertion site in the recipient DNA. Remarkably, both the RNA and protein function catalytically in DNA cleavage, with the intron RNA cleaving the sense strand of the recipient DNA by a partial or complete reverse splicing reaction at the intron insertion site and the intron-encoded protein cleaving the antisense strand at position +9 or +10 of the 3′ exon, depending on the endonuclease. The intron-encoded protein then uses the 3′ end of the cleaved antisense strand as a primer for reverse transcription of either unspliced precursor RNA or intron RNA that had reverse spliced into the sense strand of the recipient DNA. Since the cleaved DNA target site is used as a primer for reverse transcription, this step is described as target DNA-primed reverse transcription (TPRT). Homing is highly efficient, occurring at frequencies approaching 100% for fungal mtDNA group II introns and 10% for bacterial introns (*1*−*3*).

The RNA splicing or maturase activity of the intron-encoded protein was first demonstrated genetically for the yeast mtDNA introns aI1 and aI2. Mutations in the intron ORF were shown to inhibit splicing of the intron, which could then be restored by providing the intron-encoded protein in trans (*10*, *11*). The aI1 protein could not rescue splicing defects in the aI2 intron and vice versa, indicating that the aI1 and aI2 proteins are intron-specific splicing factors. Proteins encoded by bacterial group II introns have also been shown to exhibit maturase activity, but it has not yet been studied in detail (*12*−*14*). By analogy with well-studied group I intron splicing factors, such as the *Neurospora crassa* CYT-18 protein and the yeast CBP2 protein, group II intron maturase activity has been presumed to reflect the fact that binding of the protein to unspliced precursor RNA promotes the formation of or stabilizes the catalytically active structure of the intron RNA (*15*). Group I intron

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[1] Abbreviations: CBD, chitin-binding domain; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; DTT, dithiothreitol; E1, 5′ exon; E2, 3′ exon; EDTA, (ethylenedinitrilo)tetraacetetic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactoside; IVS, intervening sequence; mt, mitochondrial; ORF, open reading frame; MES, 2-(*N*-morpholino)ethanesulfonic acid; phenol/CIA, phenol/chloroform/isoamyl alcohol (25:24:1); PMSF, phenylmethanesulfonyl fluoride; RNP, ribonucleoprotein; RT, reverse transcriptase; TAPS, *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; TPRT, target DNA-primed reverse transcription; Tris, tris(hydroxymethyl)aminoethane; 100 or 450 NMT, 100 or 450 mM NaCl, respectively, 5 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), 100 μg/mL BSA, 5 mM DTT, and 1 unit/μL RNasin.

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maturases, which are proteins unrelated to group II intron maturases, are also believed to promote splicing by facilitating RNA folding (*16*).

It remains unclear whether the group II intron splicing and mobility reactions require additional components. The yeast mt RNP particle preparations used initially to demonstrate the mobility reactions were relatively impure, and genetic analysis suggests that nuclear gene products may also contribute to group II intron splicing (reviewed in ref *15*). The latter include MSS116p, a putative RNA helicase, and MRS2p, a putative membrane-anchored protein with a soluble N-terminus that extends into the mitochondrial matrix (*17*, *18*; R. J. Schweyen, University of Vienna, Vienna, Austria, personal communication). While it has not yet been demonstrated that these additional components function directly in group II intron splicing, the genetic data could be pointing to the involvement of a larger complex, analogous to the eukaryotic spliceosome.

Recently, we developed an efficient *Escherichia coli* expression system for the *L. lactis* group II intron Ll.LtrB (*13*). By using this expression system, we showed that the intron-encoded protein, LtrA, has RT, RNA maturase, and site-specific DNA endonuclease activities, similar to those of the yeast mtDNA introns. The maturase activity was demonstrated genetically by showing that mutations in the intron-encoded protein inhibit splicing in *E. coli*, as well as biochemically by showing that the LtrA protein in RNP particle preparations promotes the splicing of the Ll.LtrB intron with 5 mM Mg^{2+}, where the intron is incapable of self-splicing (*13*). The homing of the Lactococcal intron in both *L. lactis* and *E. coli* was shown to occur by reverse splicing of the intron RNA into the DNA target site, followed by TPRT of the inserted intron RNA, with the cDNA copy of the intron integrated by a repair event independent of homologous recombination (*9*).

The *E. coli* expression system for the Ll.LtrB intron provides the means for studying the splicing and mobility reactions in detail. Here, we used this expression system to develop procedures for obtaining purified LtrA protein and RNP particles having DNA endonuclease activity. By using purified constituents, we demonstrate that the LtrA protein is by itself sufficient for maturase activity. Furthermore, RNP particles reconstituted with only the LtrA protein and the excised intron RNA by themselves carry out the site-specific DNA endonuclease and TPRT reactions required for intron mobility. Detailed analysis of the splicing reaction provides the first insight into how the maturase functions.

MATERIALS AND METHODS

E. coli Strains and Growth Conditions. E. coli BL21(DE3) *lon*$^-$ *ompT*$^-$ was used for expression of the LtrA protein and DH5α (Life Technologies, Gaithersburg, MD) for preparation of plasmid DNAs. The strains were grown in LB or SOB (*19*) with 100 μg/mL ampicillin added for selection of recombinant plasmids.

Recombinant Plasmids. Plasmid pImp-1P contains the LtrA ORF from pLI1 (*13*) recloned behind the tac promoter in pCYB2 (New England Biolabs, Beverly, MA), such that the C-terminus of the ORF is fused in-frame to a cassette containing the *Saccharomyces cerevisiae VMA1* intein and the *Bacillus circulans* chitin-binding domain (CBD). To construct pImp-1P, the LtrA ORF from pLI1 was amplified by PCR using the 5′ primer expr5′ (5′-AAAACCTC-CATATGAAACCAACAAATG) and the 3′ primer ltr impact (5′-TAACTTCCCGGGCTTGTGTTTATGAATCAC). The latter primer deletes the termination codon and introduces a *Sma*I site. The PCR product was digested with *Nde*I and *Sma*I and then cloned between the corresponding sites of pCYB2. As a result of the construction and intein cleavage, the LtrA protein expressed from pImp-1P contains two extra amino acids (Pro and Gly) appended to the C-terminus. The construct used in some early experiments was found to have a conservative N536 → D substitution introduced during PCR, but was verified to have the same characteristics as a construct with the wild-type amino acid at this position. Plasmid pImp-1Myc is a derivative of pImp-1P that produces LtrA protein with the C-terminal c-myc epitope tag EQK-LISEEDL followed by the extra PG. The splicing activity of the myc-tagged protein was indistinguishable from that of the wild-type protein.

Plasmid pGMΔORF was used to synthesize in vitro transcripts containing the Ll.LtrB intron. The plasmid contains a 902-nucleotide ΔORF derivative of the Ll.LtrB intron, cloned behind the phage T3 promoter in pBSKS+ (Stratagene, LaJolla, CA). The construct was derived from pLI2-ΔORF (*13*) by correcting nucleotide changes in domain I (G238 → A and A380 → C) that were introduced during the original PCR and by introducing a *Hin*dIII site (U208 → C and A341 → G) to facilitate further mutagenesis of the intron. In vitro transcription of pGMΔORF linearized with *Bam*HI gives a 1214-nucleotide transcript (denoted Ll.LtrB RNA) having a 214-nucleotide 5′ exon, a 902-nucleotide intron, and a 98-nucleotide 3′ exon. Plasmid pMMG3A is a derivative of pGMΔORF with a mutation of the highly conserved G3 position in intron domain V (G2399A; referred to as G3A).

Plasmids used for in vitro transcription of other group II introns were as follows: pJD20, yeast aI5γ (*20*); pJVM159, yeast aI1 (*21*); pSZD2, a 1683-nucleotide ΔORF derivative of the yeast aI2 intron (S. Zimmerly and A. M. Lambowitz, unpublished); pGMIntB, a 709-nucleotide ΔORF derivative of *E. coli* IntB (G. Mohr and A. M. Lambowitz, unpublished); and pGMCal, a 846-nucleotide ΔORF derivative of the *Calothrix* X1 intron (G. Mohr and A. M. Lambowitz, unpublished). Plasmid pBD5A was used to obtain an in vitro transcript containing a 388-nucleotide ΔORF derivative of the *N. crassa* mt LSU group I intron (*22*).

Plasmid pLHS contains a 70-nucleotide sequence corresponding to the ligated E1−E2 junction of the *ltrB* gene (−35 to +35 from the intron insertion site), cloned in pBSKS+ (*13*).

Expression and Purification of the LtrA Protein. E. coli BL21(DE3) was transformed with the expression plasmid pImp-1P and plated on LB medium containing ampicillin. For starter cultures, a single colony from the LB plate was inoculated into 50 mL of SOB medium containing ampicillin and incubated overnight at 37 °C. Ten milliliters of the overnight culture was then inoculated into 0.5 L of SOB containing ampicillin in a 4 L flask and shaken vigorously at 37 °C, until the OD_{595} equaled 0.8. The cultures were then shifted to 25 °C and induced with 1 mM IPTG for 6 h. All subsequent steps were carried out at 4 °C. The cultures were harvested by centrifugation in a Beckman JA-14 rotor (4000

rpm for 10 min), and the resulting bacterial pellet was resuspended in 40 mL of ice-cold 150 mM NaCl and recentrifuged. The final pellet was resuspended in 10 mL of column buffer (CB) [50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1% NP-40, and 1 mM PMSF] containing 0.5 M NaCl, and lysozyme was added to a final concentration of 0.1 mg/mL. Cells could then be stored at −70 °C for months. Cells to be lysed were thawed and incubated on ice for 1 h and then subjected to two or three cycles of freezing−thawing between −70 and 25 °C, followed by sonication (Branson 450 Sonifier, Branson Ultrasonics Inc., Danbury, CT; four 10 s bursts at amplitude 60, with 10 s between bursts). After sonication, insoluble material was removed by centrifugation in a Beckman JA20.1 rotor (11 000 rpm for 15 min).

For chromatography, the cleared lysate was diluted to 30 mL with CB and loaded on a chitin column (New England Biolabs; 2.5 cm × 5 cm plastic column with a 5 mL bed volume), which had been washed with at least 20 mL of CB containing 0.5 M NaCl at a flow rate of 0.5 mL/min. After being loaded, the column was washed with at least 100 mL of 0.5 M NaCl in CB, and then with 50 mL of 0.75 M NaCl in CB, the latter wash being necessary to remove minor contaminants that otherwise adhere to the column and coelute with the LtrA protein. After these washes, the LtrA−intein junction was cleaved by addition of 20 mL of 30 mM DTT in CB containing 0.5 M NaCl. The column flow was stopped, and the column was incubated with the DTT overnight at 4 °C. The freed LtrA protein was then recovered by washing the column with 0.5 M NaCl in CB. Fractions of 1.5 mL were collected, and the LtrA protein was recovered in the first three or four fractions. For storage, pooled fractions were dialyzed against CB containing 0.5 M NaCl and 50% glycerol, concentrating the protein 4−7-fold. The protein remained active for months when stored in 50% glycerol at −70 °C.

