Escherichia coli proteins, including ribosomal protein S12, facilitate in vitro splicing of phage T4 introns by acting as RNA chaperones

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To address the effect of host proteins on the self-splicing properties of the group I introns of bacteriophage T4, we have purified an activity from *Escherichia coli* extracts that facilitates both *trans*- and *cis*-splicing of the T4 introns in vitro. The activity is attributable to a number of proteins, several of which are ribosomal proteins. Although these proteins have variable abilities to stimulate splicing, ribosomal protein S12 is the most effective. The activity mitigates the negative effects on splicing of the large internal open reading frames (ORFs) common to the T4 introns. In contrast to proteins shown previously to facilitate group I splicing, S12 does not bind strongly or specifically to the intron. Rather, S12 binds RNA with broad specificity and can also facilitate the action of a hammerhead ribozyme. Addition of S12 to unreactive *trans*-splicing precursors promoted splicing, suggesting that S12 can resolve misfolded RNAs. Furthermore, incubation with S12 followed by its proteolytic removal prior to the initiation of the splicing reaction still resulted in splicing enhancement. These results suggest that this protein facilitates splicing by acting as an RNA chaperone, promoting the assembly of the catalytically active tertiary structure of ribozymes.

Bacteriophage T4 contains three self-splicing group I introns located in the structural genes for thymidylate synthase (*td*), ribonucleotide reductase (*nrd*B), and a putative anaerobic ribonucleotide reductase (*sun*Y) (Chu et al. 1984; Gott et al. 1986; Shub et al. 1987; Sun et al. 1993). These introns splice by the typical group I pathway, via two transesterification reactions initiated by nucleophilic attack of guanosine at the 5' splice site. This process depends on conserved secondary and tertiary structures that direct folding of the intron such that the 5' and 3' splice sites are juxtaposed to the guanosine-binding site within the intron's catalytic core (Cech 1990; Michel and Westhof 1990; Cech et al. 1992; Saldanha et al. 1993).

Although a number of group I introns (including the T4 introns) self-splice in vitro, evidence points to involvement of accessory factors during in vivo splicing.

For example, the canonical *Tetrahymena* intron splices 50-fold more efficiently in vivo than in vitro (Brehm and Cech 1983). In addition, two groups of accessory proteins that regulate splicing of fungal mitochondrial introns have been defined by genetic criteria (for review, see Burke 1988; Lambowitz and Perlman 1990). First are the intron-encoded proteins called maturases that facilitate splicing of their cognate introns (Lazowska et al. 1980, 1989; Anziano et al. 1982; De La Salle et al. 1982). Second are the nuclear-encoded accessory splicing proteins (Burke 1988; Lambowitz and Perlman 1990). The most well-characterized of these is found in *Neurospora*, where a mutation in the *cyt18* gene blocks splicing of a number of group I introns in vivo, including the COB1 intron, which can self-splice in vitro (Garriga and Lambowitz 1984). CYT-18 protein, which corresponds to mitochondrial tyrosyl tRNA synthetase (Akins and Lambowitz 1987), binds specifically to the mitochondrial large subunit rRNA (LSU) intron with high affinity (Guo and Lambowitz 1992). CYT-18 can also promote splicing of mutant T4 *td* introns both in vivo and in vitro (Mohr

et al. 1992), suggesting that the protein acts by stabilizing the correct tertiary structure of the intron (Guo and Lambowitz 1992).

A role for protein factors in modulating splicing of the T4 introns in vivo is suggested by several observations. First, both the *sun*Y and *nrd*B introns require elevated monovalent ion concentrations to splice efficiently in vitro (Hicke et al. 1989). Second, utilization of a cryptic 5′ splice site by the *td* intron is reduced in vivo compared with in vitro (Chandry 1991). Third, the *td* intron splices more efficiently in vitro at elevated temperature (Chu et al. 1987). Fourth, analysis of a *td* trans-splicing reaction revealed that whereas the in vitro reaction occurs efficiently only at high temperatures (55°C), the *td* intron is capable of *trans*-splicing in vivo at physiological temperatures (37°C) (Galloway-Salvo et al. 1990). These findings suggested that facilitatory factors may be present within the cell, prompting attempts to purify these putative factors. Using stimulation of *trans*-splicing as an initial in vitro assay, we have identified an activity that facilitates both *trans*- and *cis*-splicing of the *td* intron. The activity, which also promotes splicing of the *nrd*B and *sun*Y introns, comprises multiple proteins that have different activities for splicing enhancement. Several of these are ribosomal proteins, with ribosomal protein S12 being the most active. Functional analyses suggest that these proteins are mechanistically distinct from those that specifically enhance group I splicing and, rather, serve to facilitate formation of the active conformation of the intron by acting as RNA chaperones.

Results

Identification of an Escherichia coli activity that facilitates trans-splicing

Previously, we described construction and characterization of a *trans*-splicing assay for the *td* intron (Galloway-

Salvo et al. 1990). In this assay the intron is separated into two halves that must associate for splicing to occur (Fig. 1A; Materials and methods). Although this reaction occurs readily at 55°C in vitro, *trans*-splicing is extremely inefficient at 37°C (Fig. 1B, lanes 1,2). To determine whether cellular factors may be capable of promoting the reaction in vitro, we fractionated a crude *E. coli* extract on carboxymethylcellulose and tested the bound fraction (EXT) for its ability to promote *trans*-splicing at 37°C. In the presence of EXT, *trans*-splicing was stimulated as evidenced by appearance of ligated exons (E1–E2) (Fig. 1B, lanes 3,4). We also observed the appearance of the linear homolog of the cyclized intron ("c″I) (Fig. 1A; Galloway-Salvo et al. 1990), indicating that the precursors were competent for all three steps of the group I splicing pathway, 5′ and 3′ splice site cleavage and cyclization.