Protein Analysis. LtrA protein concentrations were determined from A_{205} measurements, using an extinction coefficient based on peptide bond absorbance (*23*), and from A_{280} measurements, using an extinction coefficient calculated from the amino acid sequence (ϵ_{280} = 77 820 M^{-1} cm^{-1}). Prior to the measurement of absorbance, samples were precipitated with chloroform/methanol (*24*) to remove NP-40, which was present during the purification. The protein pellet was dried and dissolved in 1 mL of 6 M guanidine hydrochloride and 0.02 M sodium phosphate (pH 6.5), and absorbance was measured with a Perkin-Elmer Lambda Bio20 spectrophotometer. Most preparations were substantially free of nucleic acid, but if nucleic acids were present, the A_{280} values were corrected by the method of Warburg and Christian (*25*). Protein determinations by the Bradford method using Bio-Rad protein assay reagent with BSA as a standard systematically overestimated LtrA concentrations (Bio-Rad Bulletin 1069, Hercules, CA). All protein concentrations in the text refer to LtrA monomer.

Proteins were analyzed by electrophoresis in 0.1% SDS−8% polyacrylamide gels, which were stained with Coomassie blue. For immunoblot analysis of c-myc-tagged LtrA, gels were electroblotted to nitrocellulose membranes using a Hoefer TE77 semidry electroblotter (Amersham Pharmacia Biotech, Piscataway, NJ). The blots were blocked with 2% nonfat dry milk in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20 (TBST), and then incubated for 1 h at room temperature with 2 μg/mL mouse monoclonal antibody directed against the c-myc epitope tag (clone 9E10; Boehringer Mannheim, Indianapolis, IN). The blots were washed sequentially for 15 min each with TBST containing 0.01% SDS and with TBST, and then incubated with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1:3000 dilution; Bio-Rad) for 1 h at room temperature. After washes like those described above, the LtrA−antibody complex was detected by chemiluminescence using the Immuno-Star system (Bio-Rad).

Reconstitution of RNP Particles with the LtrA Protein and in Vitro-Synthesized Intron RNA. RNP particles having DNA endonuclease and reverse splicing activity were reconstituted from purified LtrA protein and excised intron RNA obtained by self-splicing of Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript). The self-splicing reaction was carried out in 400 μL of 1.5 M NH_4Cl, 50 mM $MgCl_2$, 50 mM Tris-HCl (pH 7.5), and 10 mM DTT for 1.5 h at 37 °C. Intron lariat RNA was purified by electrophoresis in a denaturing 4% polyacrylamide gel, and then renatured by heating to 80 °C for 5 min, followed by cooling on ice. For reconstitution, 0.1 μg (1.4 pmol) of the purified LtrA protein was incubated with either 0.5 μg (1.2 pmol) of self-spliced Ll.LtrB RNA or 0.5 μg (1.6 pmol) of gel-purified lariat RNA in 4 μL of 50 mM $MgCl_2$ for 10 min at room temperature, and then added to the reactions described below.

RT Assays. RT assays with poly(rA)/oligo$(dT)_{18}$ were as described previously (*13*). TPRT reactions were carried out by incubating 1 μg of unlabeled target plasmid with either 0.025 OD_{260} unit of pLI1 RNP particles or 4 μL (0.025 OD_{260} unit) of reconstituted RNP particles in 20 μL of reaction medium [10 mM KCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.5)] containing dATP, dGTP, and dCTP (0.2 mM each), and 20 μCi of [α-^{32}P]dTTP (3000 Ci/mmol; New England Nuclear, Boston, MA). The reactions were initiated by addition of the RNP particles, and the mixtures were incubated for 20 min at 37 °C and chased with 0.2 mM dTTP for an additional 10 min. Products were analyzed in a 1% agarose gel, which was dried and autoradiographed or quantitated with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Reverse Splicing and DNA Endonuclease Assays. Reverse splicing and DNA endonuclease reactions were carried out with a 129 bp ^{32}P-labeled DNA substrate containing the ligated E1−E2 junction of the *ltrB* gene (*13*). The DNA substrate was generated from pLHS by PCR with primers KS and SK, which are complementary to vector sequences flanking the 70 bp E1−E2 insert. For the assays, the labeled DNA substrate (150 000 cpm, ∼1.25 nM) was incubated with pLI1 RNP particles (0.025 OD_{260} unit) or reconstituted RNP particles (4 μL, 0.025 OD_{260} unit) in 20 μL of 10 mM KCl, 10 mM $MgCl_2$, and 50 mM Tris-HCl (pH 7.5). The reactions were initiated by addition of RNP particles, the mixtures incubated for 20 min at 37 °C, and then the reactions terminated by phenol/CIA extraction and ethanol precipitation. Reverse-spliced products were glyoxylated and analyzed in a 1.2% agarose gel, and endonuclease products were analyzed in a denaturing 6% polyacrylamide gel.

RNA Binding and Splicing Assays. Unless specified otherwise, RNA binding and splicing assays were carried out in a reaction medium containing 450 or 100 mM NaCl,

5 mM $MgCl_2$, 40 mM Tris-HCl (pH 7.5), 100 μg/mL BSA, 5 mM DTT, 1 unit/μL RNasin (Amersham, Arlington Heights, IL), and 5% glycerol (450 or 100 NMT). Incubations were carried out at 30 °C, a temperature at which the purified LtrA protein is relatively stable ($t_{1/2}$ > 6 h in 450 NMT; $t_{1/2}$ = 15 min in 100 NMT). Although LtrA is even more stable at 22 °C, the rate and extent of splicing were substantially decreased, so 30 °C was adopted as the compromise condition.

For k_{on} measurements, the LtrA protein (20−120 pM) was added to 5 pM ^{32}P-labeled RNA (2000−3000 cpm) in 100 μL of reaction medium and incubated at 30 °C. After times ranging from 10 s to 120 min, the mixture was diluted into an equal volume of reaction medium containing 0.5 mg/mL heparin, incubated for 10 s to bind any unassociated or loosely bound protein, and then filtered through a nitrocellulose filter (Schleicher & Schuell, Keene, NH), which binds RNP complexes. The filters were washed three times with 0.8 mL of reaction medium, air-dried, and counted for Cerenkov radioactivity.

For k_{off} measurements, 40 nM ^{32}P-labeled RNA (20000−40000 cpm) was bound to 20 nM LtrA protein in 0.1 mL of reaction medium for 60 min at 30 °C. The mixture was then diluted into 2 mL of reaction medium containing 0.5 mg/mL heparin, and 90 μL portions were withdrawn at times ranging from 2 to 180 min. In some experiments, complexes were formed with 20 nM ^{32}P-labeled RNA and 10 nM LtrA, and then diluted into 2 mL of reaction medium containing excess (100 nM) unlabeled Ll.LtrB RNA. The amount of complex remaining at each time point was quantitated by nitrocellulose filter binding, as described above.

For saturation binding, 5 pM ^{32}P-labeled RNA (2000−4000 cpm) was incubated with increasing amounts of LtrA protein in 200 μL of reaction medium for 1 h at 30 °C prior to nitrocellulose filter binding.

The stoichiometry of binding was determined by incubating 50 nM LtrA protein with increasing concentrations of ^{32}P-labeled Ll.LtrB RNA (1−300 nM; 3000−900000 cpm) or by incubating 50 nM ^{32}P-labeled Ll.LtrB RNA with increasing concentrations of LtrA (1−300 nM) in 40 μL of reaction medium for 1 h at 30 °C. The samples were then diluted into 40 μL of reaction medium containing 0.5 mg/mL heparin and filtered through nitrocellulose, as described above.

RNA splicing reactions were carried out by incubating 20 nM gel-purified ^{32}P-labeled Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript) with different concentrations of the LtrA protein in 250 μL of 450 or 100 NMT (see above). Prior to splicing, 300 nM RNA in 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA was renatured by heating to 90 °C for 1 min and immediately diluting 8-fold into 100 NMT at 30 °C. After 1 min, the salt concentration was adjusted to 450 mM NaCl if necessary to match the reaction medium. This renaturation protocol yielded a higher proportion of rapidly reacting RNA than those in which the RNA was slow cooled from 55 °C in the presence of salts and Mg^{2+}. Reactions were initiated by addition of LtrA protein, the mixtures incubated at 30 °C for the times indicated for individual experiments, and the reactions terminated by phenol/CIA extraction. The products were analyzed in a denaturing 4% polyacrylamide gel, which was dried, and radioactivity was quantitated with a PhosphorImager. Splicing reactions at different pHs were carried out as described above, except that instead of Tris-HCl, the reaction medium contained 50 mM MES (pH 5.0−7.5), HEPES (pH 6.5−8.5), TAPS (pH 7.5−8.5), or CHES (pH 9.0 and 9.5), and the protein was prediluted into solutions containing the desired buffer at a concentration of 100 mM.

RNA concentrations were determined by both radioactivity and OD_{260} measurements.

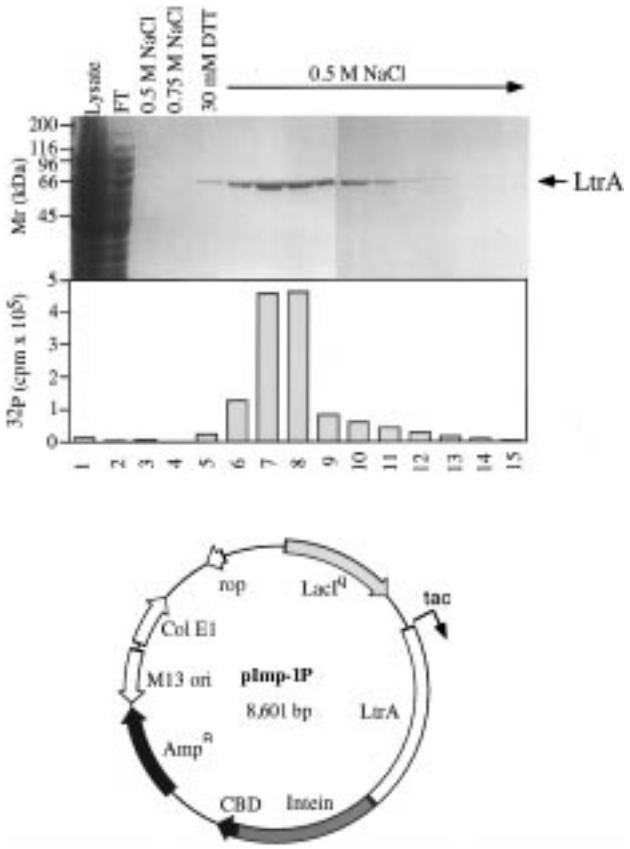

FIGURE 1: Purification of the LtrA protein. The pImp-1P construct (bottom) contains the LtrA ORF with the C-terminus fused in-frame to a cassette consisting of the *S. cerevisiae VMA1* intein and a chitin-binding domain (CBD). After expression in *E. coli* BL21(DE3), the LtrA protein was purified from a cleared lysate by binding to a chitin affinity column, followed by thiol-induced self-cleavage of the intein. Fractions (1.5 mL) were collected, with 2 μL used for RT assays and 10 μL used for SDS−PAGE: fraction 1, cleared lysate; fraction 2, column flow through (FT); fractions 3 and 4, washes with 0.5 and 0.75 M NaCl in column buffer (CB), respectively; fraction 5, addition of 30 mM DTT in CB containing 0.5 M NaCl and overnight incubation to induce cleavage of the intein; and fractions 6−15, elution of LtrA protein by washing with CB containing 0.5 M NaCl. The top panel shows a SDS−polyacrylamide gel stained with Coomassie blue, and the bottom panel shows RT assays with poly(rA)/oligo$(dT)_{18}$. Numbers to the left of the gel indicate the sizes (kilodaltons) of molecular mass markers.

RESULTS

Expression and Purification of the LtrA Protein. To purify the LtrA protein, we used an intein-based affinity purification system (IMPACT, New England Biolabs). Figure 1 shows results from an experiment with construct pImp-1P, which expresses the LtrA protein for purification by this system. The construct contains the LtrA ORF cloned behind the tac promoter in the expression vector pCYB2, with the C-terminus of the ORF fused in-frame to a cassette consisting

of the *S. cerevisiae VMA1* intein and a chitin-binding domain (CBD). After induction with IPTG, the LtrA protein was purified directly from a cleared lysate by binding to a chitin affinity column, followed by thiol-induced self-cleavage of the intein.

Remarkably, this simple one-step procedure gave large amounts of highly purified LtrA protein, which was detected as a single band ($M_r = 70$ kDa) in a Coomassie blue-stained gel. RT activity assayed with poly(rA)/oligo$(dT)_{18}$ copurified with the expressed LtrA protein, and mass spectrometry confirmed that the protein has the molecular mass expected for expression of the full-length ORF (not shown). The purification procedure routinely gave LtrA protein that is >97% pure at a yield of 1−5 mg/L of culture. Chemical cross-linking with glutaraldehyde yielded a mixture of monomers and dimers, with smaller amounts of higher-order oligomers (not shown). Sucrose gradient analysis showed that the purified LtrA protein was at least 95% active in RNA binding in buffer containing 450 mM NaCl and 5 mM Mg^{2+}, conditions shown below to favor the formation of the specific complex with the intron-containing RNA (Figure 2).

Reconstituted RNP Particles Have Reverse Splicing and Site-Specific DNA Endonuclease Activities. We previously coexpressed the LtrA protein with the intron RNA by using a construct pLI1, which contains the full-length intron and flanking exons (*13*). In this construct, the LtrA ORF, which is located in the loop of intron domain IV, is translated in *E. coli* from its own Shine-Dalgarno-like sequence. The LtrA protein promotes the splicing of the Ll.LtrB intron in *E. coli* and remains associated with the excised intron to form RNP particles having site-specific DNA endonuclease activity. To determine if the LtrA protein and excised intron RNA are the only components necessary for the reverse splicing and DNA endonuclease activities, RNP particles were reconstituted with the purified protein and in vitro-synthesized intron RNA. The intron RNA used for this purpose was gel-purified lariat obtained by self-splicing of an in vitro transcript of pGMΔORF/*Bam*HI, which contains a 0.9 kb derivative of the Ll.LtrB intron with most of the intron ORF deleted (see Materials and Methods). The pGMΔORF/*Bam*HI in vitro transcript, termed Ll.LtrB RNA, was used for most of the experiments in this work.

For reverse splicing assays, the RNP particle preparations were incubated with a small (129 bp) ^{32}P-labeled DNA substrate containing the intron insertion site in the *ltrB* gene, and the products were denatured with glyoxal and analyzed by agarose gel electrophoresis to detect intron RNA covalently linked or inserted into the small, labeled DNA substrate. Figure 3A shows that the reconstituted RNP particles are in fact capable of reverse splicing efficiently into the DNA substrate (lane 3), with the product migrating at ~1 kb, the size expected for insertion of the intact Ll.LtrB-ΔORF intron. The product band contains a mixture of partially and fully reverse spliced products (see the schematic in Figure 3). By comparison, pLI1 RNP particles isolated from *E. coli* also reverse splice into the DNA target site (lane 2), but the product is smaller than the 2.5 kb expected for the full-length intron, reflecting partial degradation of the intron RNA by *E. coli* nucleases (see ref *13*). Neither the purified LtrA protein nor the intron RNA by itself has reverse splicing activity (lanes 4 and 5).

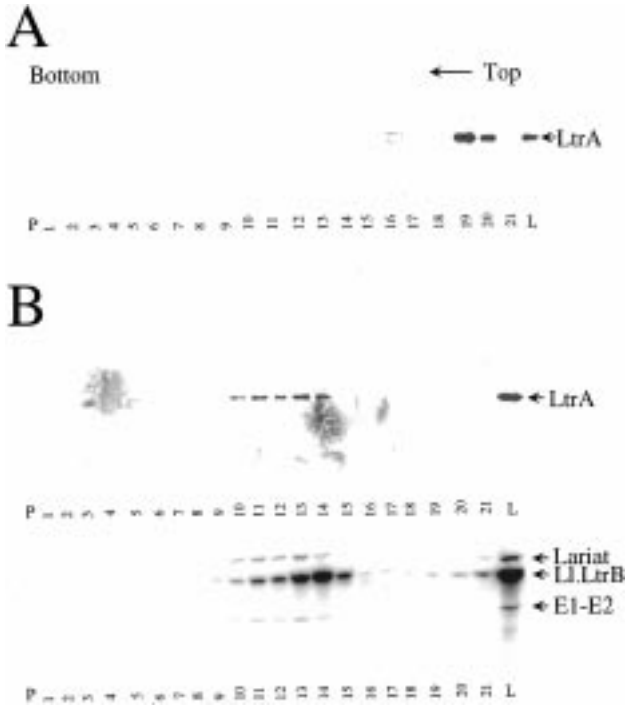

FIGURE 2: Sucrose gradient assay of RNA binding activity of the LtrA protein. c-myc-tagged LtrA protein (200 nM) was incubated (A) by itself or (B) with 400 nM ^{32}P-labeled Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript) in 500 μL of 450 mM NaCl, 5 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.5) for 15 min at 30 °C. To assess complex formation, the mixture was centrifuged through a linear 5 to 20% sucrose gradient containing the same buffer (Beckman SW 41 rotor, 40 000 rpm for 5 h at 4 °C), and 0.55 mL fractions were collected. To detect ^{32}P-labeled Ll.LtrB RNA, 10 μL portions of the gradient fractions were electrophoresed in denaturing 6% polyacrylamide gels, which were dried and analyzed with a Phosphorimager (part B, bottom panel). Some of the Ll.LtrB RNA was spliced, leading to production of ligated exons (E1−E2) and intron lariat products. To detect the LtrA protein, 0.5 mL portions of the gradient fractions were ethanol-precipitated, using linear polyacrylamide as a carrier, and electrophoresed in a 0.1% SDS−8% polyacrylamide gel. The LtrA protein was detected by immunoblotting with mouse anti-c-myc monoclonal antibody, as described in Materials and Methods. The free protein remains at the top of the gradient (panel A, fractions 19 and 20), whereas the bound protein sediments with the Ll.LtrB RNA (panel B, fractions 10−14). Quantitation indicates that at least 95% of the protein is active in binding Ll.LtrB RNA. Similar results were obtained with untagged LtrA protein detected by silver staining (not shown). Abbreviations: P, pellet; L, load.

For DNA endonuclease assays, the RNP particles were incubated with the 129 bp DNA substrate labeled at the 5′ end of the sense or antisense strand, and the cleavage products were analyzed in a denaturing polyacrylamide gel. Panels B and C of Figure 3 show that the reconstituted RNP particles also have DNA endonuclease activity that cleaves the sense strand of the DNA substrate at the intron insertion site and the antisense strand at position +9 of the 3′ exon (lanes 2), the same as pLI1 RNP particles (lanes 1). The 5′ exon fragment detected in the sense strand cleavage reaction results from partial reverse splicing. The additional lighter bands in the sense strand reaction (<10% of product by quantitation) were not seen reproducibly and may reflect nonspecific degradation of the DNA substrate and/or the fully reverse spliced product, which migrates above the substrate in the gels. Again, neither the purified LtrA protein nor the