We found that pretreatment of EXT with proteinase K abolished the activity (Fig. 1C, lane 3), indicating that a protein component was likely to be responsible for the activity. Interestingly, boiling of EXT for 10 min did not affect its ability to promote *trans*-splicing (Fig. 1C, lane 5). Thus, although the activity appeared to be proteinaceous, it was surprisingly heat stable. Furthermore, preliminary gel-filtration analysis and SDS-PAGE had shown that EXT consisted largely of proteins of molecular mass <18 kD (P. DiMaria and M. Belfort, unpubl.). Additional experiments revealed that the activity bound to heparin–agarose and other similar cation exchangers and was resistant to *N*-ethylmaleimide (P. DiMaria and M. Belfort, unpubl.). These biochemical properties are reminiscent of *E. coli* histone-like proteins IHF and HU (Drlica and Rouviere-Yaniv 1987). However, we found that neither protein stimulated *trans*-splicing to any appreciable extent (data not shown). Furthermore, ribosomal protein L14, T4 gene 32 protein, bovine serum albumin (BSA), lysozyme, and polyethylene glycol also failed to stimulate *trans*-splicing (data not shown).

Figure 1. *Trans*-splicing enhancement by an *E. coli* extract. (*A*) *Trans*-splicing pathway. Precursor RNAs H1 and H2 associate in *trans* (see Materials and methods), and splicing is initiated by an exogenous guanosine cofactor (G) resulting in splice products (ligated exons, E1–E2; excised intron sequences from H1 and H2, I1 and I2, respectively; and a linear homolog of the cyclized intron, "c″I. The sizes of the various RNAs are shown in nucleotides. (*B*) Splicing enhancement. *E. coli* extracts were fractionated by cation exchange chromatography, and the protein fraction that bound to the column (EXT) (representing 1–5% of the soluble protein in crude lysate) tested for its ability to promote *trans*-splicing. Equimolar quantities of uniformly α-^{35}S-labeled CTP precursor RNA synthesized from pTZH1 (H1) and pTZH2 (H2) were incubated at 55°C (lane *1*) or at 37°C (lanes *2–4*) in the absence (lanes *1,2*) or presence of two independent EXT preparations (lanes *3,4*). Splicing reactions were separated on a 5% acrylamide (39:1 acrylamide/bisacrylamide)/8 M urea gel. E1–E2 and "c″I are indicators of splicing. (*C*) Extract pretreatment. Stimulation of *trans*-splicing was monitored in the absence of EXT (lane *1*), with untreated EXT (lane *2*), EXT subjected to proteinase K treatment for 10 min (lane *3*), EXT treated with heat-inactivated proteinase K (lane *4*), and EXT that had been boiled for 10 min (lane *5*).
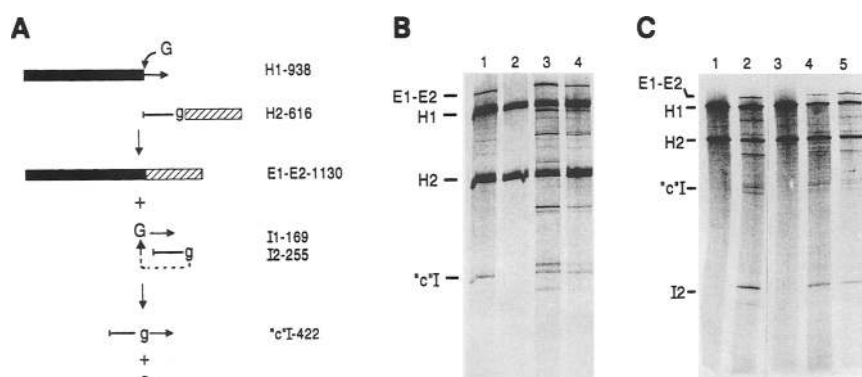
EXT stimulates cis-*splicing of all three phage introns*

We then examined the ability of EXT to stimulate *cis*-splicing. For this purpose we selected the *td*–CAT intron, in which the intron open reading frame (ORF) that encodes a highly toxic endonuclease had been replaced with the comparably sized chloramphenicol transacetylase gene. Not only are transcription templates more readily prepared from this construct, but because splicing is somewhat compromised relative to the wild type, unspliced precursors are more easily isolated. As expected with the *cis*-acting RNA, self-splicing occurred in the absence of EXT (Fig. 2A, lane 3). However, the addition of EXT enhanced the extent of exon ligation three- to sixfold above background self-splicing (Fig. 2A, lane 4; Fig. 2B), indicating that the ability of EXT to stimulate splicing was not unique to *trans*-splicing. Furthermore, the reaction went to completion in the presence of EXT, whereas in the absence of EXT, a substantial fraction of precursor remained even after lengthy incubation, suggesting that these RNA molecules are trapped in misfolded conformations (Fig. 2B; see also Fig. 7, below).

To test further the generality of the activity present in EXT, splicing of the wild-type T4 introns, as well as the *Saccharomyces* COB5 and *Neurospora* LSU introns, was assayed in EXT using a GTP incorporation assay. This assay exploits the fact that the guanosine cofactor becomes covalently linked to the intron in the first step of splicing (Garriga and Lambowitz 1984). When radiolabeled GTP is mixed with unlabeled pre-mRNA under

splicing conditions, two splice products usually appear. These are the intron–exon 2 (I–E2) intermediate, which results from cleavage at the 5′ splice site by GTP, and the smaller linear intron (I), which is released after exon ligation. EXT again promoted splicing of all three T4 introns two- to fourfold above self-splicing levels, consistent with the *td*–CAT results (Fig. 2C). However, EXT was not able to promote splicing of the *Neurospora* LSU rRNA intron or the COB5 intron of *Saccharomyces*, both of which require specific proteins to splice in vitro and in vivo (data not shown) (Garriga and Lambowitz 1984, 1986; Gampel and Tzagoloff 1987; Partono and Lewin 1988). This result is consistent with the finding that these heterologous introns do not splice in *E. coli* (Mohr et al. 1992).

EXT consists of multiple proteins

The biochemical properties of EXT, namely heat stability, small size, and affinity for cation exchangers, are reminiscent of the ribosomal proteins. This possibility was explored by analyzing both EXT and total 70S ribosomal proteins by two-dimensional gel electrophoresis. EXT proteins exhibited a remarkably similar electrophoretic profile to ribosomal proteins (Fig. 3), with at least 15 spots comigrating. On the basis of this striking similarity, we used reverse-phase HPLC to further resolve EXT, because this method allows purification of individual ribosomal proteins capable of reassembling into ac-
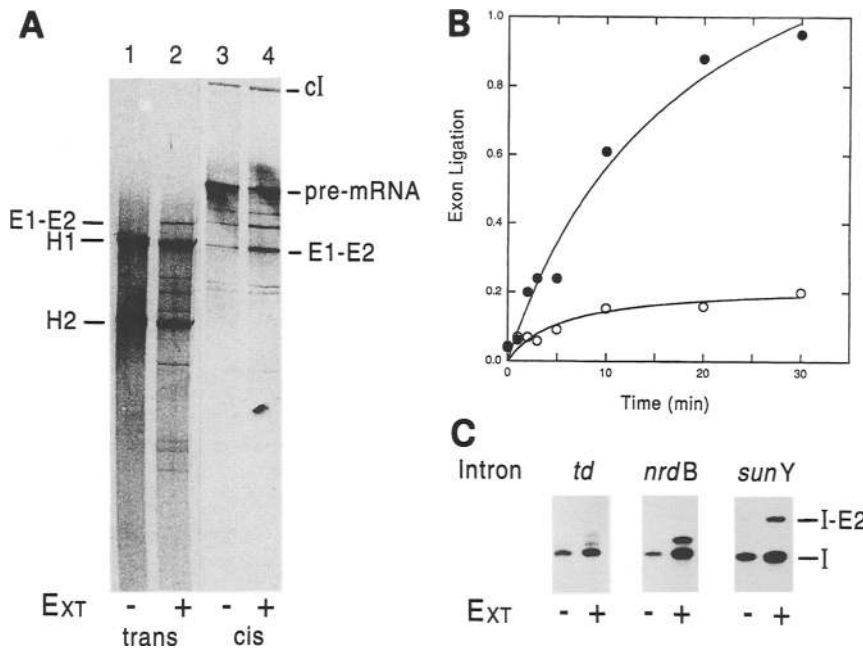

Figure 2. *Cis*-splicing with EXT. (*A*) *Trans*- and *cis*-splicing. Uniformly α-^{35}S-labeled CTP H1 and H2 (lanes *1,2*, as in Fig. 1) and *cis* pre-mRNA synthesized from linearized plasmid pTZ*td*CAT (lanes *3,4*) were incubated in the absence (lanes *1,3*) or presence (lanes *2,4*) of EXT for 30 min and separated as described in Fig. 1. The band marked cI corresponds to cyclized intron. (*B*) Quantitation of *cis*-splicing. Pre-mRNA was generated from linearized pTZ*td*CAT and incubated in the presence (●) or absence (○) of EXT at 37°C. Following phenol extraction, reaction mixtures were applied to Hybond (Amersham) membranes on a slot blotter. Membranes were probed with splice-junction (E1–E2) and intron-specific probes. Splicing was quantitated by scanning densitometry of autoradiographs. For each time point the amount of exon ligation was normalized to the signal for the intron probe, which is constant regardless of splicing. (*C*) *Cis*-splicing for the three phage introns. Wild-type *td*, *nrd*B, and *sun*Y pre-mRNA was incubated with (+) or without (−) EXT and splicing was monitored by [^{32}P]GTP incorporation. Incubations were for 30 min at 37°C for *td* and 30°C for *sun*Y and *nrd*B RNAs. Reaction products were separated as described in Fig. 1. Bands correspond to intron–exon 2 (I–E2) and linear intron (I). Splicing of *td*, *nrd*B, and *sun*Y was stimulated by EXT by factors of 2-, 4.1- and 2.5-fold over background, respectively, as determined by scanning densitometry.
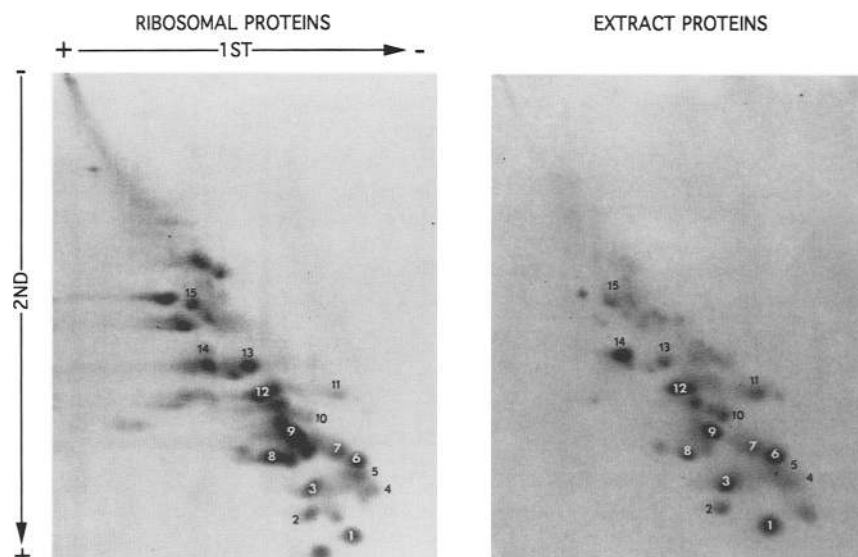
Figure 3. Electrophoretic analysis of EXT. Total ribosomal proteins and EXT were separated in two dimensions, as described by Datta et al. (1988), and stained with Coomassie blue. Spots with similar mobilities are numbered.

tive ribosomes (Kerlavage et al. 1983, 1984). By adapting the approach used by Ferris et al. (1984), we separated EXT into 26 distinct peaks (Fig. 4A) containing at least 36 proteins (data not shown). Those peaks that contained single proteins (Fig. 4B) were assayed for enhancement of *trans*-splicing (Fig. 4C). The degree to which individual proteins simulated splicing varied greatly, with protein 6 being the most active (Fig. 4C). From amino-terminal sequence determination it was established that this protein corresponds to ribosomal protein S12 (Fig. 4D). Protein numbers 7 and 20, weak enhancers of splicing, were found to correspond to ribosomal protein L35 and histone-like protein HLP-1, respectively (Fig. 4D).