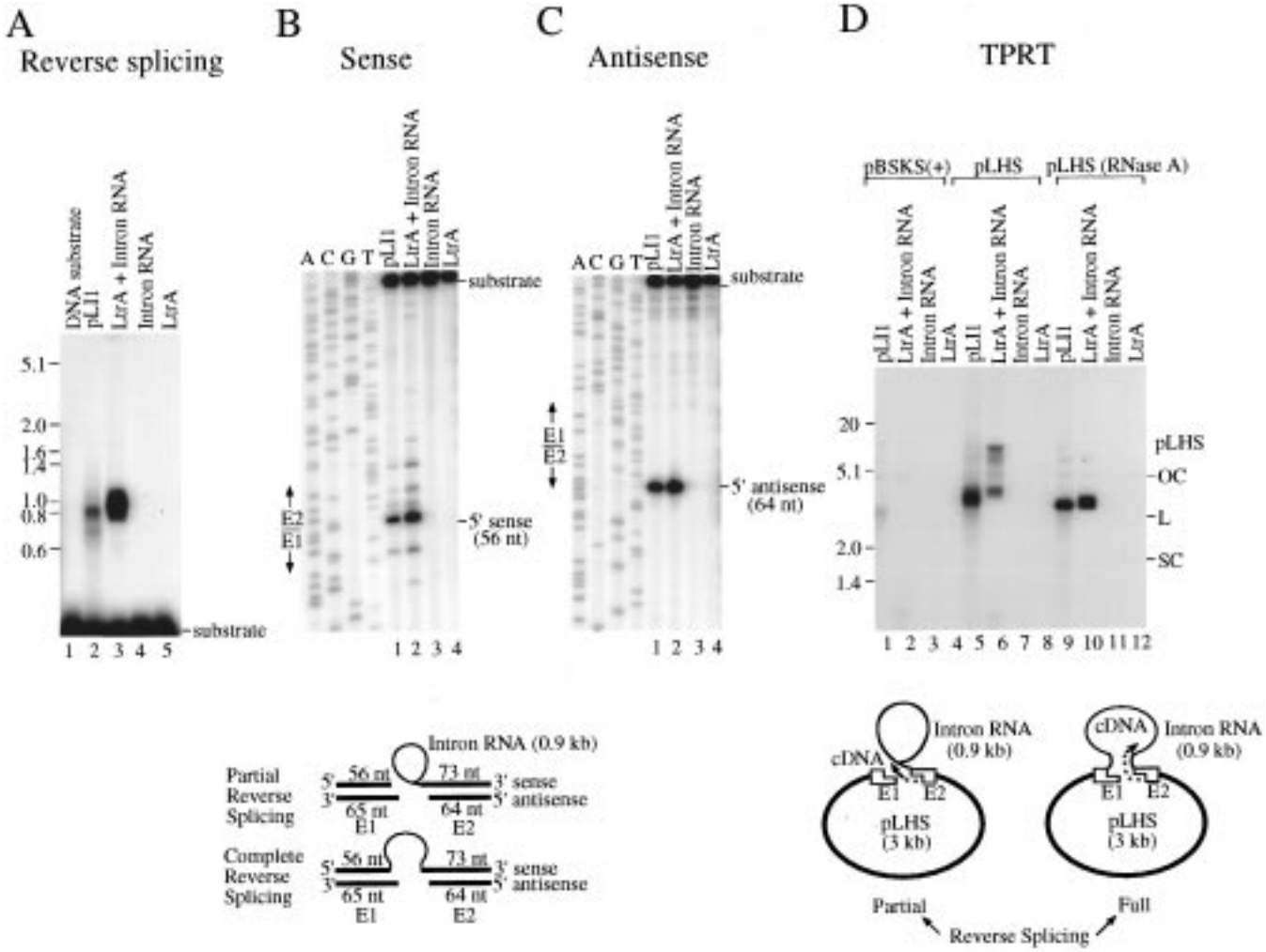

FIGURE 3: Reconstituted RNP particles exhibit reverse splicing, DNA endonuclease, and TPRT activities. (A) Reverse splicing assays. RNP particle preparations were incubated with a 129 bp internally labeled DNA substrate, which contains the E1−E2 junction of the *ltrB* gene, and the products were denatured with glyoxal and analyzed in a 1.2% agarose gel. ^{32}P-labeled DNA substrate was incubated alone (lane 1) or with native pLI1 RNP particles (lane 2), reconstituted RNP particles containing purified LtrA protein and gel-purified lariat RNA (lane 3), purified lariat RNA (lane 4), or purified LtrA protein (lane 5) in equivalent amounts. (B and C) Endonuclease assays. Reactions were carried out as described for panel A using 129 bp DNA substrates labeled at the 5′ end of the sense and antisense strands, respectively. Products were analyzed in a denaturing 6% polyacrylamide gel alongside a sequencing ladder generated from pLHS using the same 5′-end-labeled primer used to generate substrate. (D) TPRT assays. The same preparations as described above were incubated with unlabeled target plasmids in the presence of [α-^{32}P]dTTP and other dNTPs to support reverse transcription, and the products were analyzed in a 1% agarose gel. Lanes 1−4 show reactions with the vector pBSKS+, and lanes 5−12 show reactions with pLHS, which contains the 70 bp E1−E2 junction of the *ltrB* gene. In lanes 9−12, the products were incubated with RNase A prior to gel electrophoresis. The numbers to the left denote molecular mass markers (*Eco*RI/*Hin*dIII fragments of phage λ DNA). The labels at the right denote the positions of open circular (OC), linear (L), and supercoiled (SC) pLHS. Schematics at the bottom show structures expected for products of reverse splicing and DNA endonuclease reactions (left) and TPRT (right): thick lines and open boxes, DNA; thin lines, RNA; asterisks, cDNA; arrowhead, direction of cDNA synthesis. The schematics are not drawn to scale.

intron RNA by itself has DNA endonuclease activity (lanes 3 and 4).

Additional experiments showed that the purified LtrA protein in the absence of intron RNA had no detectable antisense strand cleavage activity at 22, 30, or 37 °C, either in the standard reaction medium or at higher salt concentrations (450 mM NaCl), found below to stabilize the free protein (not shown). In addition, the LtrA protein had no endonuclease activity when bound to precursor RNA having a point mutation in intron domain V (G3A) that results in the loss of splicing activity (not shown). These results suggest that LtrA is functional as an endonuclease only when complexed with the intron lariat RNA.

Reconstituted RNP Particles Have TPRT Activity. The complete TPRT reaction requires recognition of the DNA target site followed by reverse splicing of the intron RNA, cleavage of the antisense strand, and initiation of reverse transcription. To assay TPRT activity, an unlabeled target plasmid pLHS, which contains a 70 bp sequence encompassing the *ltrB* intron insertion site, was incubated with RNP particle preparations in the presence of [α-^{32}P]dTTP and other dNTPs. The products were then analyzed by agarose gel electrophoresis and autoradiography for detection of ^{32}P-labeled plasmid bands that have incorporated nascent cDNAs. Figure 3D shows that the reconstituted RNP particles have TPRT activity comparable to that in unpurified pLI1 RNP particles (lanes 5 and 6). Controls showed that neither the excised intron RNA nor purified LtrA protein by itself has TPRT activity (lanes 7 and 8), and no reaction was observed with the vector pBSKS+, which lacks the *ltrB* insertion site

(lanes 1−4).

The native pLI1 RNP particles, which contain degraded intron RNA, gave a single, major TPRT product, which migrates slightly behind linear plasmid DNA due to residual attached RNA and cDNA products (Figure 3D, lane 5). By contrast, the reconstituted RNP particles, which contain intact intron RNA, gave two bands, reflecting different forms of the intron RNA reverse spliced into the pLHS target plasmid (lane 6; see the schematic at the bottom of Figure 3D). The more slowly migrating band contains fully reverse-spliced intron RNA. Since the sense strand is covalently closed in this product, it migrates as an open circle, behind the open circular target plasmid in agarose gels. The presence of circular molecules was confirmed by restriction enzyme cleavage at a single site, which converted the band to one migrating at the position expected for a linear molecule (not shown). The more rapidly migrating TPRT product contains partially reverse-spliced or nicked intron RNA and therefore migrates more as a linear molecule in the agarose gels. Both TPRT products were sensitive to RNase digestion, resulting in bands migrating closer to but still behind linear plasmid, likely reflecting incomplete RNase digestion or residual attached cDNA products (lane 10). The proportion of fully reverse-spliced product, quantitated in experiments in which cDNA synthesis was limited to four nucleotides by incorporation of a ddGTP, ranged from 23 to 43% (not shown). Additionally, we confirmed by direct dideoxy sequencing that the nascent cDNA begins at position +9 of the 3′ exon and extends into the reverse-spliced intron RNA (not shown). Together, these findings show that the purified RNP particles containing only the LtrA protein and excised intron RNA have all the activities necessary for TPRT.

The LtrA Protein Is Stabilized by Substrate Binding. In the previous work, the LtrA protein coexpressed with intron RNA from pLI1 appeared to be more soluble and have higher RT activity than that expressed in the absence of intron RNA (*13*). The availability of purified LtrA protein enabled us to demonstrate directly that the protein is stabilized by substrate binding. Panels A and B of Figure 4 show results from experiments in which the purified LtrA protein was preincubated in RT reaction medium at 37 °C for different times in the presence or absence of RT or splicing substrates and then assayed for RT activity with poly(rA)/oligo(dT)$_{18}$. The RT assays were carried out in the presence of RNase A to degrade the bound intron RNA present in some reaction mixtures. The bound intron RNA otherwise inhibits RT activity, presumably by blocking accessibility to RT substrates (*2*; J. SanFilippo and A. M. Lambowitz, unpublished). The plots show that the purified LtrA protein by itself was unstable in RT reaction medium at 37 °C with the RT activity decaying as a single exponential ($t_{1/2}$ = 0.61 min). Other experiments showed that the inactivation of the RT activity was temperature-dependent ($t_{1/2}$ = 15 min at 30 °C and 46 min at 22 °C; not shown). Since SDS−PAGE showed that the protein remained intact during the incubations (not shown), we infer that the inactivation of RT activity is due to denaturation.

The LtrA protein could be stabilized against denaturation by adding either the RT substrate poly(rA)/oligo(dT)$_{18}$ ($t_{1/2}$ = 31 min) or the Ll.LtrB RNA ($t_{1/2}$ = 51 min) (Figure 4A,B). The level of stabilization by the intron RNA increased at 450 mM NaCl, which is shown below to favor the formation

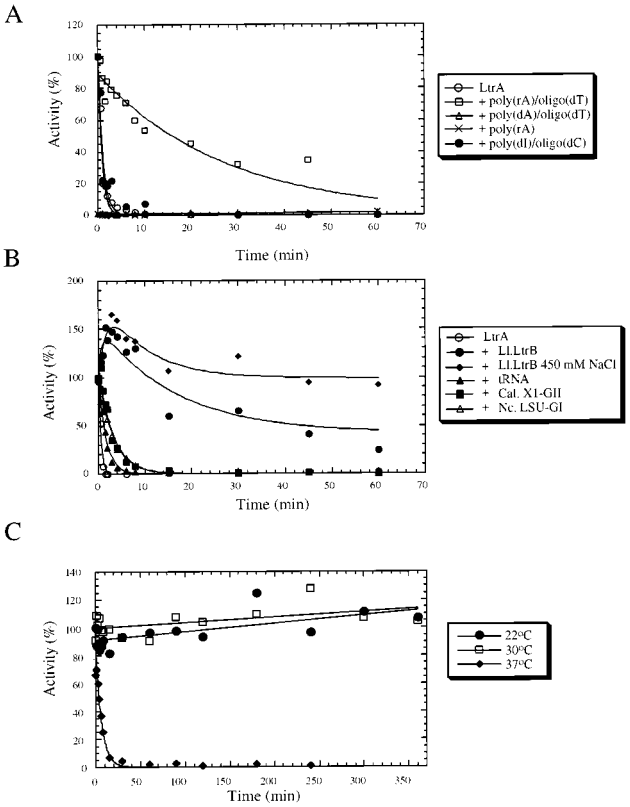

FIGURE 4: LtrA protein is stabilized by binding to an RT substrate or the Ll.LtrB intron RNA. (A and B) Purified LtrA protein (40 nM) was preincubated for the indicated times in RT reaction medium at 37 °C in the presence or absence of RT or RNA splicing substrates and then assayed for RT activity with poly(rA)/oligo(dT)$_{18}$. In panel A, LtrA protein was preincubated by itself (○) or with poly(rA)/oligo(dT)$_{18}$ (□), poly(dA)/oligo(dT)$_{18}$ (△), poly(dI)/oligo(dC)$_{18}$ (●), or poly(rA) (×). Substrates were added at 1 μg/10 μL of reaction mixture. In panel B, LtrA protein was preincubated by itself (○) or with Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript) (●), Ll.LtrB RNA in reaction medium containing 450 mM NaCl (◆), *E. coli* tRNA (▲), *Calothrix* (Cal) group II intron X1 (pGMCal/*Eco*RV in vitro transcript) (■), or *N. crassa* (Nc) mt LSU group I intron (pBD5A/*Ban*I in vitro transcript) (△). RNAs were added at a concentration of 80 nM. The plots show the percent initial activity as a function of time, with the data fit to a single-exponential Ae^{-kt}. Preincubation with the Ll.LtrB RNA results in a small increase in activity at early time points, which was omitted for curve fitting. (C) Purified LtrA protein (100 nM) was preincubated in 450 NMT at 22 (●), 30 (□), or 37 °C (◆) for the indicated times, and splicing was assayed by incubating the preincubated protein (10 nM) with ^{32}P-labeled Ll.LtrB RNA (20 nM) for 10 min at 30 °C, as described in Materials and Methods. The plot shows the percent initial activity as a function of time, with the data fit to a single exponential.

of the specific complex ($t_{1/2}$ > 250 min). Consistent with the requirement for formation of a specific complex, the RT activity was not stabilized by a noncognate bacterial group II intron RNA (*Calothrix* X1) or other nonspecific RNAs (*E. coli* tRNA or *N. crassa* mt LSU group I intron RNA). Likewise, neither poly(rA) nor poly(dI)/oligo(dC)$_{18}$ stabilized the activity, while poly(dA)/oligo(dT)$_{18}$ inhibited the activity, even without preincubation.

Figure 4C shows that LtrA preincubated in splicing reaction medium (450 NMT) containing 450 mM NaCl and 5 mM $MgCl_2$ also undergoes temperature-sensitive decay of splicing activity at 37 °C ($t_{1/2}$ = 3.5 min). Fortuitously, the splicing activity of the LtrA protein was stabilized by a combination of the high-salt reaction medium and lower

temperatures (100% active after 6 h at 22 or 30 °C). The RT activity was stabilized similarly under these conditions (not shown). This stabilization permits the quantitative binding and splicing measurements described below. The LtrA protein could also be stabilized by high concentrations of glycerol (50%) and sucrose (50%) ($t_{1/2}$ for RT activity = 60 min at 37 °C).