Relationship of the intron ORF to splicing enhancement by EXT

To begin to address possible mechanisms for splicing enhancement, we wished to determine the effect of the intron ORFs, natural residents of all three phage introns, on splicing in the presence and absence of EXT. The wild-type *sun*Y intron contains a 774-nucleotide ORF looped out of P9.1 (Fig. 5B inset). The wild-type *sun*Y intron and three *sun*Y derivatives that had 290, 413, and 593 nucleotides deleted from the ORF were tested for splicing activity in the absence and presence of EXT. As expected, all four substrates were able to self-splice (Fig. 5A). However, the extent of self-splicing was inversely related to ORF length, with two- to threefold more splice products observed with the Δ593 intron compared with the full-length wild-type intron (Fig. 5A). Conversely, the ability of EXT to promote splicing was directly related to ORF size. In the case of the wild-type intron, splicing was stimulated up to fivefold (cf. WT lanes 1, +/− EXT), whereas the effect decreased with reduced ORF size (Fig. 5B). These results and those described below suggest that EXT acts by reversing negative interactions imposed by the ORF.

RNA binding by S12 protein

To test whether S12 binds RNA specifically, like other proteins that have been found previously to enhance group I splicing, filter-binding assays were performed with a range of different RNAs. In these experiments, radiolabeled intron required similar concentrations of S12 to be retained on nitrocellulose filters as did exons and ORF RNAs, with essentially complete retention of the different RNAs at S12 concentrations of ∼1 μM (4°C, 150 mM NaCl; data not shown). Although these results provided no indication of a binding preference for the intron, as expected for a protein that would specifically stabilize the catalytic conformation of the intron, the experiments did not give well-behaved binding curves. A number of factors might contribute to such multiphasic behavior for nonspecific RNA-binding proteins, including the presence of multiple protein-binding sites on each RNA and different retention efficiencies of RNAs with different numbers of protein molecules bound. Therefore, to learn more about the RNA-binding properties of the S12 protein, the ability of poly(rC) to compete with these different RNAs for binding to S12 was determined. At both 75 nM S12 (Fig. 6A) and 300 nM S12 (data not shown), poly(rC) was more efficient at competing with tRNA and intron than with ORF or exon RNA. These results are consistent with S12 having a small binding preference for unstructured RNAs (exon and ORF), relative to structured RNAs (intron and tRNA). However, the biphasic nature of the curves implies that S12 has complex binding properties and suggests that the protein recognizes both strong and weak sites on all the RNAs.

To investigate further the RNA-binding properties of S12, labeled intron or exon RNA was used as an S12-binding substrate with exon, ORF, intron, and tRNA as competitors, each at two different concentrations (Fig. 6B). At both concentrations the exon substrate was less effectively competed than the intron substrate by the

four competitor RNAs. Furthermore, the exon and ORF RNAs tended to be more effective competitors than tRNA for both intron and exon substrates. These results suggest that binding sites in well-folded RNAs may generally be less accessible to S12 than those in RNAs that are poorly folded, the latter of which would therefore tend to bind the protein with somewhat higher affinity. Regardless, the ability of exon and ORF RNA to compete at least as well as intron for S12 filter binding with either labeled exon or intron again argued strongly against specific binding of S12 to the intron.

S12 has properties of an RNA chaperone

The *cis*-splicing experiment presented in Figure 2B suggests a protein-assisted increase in the fraction of reacting molecules, rather than an increase in the rate of the reaction. Because this issue bears on the potential of S12 to act as an RNA chaperone, we wished to address the question more directly. The *trans*-splicing reaction was therefore allowed to proceed in the absence of S12 until a plateau was reached in the fraction of reacted precursor (Fig. 7, ○). After the addition of S12 (Fig. 7, ▼), the re-
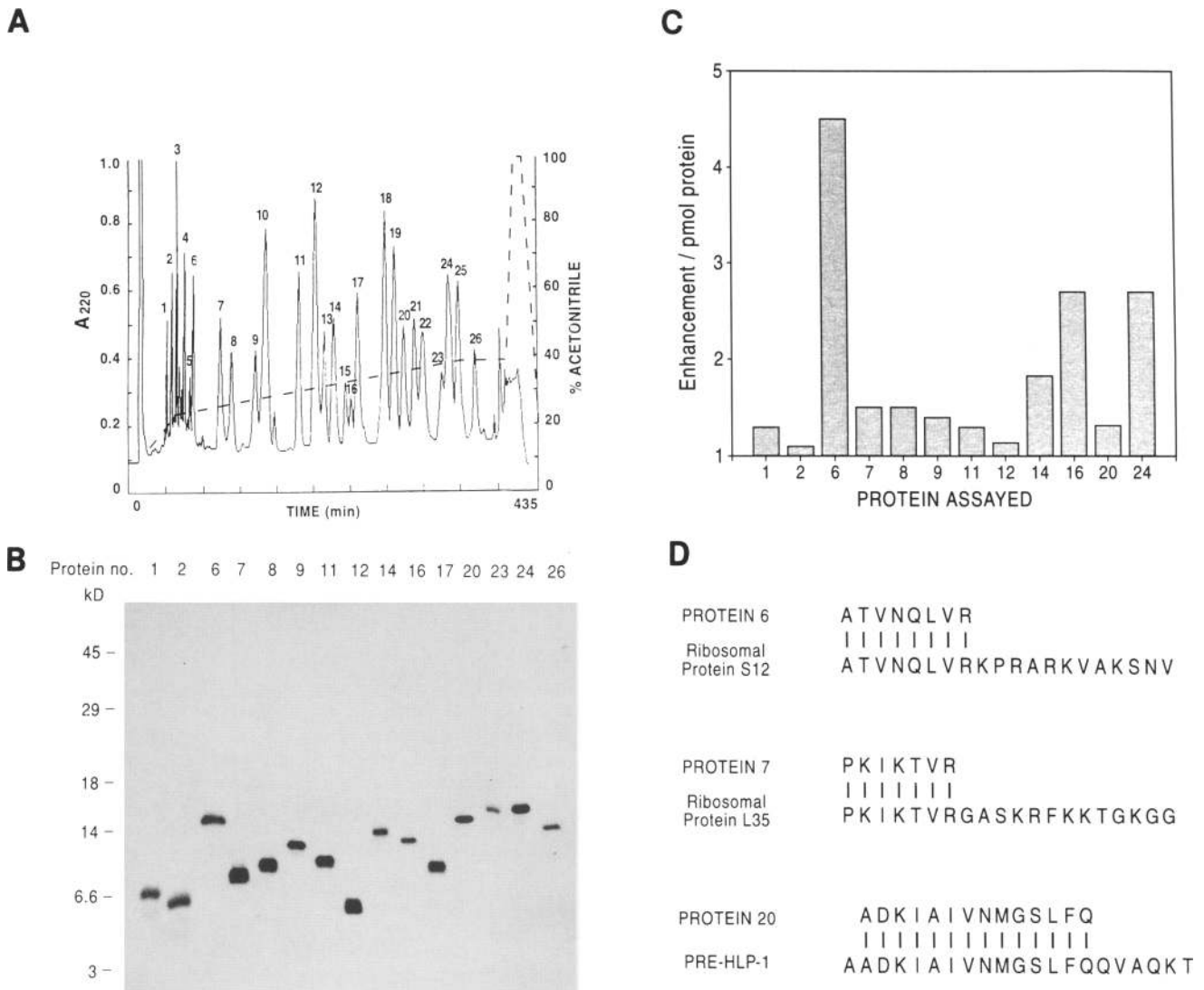

Figure 4. Purification and analysis of EXT proteins. (*A*) Reverse-phase HPLC chromatograph of EXT. Separation was as described in Materials and methods, with assigned peak numbers indicated. The broken line indicates the gradient used. (*B*) SDS-PAGE of peaks containing individual EXT proteins. Numbers assigned to each protein correspond to peak assignment in *A*. Missing numbers reflect peaks containing multiple protein bands. (*C*) Enhancement of *trans*-splicing. The splicing enhancement of individual proteins is indicated as the counts per minute (cpm) of GTP incorporated in the presence divided by that in the absence of protein, per pmole of protein. Because, typically, the amount of GTP incorporated increased and then decreased with increasing protein concentration, the ratio was obtained using the amount of protein yielding maximal splicing enhancement. (*D*) Identities of selected EXT proteins. The amino-terminal sequences of protein 6, 7, and 20 are shown aligned with the published sequences of S12, L35, and HLP-1, respectively. Attempts to sequence proteins 16 and 24 were unsuccessful.

sidual precursors reacted to an extent similar to that when S12 was added at the beginning of the reaction (Fig. 7, ●). These results argue that S12 can assist the formation of productive structures by resolving misfolded RNAs.

To test whether S12 may be dispensable during the catalytic step, S12-promoted assembly of *trans*-splicing precursors was allowed to occur in the absence of GTP. Subsequently, proteinase K was added, and thereafter, splicing was initiated by addition of [^{32}P]GTP (Table 1). Addition of the protease after the S12-promoted assembly step had no inhibitory effect on *trans*-splicing (Table 1, cf. lines 4 and 6), in contrast to pretreatment of S12 with proteinase K (line 7). No S12 was observed in proteinase K-treated reactions analyzed in Western blots with S12 antibody, where 10% (1 pmole) of the amount of S12 required to promote splicing (10 pmoles) was
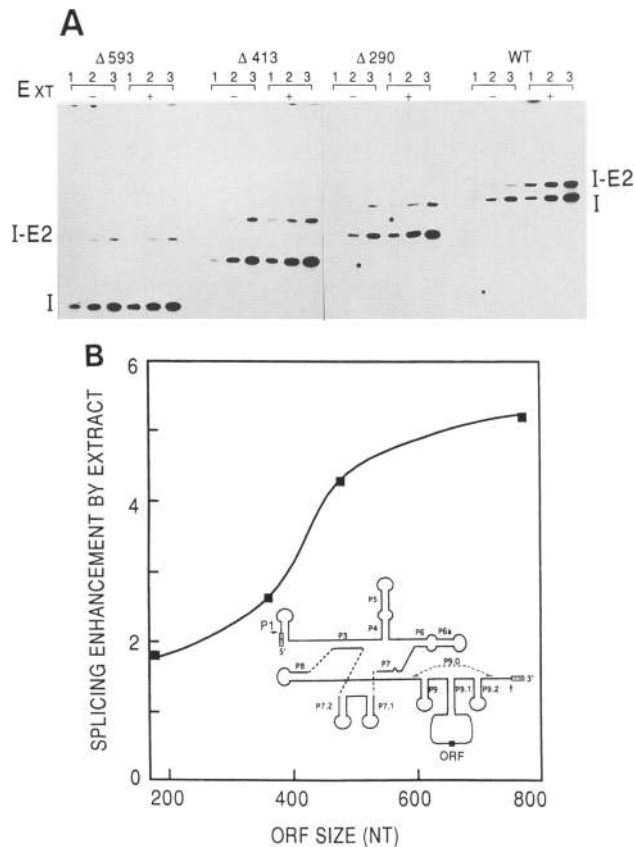