Specific Binding of the LtrA Protein to the Ll.LtrB Intron Is Favored by 450 mM NaCl. In previous work, we showed that the LtrA protein released from RNP particle preparations by micrococcal nuclease digestion promotes the splicing of the Ll.LtrB intron in reaction medium containing 100 mM NaCl and 5 mM Mg^{2+}, where the intron is not self-splicing (*13*). NaCl was chosen for the splicing reactions in initial experiments because of the appearance of aberrant products in KCl (*13*). To establish optimal conditions for the specific binding of LtrA to the Ll.LtrB intron, we carried out the competition binding assay shown in Figure 5A. In this experiment, a limiting amount of purified LtrA protein (5 nM) was incubated with excess Ll.LtrB RNA and *N. crassa* mt LSU group I intron RNA (50 nM each) in reaction medium containing 5 mM Mg^{2+} and increasing concentrations of NaCl. After incubation for 30 min, the mixture was filtered through nitrocellulose, and the bound RNAs were recovered by phenol/CIA extraction and analyzed by gel electrophoresis to determine the ratio of specific to nonspecific binding.

Surprisingly, the level of specific binding of the Ll.LtrB intron increased dramatically at higher salt concentrations, with maximum binding at 450 mM NaCl. At this salt concentration, the discrimination between the specific and nonspecific RNA was at least 20-fold (Figure 5B). The Ll.LtrB RNA does not self-splice under any of the salt conditions tested, but does splice in the presence of LtrA protein (see below). The gel shows that along with unspliced precursor RNA, splicing products (ligated exons and excised lariat RNA) and intermediates (E1 and lariat−E2) were also recovered from the nitrocellulose filter. Since ligated exon RNA does not by itself bind LtrA protein in the reaction medium containing 450 mM NaCl (not shown), these findings suggest that ligated exons remain bound to the complex after splicing.

The effect of salt on the specificity of binding was confirmed by the experiments whose results are shown in panels C and D of Figure 5. In these experiments, 5 pM ^{32}P-labeled Ll.LtrB or other intron RNAs were incubated with increasing amounts of the LtrA protein, and binding was assayed by retention of the complex on a nitrocellulose filter. At 100 mM NaCl, LtrA bound the Ll.LtrB intron with a $K_{1/2}$ of 0.86 nM, but also bound noncognate group II introns and the *N. crassa* mt LSU group I intron relatively tightly ($K_{1/2}$ = 5−12.5 nM). By contrast, at 450 mM NaCl, LtrA bound the Ll.LtrB intron with a $K_{1/2}$ of 0.17 nM, while tight binding to the noncognate intron was abolished ($K_{1/2}$ > 42 nM). We show below that the binding of LtrA to the Ll.LtrB RNA is essentially stoichiometric, with very low K_d values (picomolar) that are not equivalent to the $K_{1/2}$ values measured above (see ref *26*). Calculated by k_{off}/k_{on}, the K_d for the specific complex at 450 mM NaCl is ≤0.12 pM (see below), compared to 3 nM for nonspecific binding of the group I intron at 100 mM NaCl (not shown).

In addition to the specificity of binding, the higher salt concentration also appears to increase the amount of Ll.LtrB intron bound by limiting amounts of LtrA (Figure 5A,B). We thought initially that this enhanced binding might reflect the fact that high salt favors the formation of an RNA structure recognized by the protein. We were surprised to find, however, that the increased level of binding reflects primarily the fact that the higher salt level is required for maximum activity of the LtrA protein. This is demonstrated in Figure 5E, which shows the amount of complex obtained by incubating a fixed amount of LtrA protein (50 nM) with increasing amounts of ^{32}P-labeled Ll.LtrB RNA. At 450 mM NaCl, the curve plateaus at 25 nM bound RNA (stoichiometry of 2:1), whereas at 100 mM NaCl, the curve plateaus at only 5 nM bound RNA. Since the sucrose gradient analysis indicated that the purified LtrA is at least 95% active in RNA binding under the high-salt conditions (see Figure 2), the stoichiometry of 2:1 under these conditions suggests that the protein binds the intron RNA as a dimer. The same salt behavior was exhibited by LtrA with the natural C-terminus released from pLI1 RNP particles by micrococcal nuclease digestion (not shown). The inactivity and instability of the purified LtrA protein at low salt concentrations in vitro could reflect a requirement for an additional protein in vivo.

Quantitative Analysis of RNA Binding. On the basis of the above experiments, the binding of LtrA protein to the intron RNA was analyzed quantitatively in reaction medium containing 450 mM NaCl and 5 mM Mg^{2+} (450 NMT) where the protein is fully active. Since the protein is inactivated rapidly at 37 °C, quantitative analysis of RNA binding and splicing was carried out at 30 °C, a temperature at which the protein is stable in 450 NMT for at least 6 h.

For k_{on} measurements, different concentrations of LtrA protein (20−120 pM) were incubated with 5 pM ^{32}P-labeled Ll.LtrB RNA, and the amount of heparin-stable complex at different times was assayed by retention of the ^{32}P-labeled RNA on a nitrocellulose filter (Figure 6A). The binding data at each protein concentration were best fit to a single-exponential equation. As expected for a bimolecular reaction, a plot of the rate of association versus the concentration of LtrA protein gave a straight line, with the slope corresponding to a k_{on} of 3.8×10^9 min^{-1} M^{-1} (Figure 6B). Similar k_{on} values were obtained when the complex was not challenged with heparin prior to filtration (2.8×10^9 min^{-1} M^{-1}) (not shown). Although LtrA appears to bind Ll.LtrB RNA at a stoichiometry of 2:1 (see the experiments described above and below), we cannot distinguish whether the protein binds as a dimer or dimerizes after RNA binding, as appears to be the case for the *Bombyx mori* R2 element RT (*27*). If LtrA binds as a dimer, the k_{on} values would be 2 times greater than the above values.

For k_{off} measurements, complexes were formed by incubating 10 nM LtrA protein with 20 nM ^{32}P-labeled Ll.LtrB RNA, and then diluted into a reaction medium containing a 100-fold excess of unlabeled Ll.LtrB RNA to bind unassociated or loosely bound LtrA protein. Figure 6C shows that dissociation of the complex is biphasic with k_{off} values of 0.17 min^{-1} (28% fast) and 4.0×10^{-4} min^{-1} (72% slow). Similar results were obtained when the complex was diluted into the reaction medium containing 0.5 mg/mL heparin instead of excess unlabeled intron RNA (not shown). In five repeats of the experiment, the k_{off} values for the fast and

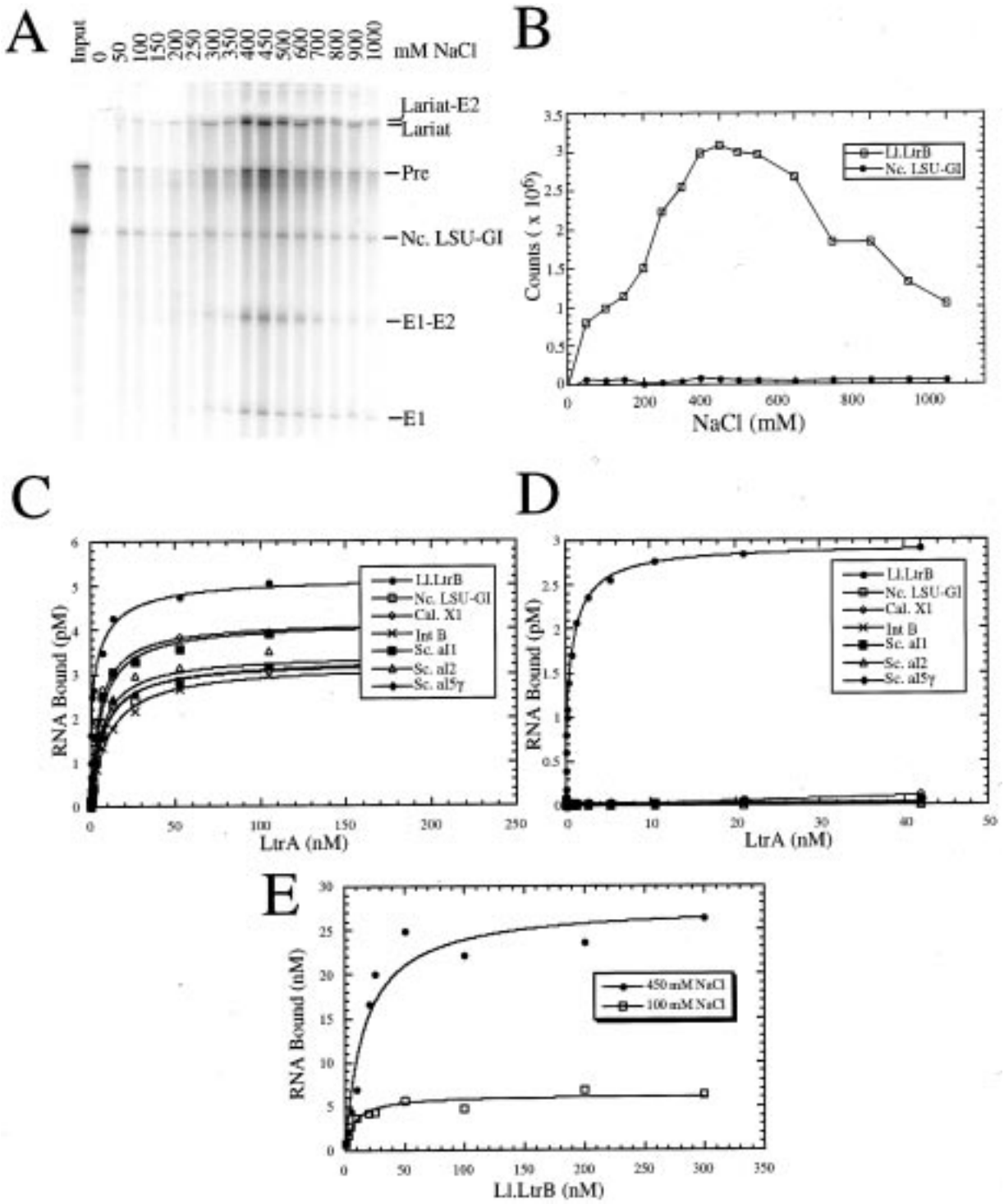

FIGURE 5: Specific binding of the Ll.LtrB intron is favored at 450 mM NaCl. (A) Competition assay. LtrA protein (5 nM) was incubated with a mixture of 50 nM ^{32}P-labeled Ll.LtrB RNA (pGMΔORF/*Bam*HI RNA) and 50 nM *N. crassa* mt LSU group I intron RNA (pBD5A/*Ban*I RNA) in reaction medium (NMT) containing different concentrations of NaCl for 30 min at 37 °C. After filtration through nitrocellulose, bound RNAs were recovered by phenol/CIA extraction and analyzed in a denaturing 4% polyacrylamide gel. Abbreviations: Pre, precursor, Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript); E1−E2, ligated exons; E1, 5′ exon; Nc. LSU-GI, *N. crassa* mt LSU group I intron (pBD5A/*Ban*I) RNA. (B) PhosphorImager quantitation of bound RNAs from panel A. PhosphorImager counts for Ll.LtrB RNA include splicing products. (C and D) Saturation binding of LtrA to the Ll.LtrB intron and noncognate introns in reaction medium containing 100 or 450 mM NaCl. Binding assays were carried out by incubating 5 pM ^{32}P-labeled Ll.LtrB RNA and increasing amounts of LtrA protein (3 pM to 210 nM in panel C and 2.5 pM to 42 nM in panel D) in NMT containing either 100 (C) or 450 mM NaCl (D). The intron RNAs that were tested were Ll.LtrB (pGMΔORF/*Bam*HI RNA) (●), *N. crassa* mt LSU group I intron (pBD5A/*Ban*I RNA) (□), *Calothrix* X1 group II intron (pGMCal/*Eco*RV RNA) (◇), *E. coli* IntB group II intron (pGMIntB/*Eco*RV RNA) (×), yeast aI1 (pJVM159/*Bst*EII RNA) (■), yeast aI2 (pSZD2/*Bst*EII RNA) (△), and yeast aI5γ (pJD20/*Hin*dIII) (◆). (E) RNA binding activity of LtrA at different salt concentrations. LtrA (50 nM) was incubated with increasing concentrations (1−300 nM) of ^{32}P-labeled Ll.LtrB RNA (pGMΔORF/*Bam*HI RNA) in NMT containing 100 (□) or 450 mM NaCl (●) for 1 h at 30 °C, and the amount of heparin-stable complex was determined by nitrocellulose filter binding.