Figure 5. Effect of ORF size on splicing stimulation by EXT. (A) Splicing of the *sunY* intron with ORF deletions. The wild-type (WT) and three deletion constructs (Δ290, Δ413, and Δ593) were monitored for splicing by GTP incorporation. Lanes *1*, *2*, and *3* correspond to 2.5-, 5-, and 10-min incubation, respectively, in the presence (+) or absence (−) of EXT. Splicing reactions were separated as described in Fig. 1. Labeled bands correspond to intron–exon 2 (I–E2) and linear intron (I). (B) EXT-mediated splicing enhancement as a function of ORF size. The 5-min time point for each intron (A, lanes 2) was used to measure splicing enhancement as in Fig. 4C, with EXT relative to that without EXT. The secondary structure of the *sunY* intron with the placement of the ORF is shown in the *inset*.
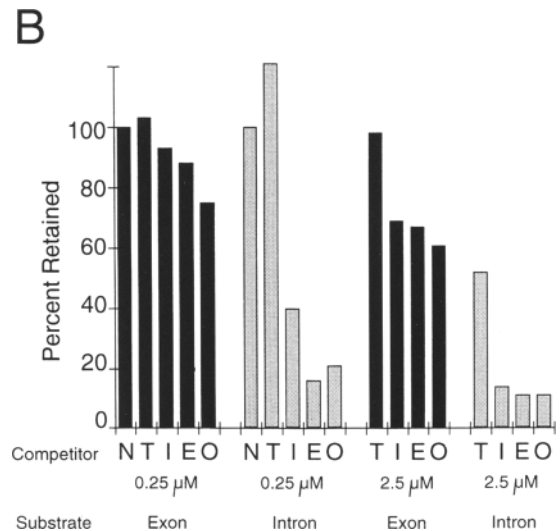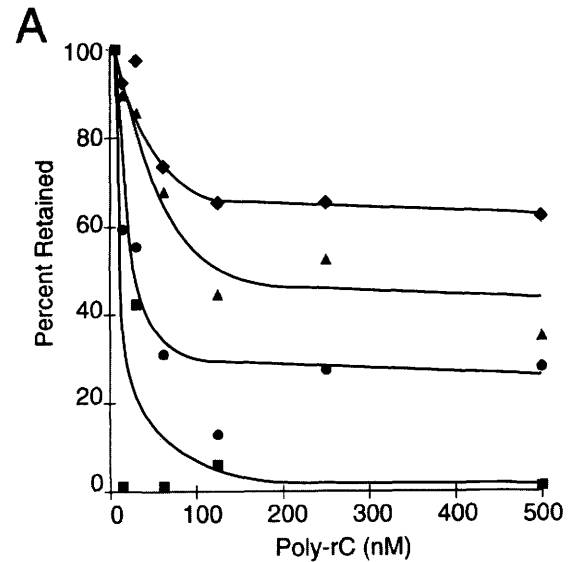

Figure 6. Filter-binding competition with S12 protein and various RNAs. (A) Poly(rC) competition with different RNAs for binding to S12. Binding of S12 (75 nM) to exon, ORF, intron, and tRNA was competed by poly(rC) at the indicated concentration (in nM nucleotide). The RNA substrates were as follows: exon (◆), 245 nucleotides of *td* exon 2; intron (●), 210 nucleotides of *td* intron, shown previously to adopt a typical group I folded structure (Heuer et al. 1991); ORF (▲), 348 nucleotides of the *sunY* intron ORF; tRNA (■), the 72-nucleotide tRNA^{Phe}. The retention is expressed relative to the amount bound in the absence of competitor, which is assigned a value of 100%. The data represent the averages of duplicate samples. The fraction of input cpm retained on the filter in the absence of competitor for each RNA was as follows: exon, 37%; ORF, 27%; intron, 29%; tRNA, 4%. (B) Different competitors of S12 binding to exon and intron RNA. The experiments were performed as in A, with labeled exon and intron substrates and unlabeled competitors, exon (E), ORF (O), intron (I), and tRNA (T), at two concentrations each (5 and 50 ng, corresponding to 0.25 and 2.5 μM in nucleotide, respectively). Retention is expressed relative to that in the absence of competitor (N, 100%), which corresponded to 45% and 22% of input RNA for exon and intron, respectively. The small increase in retention of intron RNA in the presence of low concentrations of tRNA inhibitor (>100%) was reproducible.
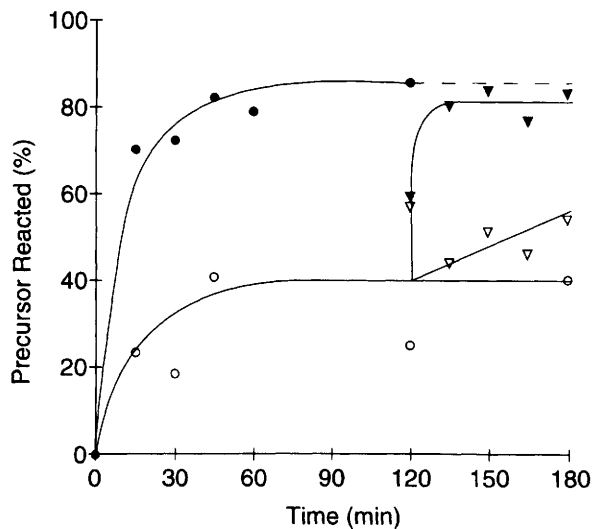
Figure 7. S12 promotes splicing by unreacted molecules. The *trans*-splicing assay was performed at 37°C with uniformly labeled precursors H1 and H2 (20 nM) in the absence (O) or presence (●) of S12 protein (1 μM). At 120 min, S12 (▼) or buffer (▽) was added to a portion of the reaction mixture lacking S12. Aliquots were removed at the indicated times into an equal volume of FDM, and the RNAs were separated on a 5% acrylamide (39:1)/8 M urea gel. The RNAs were quantitated on a Betascope 603 blot analyzer, and the amount of precursor reacted was expressed relative to that at 0 time.

readily detectable. These results suggest that S12 has properties of a molecular chaperone, as its presence is not required after formation of the desired conformation. Although we cannot rule out the possibility that <1 pmole of protein remains trapped within the complex, control experiments indicated that this amount of S12 alone cannot stimulate the splicing reaction (data not shown).

S12 also promotes a hammerhead ribozyme reaction

A detailed kinetic and thermodynamic characterization of a hammerhead ribozyme reaction, for ribozyme HH16 (Fig. 8A), established that association of the oligonucleotide substrate is rate-limiting under subsaturating conditions (e.g., single turnover reactions with low concentrations of ribozyme) and that dissociation of the oligonucleotide products is rate-limiting for multiple turnover under saturating conditions (Hertel et al. 1994). It has been shown previously that two nonspecific RNA-binding proteins, the p7 nucleocapsid protein of HIV-1 and the carboxy-terminal domain of the hnRNP A1 protein, can help to overcome these physical limitations to catalysis by facilitating both substrate association and product dissociation (Tsuchihashi et al. 1993; Herschlag et al. 1994). It was suggested that the steps in the hammerhead ribozyme reaction provide a model for a number of processes that involve RNA and that the nonspecific RNA-binding proteins act as RNA chaperones in

that they prevent the ribozyme from being kinetically trapped through the reaction cycle.

Because of the analogy to the putative role of S12 in group I splicing, we investigated the effect of S12 on catalysis by HH16. The protein did facilitate single turnover reactions (4- to 10-fold stimulation at 200 nM S12; Fig. 8B; data not shown). As expected for a nonspecific interaction, addition of single-stranded DNA (ssDNA) abolished stimulation by S12 (Fig. 8B). The results of Figure 8C suggest that 200 nM S12 has no significant effect on multiple turnover. However, higher concentrations of S12 (350–500 nM) appeared to increase multiple turnover (Fig. 8C), implying that S12 can facilitate the dissociation of products from the ribozyme. It should be noted that under these conditions only about one half of the total oligonucleotide was recovered on polyacrylamide gels, suggesting that some was lost in a complex with S12. Nevertheless, the reaction time courses were well-behaved. These results also indicate that higher concentrations of S12 are required to disrupt the HH16 product duplex than to promote duplex formation, consistent with a preference of S12 for binding to unstructured single-stranded RNAs.

Discussion

E. coli *proteins, including ribosomal proteins, can facilitate splicing*

In exploring a role for accessories that promote splicing of prokaryotic group I introns, we purified an activity from *E. coli* that has the ability to stimulate both *trans*- and *cis*-splicing of the three T4 introns (Figs. 1 and 2). The active extract consists largely of small, basic, heat-stable proteins, many of which are ribosomal proteins, capable of promoting splicing to varying degrees (Figs. 3

Table 1. *Proteinase K treatment of S12-assembled precursors*

Treatment[a]	Assay temperature (°C)	GTP incorporation[b] buffer	S12
1. None	55	100	—
2. None	37	19.8	74
3. Proteinase K	55	49.5	—
4. Proteinase K	37	14.4	52.2
5. PKB	55	49.7	—
6. PKB	37	17.2	52.5
7. S12–PK	37	—	14.5

[a]Precursors for the *trans*-splicing reaction were incubated at 37°C for 30 min with or without S12 to allow assembly and assayed directly for [^{32}P]GTP incorporation at 37°C or 55°C (lines 1 and 2) or after further incubation with proteinase K (lines 3 and 4) or proteinase K buffer (PKB, lines 5 and 6). For the experiment in line 7, S12 protein was incubated with proteinase K prior to the assembly step. The absence of S12 after proteinase K treatment was verified by Western blot as described in Materials and methods.

[b]GTP incorporation was calculated relative to that in the absence of S12 at 55°C (line 1).