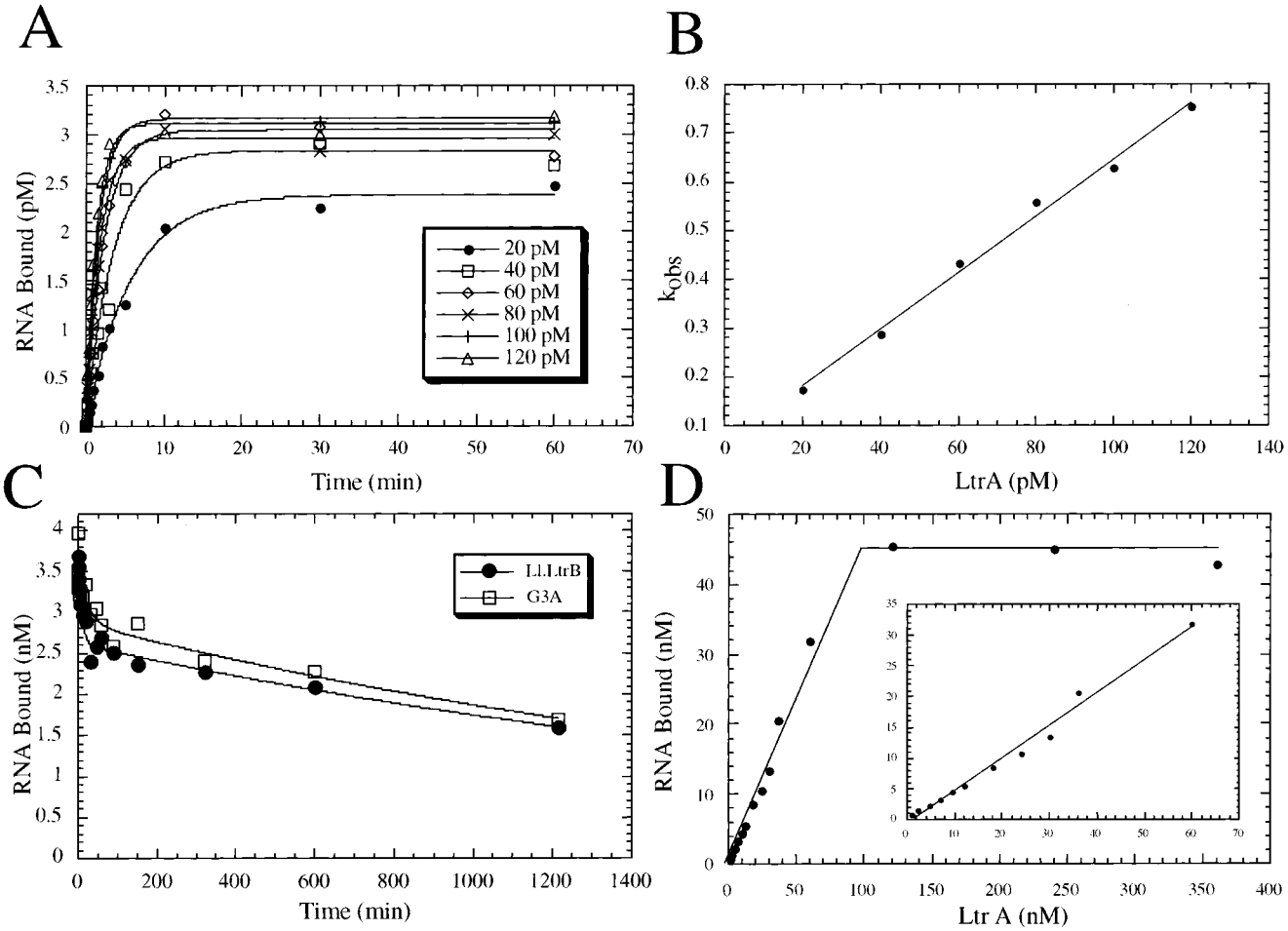

FIGURE 6: Quantitative analysis of intron RNA binding by purified LtrA protein. (A) k_{on} measurements. ^{32}P-labeled Ll.LtrB RNA (5 pM) (pGMΔORF/*Bam*HI in vitro transcript) was incubated with the indicated concentrations of LtrA protein in 450 NMT for 10 s to 60 min. The rate of formation of heparin-stable complex was measured by nitrocellulose filter binding, and the data were fit to a single exponential. (B) The rates derived from panel A are plotted as a function of concentration of LtrA monomer. The slope equal to the k_{on} is 3.8×10^9 min^{-1} M^{-1}. (C) k_{off} determination. The complex was formed by incubating 10 nM LtrA protein with 20 nM ^{32}P-labeled Ll.LtrB RNA (●) or splicing-defective Ll.LtrB RNA with the G3A mutation in domain V (□) in 0.1 mL of 450 NMT for 60 min at 30 °C. The mixture was diluted into 2 mL of reaction medium containing 100 nM unlabeled Ll.LtrB RNA, and the amount of complex remaining at different times was determined by nitrocellulose filter binding. (D) Stoichiometry. ^{32}P-labeled Ll.LtrB RNA (50 nM) was incubated with increasing concentrations of LtrA protein (1−300 nM), and the amount of heparin-stable complex was determined by nitrocellulose filter binding. The plot of the amount of complex as a function of added RNA concentration is linear with a slope of 0.53 up to a plateau at 100 nM LtrA where all of the ^{32}P-labeled RNA is bound. The inset shows the initial portion of the curve plotted on an expanded scale. The data indicate that two molecules of protein are bound per molecule of intron RNA.

slow phases were 0.18 ± 0.08 min^{-1} ($19 \pm 5\%$) and (4.7 ± 2.8) $\times 10^{-4}$ min^{-1} ($81 \pm 5\%$), respectively. The extremely slow k_{off} for the second phase corresponds to a $t_{1/2}$ of >24 h. The K_d values calculated as k_{off}/k_{on} are 48 and 0.12 pM, respectively, if LtrA binds as a monomer, and 24 and 0.06 pM, respectively, if LtrA binds as a dimer. To reflect this uncertainty, the K_d values for the fast and slow phases are listed as ≤48 and ≤0.12 pM, respectively. The biphasic k_{off} behavior does not reflect the occurrence of splicing during the incubations, since the same biphasic curve was obtained irrespective of whether the initial complex was formed for 2 or 60 min (not shown), or with the splicing-defective Ll.LtrB RNA, which has a mutation at the conserved G3 residue of domain V (G3A; Figure 6C). The stable complex is presumed to be productive since it corresponds to the proportion of spliced RNA (see below).

Finally, the stoichiometry of binding was confirmed by incubating a fixed amount of ^{32}P-labeled Ll.LtrB RNA (50 nM) with increasing amounts (1−300 nM) of the LtrA protein (Figure 6D). The plot of the amount of complex formed as a function of LtrA concentration was linear with a slope of 0.53 up to 100 nM LtrA, where essentially all of the input RNA was bound. Thus, the stoichiometry is two molecules of LtrA protein per molecule of Ll.LtrB RNA. In other experiments, the same stoichiometry was also found for both splicing-defective precursor RNA and gel-purified intron lariat RNA and in reaction medium containing a higher salt concentration (1 M NaCl) (not shown).

The Purified LtrA Protein Is by Itself Sufficient To Promote Splicing of the Ll.LtrB Intron. Figure 7A shows the time course of RNA splicing in reaction medium containing 450 mM NaCl and 5 mM Mg^{2+}, where formation of a specific complex with the LtrA protein is favored. The splicing reaction was carried out with 20 nM Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript) and a 10-fold excess of LtrA protein (200 nM). The reaction was biphasic with 74% of the spliceable RNA reacting at a rate of 1.2 min^{-1} and 26% at a rate of 0.026 min^{-1}. In independent experiments, the proportion of reactive RNA ranged from 75 to 100%, and the rate of the fast phase ranged from 0.6 to 1.5

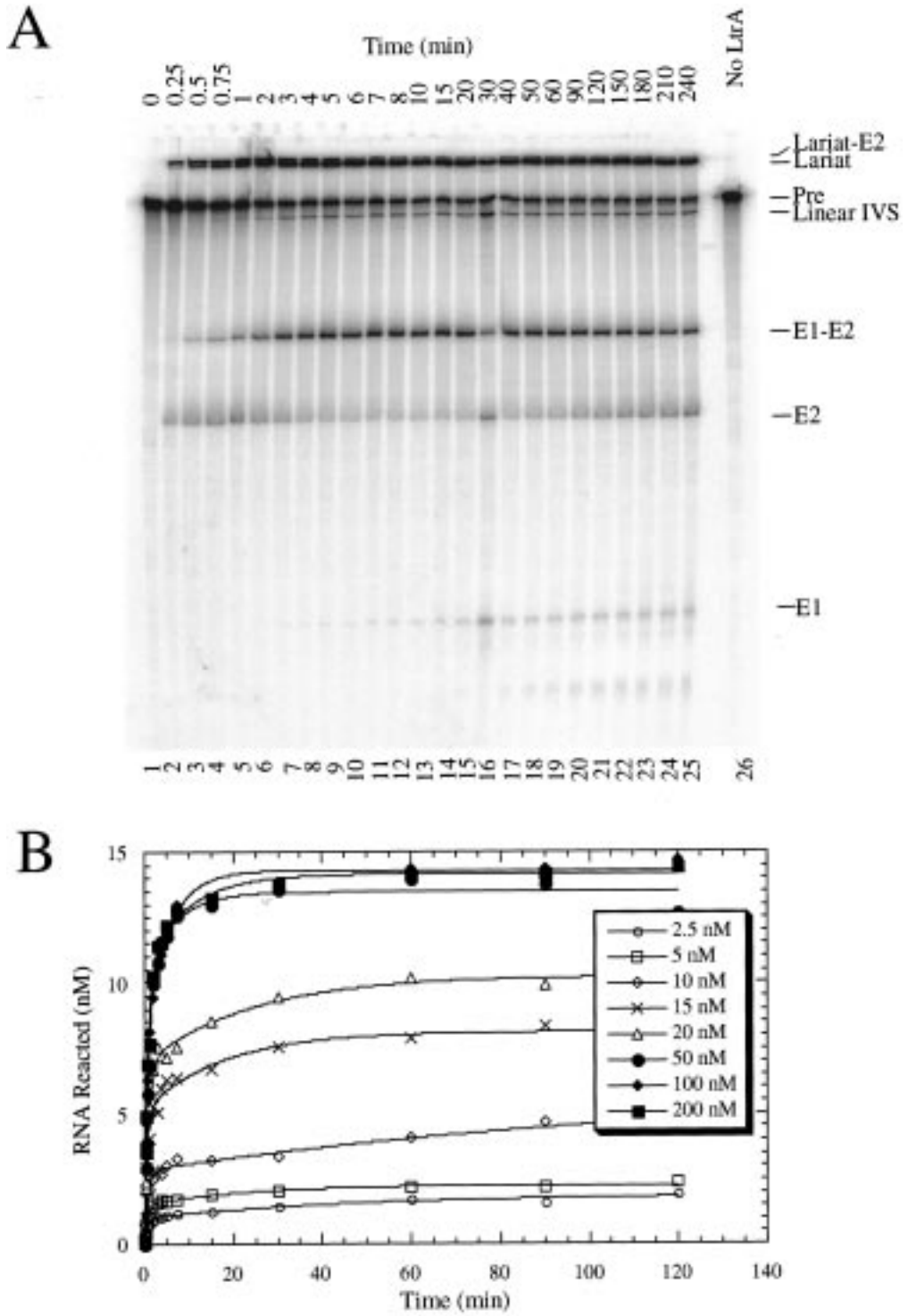

FIGURE 7: Splicing of the Ll.LtrB intron using purified LtrA protein. (A) Splicing with a protein excess. Splicing was carried out by incubating 20 nM ^{32}P-labeled Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript) with 200 nM purified LtrA protein in 450 NMT, and the products were analyzed in a denaturing 4% polyacrylamide gel. Lane 26 shows ^{32}P-labeled Ll.LtrB RNA incubated for 4 h in the same reaction medium without LtrA. Abbreviations: linear IVS, excised linear intron; E2, 3′ exon; other abbreviations as described in the legend of Figure 5. The data along with those for other protein concentrations are plotted in panel B. (B) Splicing reactions were carried out with 20 nM ^{32}P-labeled Ll.LtrB RNA and the indicated concentrations of LtrA protein. The RNA product bands labeled IVS-E2, IVS, Linear IVS, E1−E2, E1, and E2 in panel A were summed and normalized against the total amount of labeled RNA (products and precursor). The plots show the rate of appearance of the normalized products fit to a two-exponential equation.

min^{-1}. These rates are 20−50-fold faster than the fastest rate observed for self-splicing of this intron under high-salt, high-Mg^{2+} conditions (0.03 min^{-1}; not shown). The biphasic reaction and residual unreactive RNA in the presence of excess protein presumably reflect different RNA conformations, with the proportion of spliceable RNA corresponding to that bound tightly in the k_{off} experiments. The RNA by itself exhibited no detectable self-splicing when incubated in the same reaction medium for 4 h at 30 °C (lane 26).

Figure 7B shows the time course of splicing reactions with 20 nM RNA and different concentrations of the LtrA protein ranging from 2.5 to 200 nM. The reaction was again biphasic

with some proportion of the RNA remaining unreactive at the latest time points. Under conditions of RNA excess, the total amount of RNA that was spliced was equal to the amount of protein dimer, as expected if each tight binding event leads to splicing. The rate of the fast phase was approximately 1.3 min^{-1}, independent of protein concentration, indicating that the formation of the complex was not rate-limiting under any of these conditions, as expected from the RNA binding data (see above). This fast phase was followed by a slow phase (0.02 min^{-1}), which under conditions of RNA excess, could reflect the reaction of a more slowly folding RNA conformer and/or exchange of LtrA from nonproductive binding sites. The latter was found experimentally to occur at a rate of 0.03 min^{-1} by adding fresh ^{32}P-labeled Ll.LtrB RNA after initiation of a splicing reaction with excess unlabeled RNA (not shown). On the basis of the k_{off} experiments, turnover of LtrA from the excised intron RNA products is expected to occur at a rate of 4.7×10^{-4} min^{-1} (Figure 6C) and should not contribute significantly to splicing under these conditions. Additional experiments under RNA excess conditions showed that neither the rate nor extent of splicing was affected by ATP or other dNTPs (not shown).

The purified LtrA protein also promoted splicing in reaction medium containing 100 mM NaCl and 5 mM Mg^{2+}. At this lower salt concentration, however, measurements of the amount of splicing as a function of protein concentration gave stoichiometries ranging from 1:5 to 1:30 for different protein preparations, consistent with the smaller proportion of active protein under these conditions (see above). At excess protein (200 nM LtrA/20 nM RNA substrate), 40% of the RNA spliced at 0.14 min^{-1} and 40% at 0.005 min^{-1}, with 20% being unreactive. Both the fast and slow rates and the amplitude of the fast phase were significantly lower than at 450 mM NaCl (see above), suggesting that the higher salt concentration may increase the proportion of active RNA and/or the rate of productive RNA folding, in addition to activating and stabilizing the LtrA protein. NaCl at a concentration of 450 mM could also increase the rate of splicing by weakening nonproductive interactions with the intron RNA.

The pH Dependence Suggests That a Conformational Change is Rate-Limiting for Splicing. The pH dependence of the reaction rate can provide information about the rate-limiting step in a reaction. Thus, a log-linear pH dependence with a slope of 1 suggests a rate-limiting step involving the loss of one proton, most likely chemistry, while pH independence can indicate a rate-limiting conformational change (*28*, *29*). To determine the pH dependence of the LtrA-dependent splicing reaction, splicing time courses were determined with 20 nM ^{32}P-labeled Ll.LtrB RNA and 200 nM LtrA protein over a pH range of 5.0−9.5 (Figure 8). From pH 5.5 to 7.5, the rate of splicing shows a log-linear pH dependence with a slope of 1.1, whereas above pH 8.0, the rate plateaus at ~1.7 min^{-1}. We confirmed that the rate of splicing was not strongly affected by buffer concentration (tested at 25, 50, and 100 mM at pH 6.0, 7.5, and 9.0; see Figure 8) or by the identity of the buffer (pH 6.5, MES/HEPES; pH 7.5, MES/HEPES/TAPS; and pH 8.5, HEPES/TAPS; <25% difference) (not shown). Further, although pH changes could potentially perturb the binding of the LtrA protein to the RNA, the rates measured at pH 6.0 or 8.5 were unchanged when the protein concentration was decreased to 100 nM (not shown), indicating that the protein was saturating under these conditions. If it is assumed that k_{chem} is rate-limiting at low pH, the extrapolated k_{chem} at pH 7.5 is ~3 min^{-1}. The findings here are similar to those for the yeast CBP2 protein in group I intron splicing, which showed a log-linear pH dependence with a slope of 0.6 below pH 6, and a plateau with disappearance of a thio effect above pH 8.0, taken to suggest that a pH-independent conformational change becomes rate-limiting (*29*). Our results do not exclude more complicated models for the pH dependence (see ref *30*).

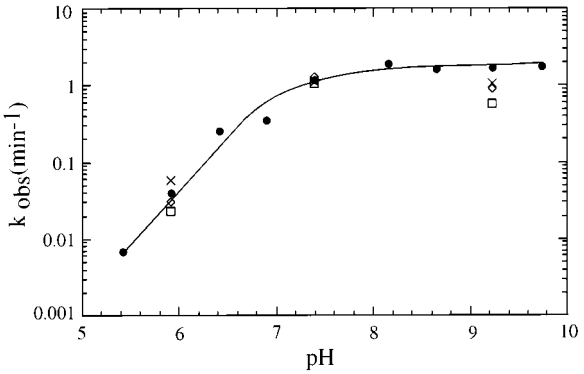

FIGURE 8: pH dependence of the LtrA-promoted splicing reaction. The rate of splicing with 200 nM LtrA protein and 20 nM ^{32}P-labeled Ll.LtrB RNA (pGMΔORF/*Bam*HI in vitro transcript) was measured as described in the legend of Figure 7 at pHs ranging from 5 to 9.5. A log plot of the initial k_{obs} as a function of pH is linear with a slope of 1.1 at pHs between 5.5 and 7.5, and plateaus above pH 8.0. Primary data (●) were obtained in reaction medium containing 50 mM MES (pH 5.0−7.5), HEPES (pH 6.5−8.5), TAPS (pH 7.5−8.5), or CHES (pH 9.0 and 9.5). In a supplementary experiment, k_{obs} at several pH values was measured in reaction medium containing 25 (□), 50 (◇), or 100 mM (×) buffer.

LtrA Is an Intron-Specific Splicing Factor. Finally, Figure 9 shows that the LtrA protein functions specifically in splicing the Ll.LtrB intron. In the experiment shown, 200 nM LtrA protein was incubated with 20 nM ^{32}P-labeled transcripts containing the Ll.LtrB intron or other group II introns (yeast mtDNA introns aI2 and aI5γ, *E. coli* IntB, and *Calothrix* X1) in reaction medium containing 100 mM NaCl and 5 mM Mg^{2+}. Under these conditions, the LtrA protein binds to the other group II introns with $K_{1/2}$ values ranging from 5 to 12.5 nM (Figure 5C). However, only the cognate intron Ll.LtrB is spliced, indicating that the binding of LtrA to the other group II introns at 100 mM NaCl is nonproductive. In reaction medium containing 450 mM NaCl and 5 mM Mg^{2+}, the LtrA protein neither binds to the other group II introns (Figure 5D) nor promotes their splicing (not shown). Control lanes show that the transcripts containing the other group II introns could self-splice under appropriate conditions (lanes 3, 6, 9, 12, and 15).

DISCUSSION

Here, we used purified constituents to show that the LtrA protein is by itself sufficient to promote splicing of the group II intron Ll.LtrB at low Mg^{2+} concentrations, and that RNP particles containing only the LtrA protein and the excised intron RNA have both site-specific DNA endonuclease and TPRT activity. Although it remains possible that additional

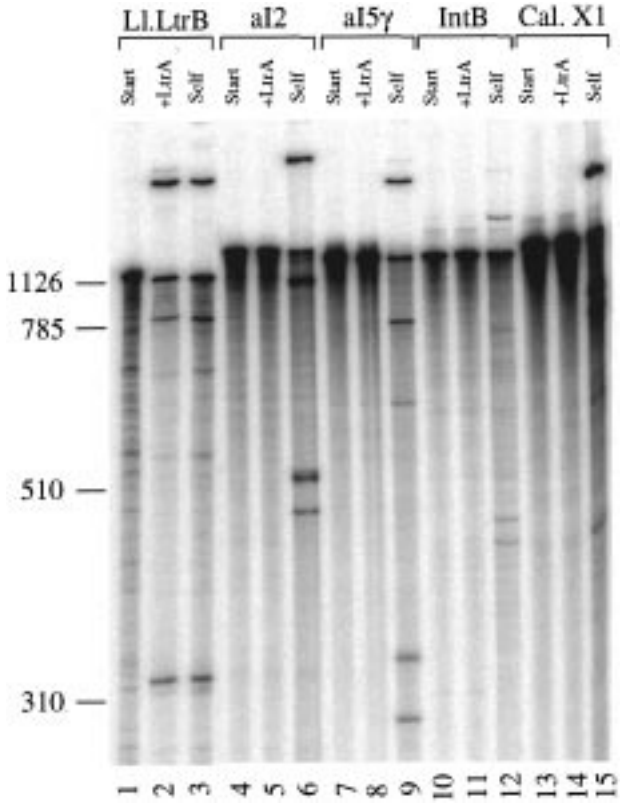

FIGURE 9: LtrA protein is an intron-specific splicing factor. ^{32}P-labeled precursor RNAs (20 nM) containing group II introns Ll.LtrB, *S. cerevisiae* aI2 and aI5γ, *E. coli* IntB, and *Calothrix* X1 were incubated for 1 h at 30 °C under three conditions: (1) 100 NMT (lanes 1, 4, 7, 10, and 13), (2) 100 NMT in the presence of 200 nM LtrA (lanes 2, 5, 8, 11, and 14), and (3) self-splicing conditions [1.5 M NH_4Cl, 100 mM $MgCl_2$, and 40 mM Tris-HCl (pH 7.5)] (lanes 3, 6, 9, 12, and 15). The products were analyzed in a denaturing 4% polyacrylamide gel. The numbers to the left indicate sizes (nucleotides) of in vitro-synthesized RNA markers.

proteins enhance the efficiency of these reactions in vivo (see below), group II introns appear to be remarkably self-sufficient in encoding RNA and protein components which, when combined in an RNP particle, have the multiple activities necessary for intron splicing and mobility. This self-sufficiency presumably facilitates the transmission of group II introns to different hosts.

Mechanism of the Maturase-Promoted RNA Splicing Reaction. Our results provide the first direct evidence that the group II intron maturase activity involves specific binding of the protein to the intron RNA. Detailed analysis of RNA splicing using purified maturase indicates that LtrA binds to unspliced precursor RNA at a stoichiometry of 2:1, suggesting that the protein either is a dimer or dimerizes upon binding to the intron-containing RNA. The binding is very tight (K_d at 30 °C $\leq$ 0.12 pM) and occurs in a rapid bimolecular reaction, which is followed by a slower unimolecular step, presumably a conformational change, required for splicing to occur. Although the nature of the rate-limiting step cannot be inferred from kinetic data, it likely involves folding of the group II intron RNA into the catalytically active structure. The finding that the protein-assisted splicing reaction occurs more rapidly than self-splicing (see the Results) suggests that binding of the protein facilitates the formation of or stabilizes at least one RNA structure that is not optimally formed with high salt or Mg^{2+}. If it is assumed that k_{chem} at pH 7.5 has the value extrapolated from the linear pH dependence at lower pH and that the RNA conformational change (I $\rightarrow$ I*) is the rate-limiting step at higher pH (Figure 8), the minimal kinetic model for the splicing reaction is

$$\mathrm{I} + \mathrm{LtrA} \underset{0.00047\ \mathrm{min}^{-1}}{\overset{\leq 3.8 \times 10^9\ \mathrm{M}^{-1}\ \mathrm{min}^{-1}}{\rightleftharpoons}} \mathrm{LtrA{\cdot}I} \xrightarrow[1.7\ \mathrm{min}^{-1}]{k_{conf}} \mathrm{LtrA{\cdot}I^*} \xrightarrow[\sim 3\ \mathrm{min}^{-1}]{k_{chem}} \mathrm{products}$$

The estimated rate of the chemical step is in agreement with previous estimates of >1 min^{-1} for the reverse branching reaction of the yeast aI5γ intron (*31*), although recent findings indicate that k_{chem} values for group II intron ribozyme reactions may increase substantially at higher Mg^{2+} concentrations or in the presence of Mn^{2+} (*32*).

The yeast aI1 and aI2 maturases have been shown genetically to be intron-specific splicing factors (*10*, *11*). Here, we show biochemically that the LtrA protein is likewise an intron-specific splicing factor and that this specificity correlates with specific binding of the Ll.LtrB RNA. Thus, at 450 mM NaCl, LtrA binds with high affinity to the Ll.LtrB intron, but not to noncognate group II introns. At 100 mM NaCl, LtrA binds nonspecifically to noncognate group II introns, as well as to other RNAs. This nonspecific binding is rather tight ($K_{1/2}$ < 12.5 nM) but is not productive for RNA splicing. The productive binding of LtrA to the Ll.LtrB RNA presumably involves recognition of unique structural features of the unspliced precursor RNA.

Studies of self-splicing group II introns have shown that the first step of splicing, cleavage at the 5′ splice site, can occur by either branch formation or hydrolysis, leading to excision of the intron as a lariat or linear RNA, respectively (*33*, *34*). For the fast phase of the protein-dependent splicing reaction, the major product is lariat RNA (ratio of lariat to linear intron of >37:1 in Figure 7A, lane 5). By contrast, in self-splicing reactions with 1.5 M NH_4Cl and 100 mM Mg^{2+}, lariat and linear intron RNA appear simultaneously at a ratio of 1.7:1 (Figure 9). Similar results were obtained when the protein was added under self-splicing conditions in a reaction medium containing 0.5 M NH_4Cl and 50 mM Mg^{2+} (not shown). These findings suggest that the intron RNA structure induced by protein binding differs from that formed under the self-splicing conditions in favoring lariat formation or suppressing hydrolysis.

An unexpected feature of the maturase-promoted splicing reaction is its salt dependence in vitro. The reaction occurs at 100 mM NaCl and 5 mM Mg^{2+}, but both the rate and extent of splicing are increased at 450 mM NaCl. Our results show that the high salt level is required primarily for maximal activity and stability of the LtrA protein (see above). Additionally, the higher salt concentration favors specific binding of the LtrA protein to the Ll.LtrB RNA (Figure 5) and may increase the proportion of active RNA or the rate of productive RNA folding, as judged by the faster splicing rate at 450 mM than at 100 mM NaCl under conditions of protein excess. In vivo, other factors, such as RNA helicases or other RNA chaperones, may facilitate formation of the productive structure, obviating the need for a high salt level.

Genetic studies indicate that the yeast MSS116 protein, a putative RNA helicase, is required for efficient splicing of the yeast mtDNA intron aI1, which is also dependent on a maturase (*17*).

The mode of action found for LtrA is similar to that of the *N. crassa* CYT-18 protein, which binds group I intron RNAs in a rapid bimolecular reaction and then induces a slower RNA conformational change required for splicing (*26*, *35*). By contrast, the yeast CBP2 protein uses a different mode of action, termed tertiary structure capture, in which binding occurs at a slow unimolecular rate that is limited by the rate of formation of RNA tertiary structure recognized by the protein (*36*). Although LtrA may recognize secondary or tertiary structure features of the group II intron-containing RNAs, the kinetic analysis indicates that the required structure would have to form rapidly in the free RNA, so as not to be rate-limiting for protein binding in our experiments.

After splicing, the LtrA protein remains tightly bound to the excised intron lariat RNA, where it constitutes the DNA endonuclease. The very tight binding to the excised intron limits turnover of the protein in vitro and suggests that additional components, such as the debranching enzyme, nucleases, or RNA helicases, may be required to facilitate turnover in vivo. Similar findings have been made for the *N. crassa* CYT-18 and yeast CBP2 proteins in group I intron splicing (*26*, *36*). In addition to recycling LtrA, turnover of the excised group II intron RNPs may be advantageous in protecting the cell against the potentially deleterious DNA endonuclease and reverse splicing activities of these RNPs.

Interdependence of the Intron-Encoded Protein and Intron RNA in RNA Splicing and Mobility Reactions. Our results emphasize that the activities of the intron-encoded protein and intron RNA are mutually dependent on their tight association in RNP particles. Thus, the LtrA protein is required to induce the formation of the catalytically active intron RNA structure, thereby promoting splicing. Conversely, we show that the RT and maturase activities of the purified LtrA protein are unstable at the normal growth temperature of 37 °C, but are stabilized by binding of the intron RNA or RT substrates. The purified protein also lacks DNA endonuclease activity unless complexed with intron lariat RNA. Stabilization by binding of an RT substrate has also been found for yeast aI2 intron RT (*2*; S. Zimmerly et al., submitted) and is likely to be a general characteristic of group II intron RTs. In this respect, the group II intron RTs resemble hepatitis B RT, where expression of the active protein requires the presence of the template RNA binding site (*37*, *38*), and the *B. mori* R2 element RT, where an RNA cofactor is required for second-strand cleavage by the associated DNA endonuclease activity (*27*, *39*). The instability of the free group II intron-encoded proteins suggests that they may preferentially function in cis by binding to the intron RNAs from which they are translated. On the other hand, a low level of trans activity is indicated for the yeast aI1 and aI2 RTs by their ability to promote the deletion of noncognate introns from genomic DNA via recombination with a cDNA copy of a spliced transcript (*40*). In addition, LtrA expressed in *E. coli* can promote the splicing of the Ll.LtrB intron in trans (B. Cousineau and M. Belfort, Wadsworth Center, Albany, NY, personal communication).

The finding that the purified LtrA protein is fully active only at 450 mM NaCl raises the possibility that it ordinarily functions in a complex with one or more other proteins that stabilize its active structure in vivo. A possible precedent for group I introns involves the maturases encoded by the closely related yeast mtDNA introns bI4 and aI4. These proteins, which are related to the LAGLIDADG family of DNA endonucleases, have been shown genetically to function in splicing by acting in concert with the mt LeuRS protein (Nam*2*p). Further, an inactive form of the aI4 maturase can be activated by suppressor mutations in Nam*2*p, suggesting a protein−protein interaction (*41*, *42*). A related LAGLIDADG protein, the yeast *Sce*I endonuclease, copurifies with a mitochondrial hsp70 that is required for maximal endonucleolytic activity (*43*). For both group I and group II introns, the requirement for a protein partner for optimal maturase function may provide a measure of host control of the splicing reaction.

In summary, the in vitro system described here faithfully recapitulates the group II intron splicing and mobility reactions and shows that all of the activities required for these steps are associated with a single species of RNP particle containing only the intron-encoded protein and the excised intron RNA. The activities required for mobility include formation of the RNP endonuclease by RNA splicing, the initial recognition of the double-stranded DNA target site by the protein component of the endonuclease, unwinding of the double-stranded DNA to permit base pairing of the intron RNA, reverse splicing of the intron into the DNA's sense strand, cleavage of the DNA's antisense strand by the LtrA protein, and use of the 3′ end of the cleaved antisense strand as a primer for reverse transcription of the reverse spliced intron RNA. Thus, the group II intron RNP particles provide a remarkable example of how protein and RNA components can coevolve to carry out a coordinated series of reactions, leading to a new activity, integration into double-stranded DNA, that is not possible for either component alone. The coordination of RNA and protein catalytic activities could also be relevant to the evolution of more complex RNP particles, like the ribosome or spliceosome.

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