Figure 8. S12 enhances hammerhead ribozyme interaction. (*A*) Ribozyme HH16 complexed with its oligoribonucleotide substrate (S). Boxed residues are conserved and presumably contribute to formation of the active site. Cleavage of S by HH16 yields two products, P1, with a 2′, 3′-cyclic phosphate, and P2, with a 5′-hydroxyl. (*B*) Single turnover assay. HH16 (3 nM) and ~0.5 nM ^{32}P-labeled S were mixed in 20 mM Tris (pH 7.5) and 10 mM MgCl$_2$ at 25°C in the absence of S12 (O), in the presence of 200 nM S12 (■), or in the presence of 200 nM S12 and 1 μM of a ssDNA 28-mer (△). Aliquots were quenched in 2 volumes of formamide–20 mM EDTA gel loading buffer, and products were separated by denaturing 20% polyacrylamide/7 M urea gel electrophoresis and quantitated using a PhosphorImager (Molecular Dynamics) (Tsuchihashi et al. 1993; Hertel et al. 1994). The lines are nonlinear least squares fits to the data, giving $k_{obs} = 0.016$ and 0.064 min^{-1} in the absence (O, △) and presence (■) of free S12, respectively. (*C*) Multiple turnover assay. Reactions of 6 nM HH16 and 30 nM ^{32}P-labeled S in 40 mM Tris (pH 7.5) and 10 mM MgCl$_2$ at 25°C in the absence of S12 (●) and in the presence of 200 nM (△), 350 nM (□), or 500 nM (◊) S12. HH16 and S were preannealed, and the reaction was initiated by addition of MgCl$_2$ and was followed as described in *B*.
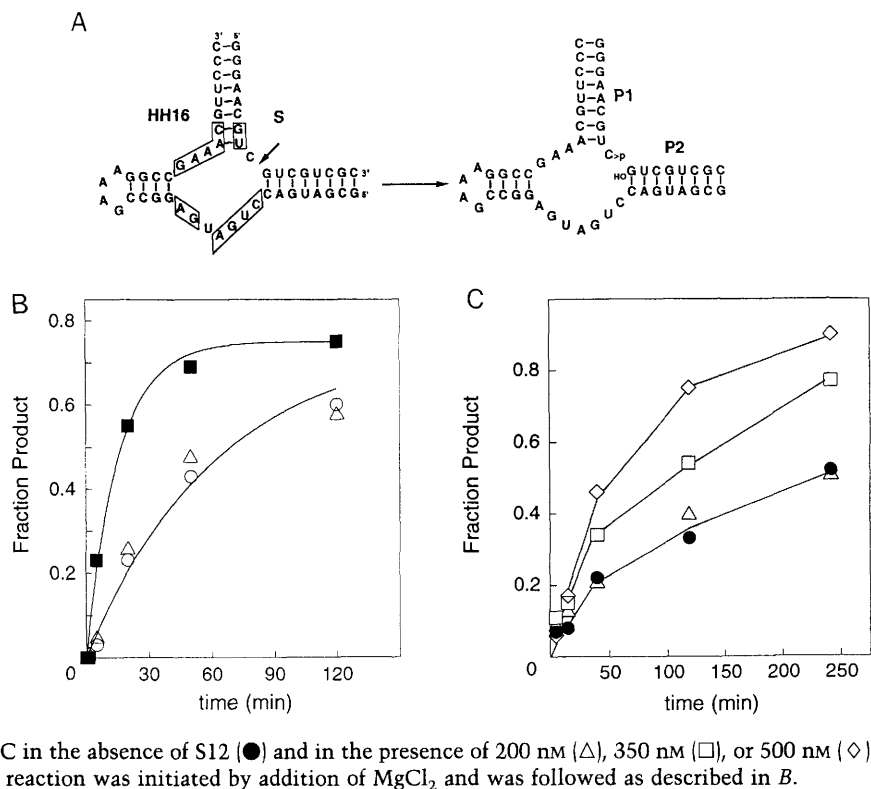
and 4). Although the activity undoubtedly reflects the aggregate contribution of a number of individual proteins, ribosomal protein S12 was most effective in stimulating splicing under our assay conditions (Fig. 4).

Although overexpression of S12 slightly reduced utilization of a cryptic splice site (T. Coetzee and M. Belfort, unpubl.), a clear-cut in vivo role for this protein remains to be established. Even if there is a physiological role in splicing, this may be difficult to prove convincingly, given that other cellular proteins (Fig. 4C) can also facilitate splicing in vitro and possibly in vivo. Phenotypes associated with manipulating levels of these proteins are likely to be subtle if this protein pool is present in saturating amounts in the cell.

Nonspecific RNA-binding proteins can act as splicing factors

Two proteins, CYT-18 and CBP2, have been shown previously to promote group I splicing in vivo and in vitro and to act by binding specifically to the intron (Garriga and Lambowitz 1986; Gampel and Cech 1991; Guo and Lambowitz 1992). Further data suggest that CYT-18 serves to thermodynamically stabilize the correct tertiary fold of the intron's catalytic core (Guo and Lambowitz 1992; Mohr et al. 1992). In contrast, our experiments suggest that the *E. coli* proteins that facilitate group I intron splicing in vitro, including ribosomal protein S12, mediate their effect by binding RNA nonspecifically. Most significantly, filter-binding studies provide no indication of strong binding interactions be-

tween S12 and the group I introns. The results are consistent with a small preference for S12 binding to unstructured RNAs, rather than the group I catalytic core (Fig. 6). Furthermore, S12 can stimulate another RNA reaction, that of a hammerhead ribozyme (Fig. 8), rendering it unlikely that this protein recognizes both of the catalytic RNAs specifically. Additionally, the multiplicity of proteins that can exert an effect (Fig. 4) and the ability of S12 to have its effect on splicing exercised even after its proteolytic removal from the RNA (Table 1) both argue against a specific effect arising from thermodynamic stabilization of the catalytic core.

Nonspecific RNA-binding proteins as RNA chaperones

The nonspecific nature of the S12 effect suggests that it and similar proteins might act as RNA chaperones, facilitating splicing by ensuring that the intron adopts the correct conformation. Analogously, the protein chaperones constitute a number of widely divergent proteins, such as GroE and nucleoplasmins, which function to inhibit misfolding and thereby facilitate correct folding of a wide range of polypeptides, without forming a part of the correctly folded structure (Ellis and Hemmingsen 1989; Ellis 1994). Like proteins, group I introns require a directed self-assembly of the catalytic core into correct secondary and tertiary structures (Cech 1990; Michel and Westhof 1990; Cech et al. 1992; Saldanha et al. 1993). This assembly can be facilitated by elevated temperature (Fig. 1) (Tanner and Cech 1985; Hicke et al.

1989). Likewise, S12 could act as an RNA chaperone, using its nonspecific RNA-binding ability to ensure that the intron adopts the correctly folded structure for splicing. Interestingly, a similar chaperone-like function has been attributed recently to a group of heterogenous nuclear ribonucleoproteins (hnRNPs) in HeLa cells, where they are suspected to affect pre-mRNA interactions (Portman and Dreyfuss 1994).

A rearrangement function for S12 and related proteins, which might promote the active conformation of the ribozyme by preventing formation of inhibitory structures or rearranging them subsequent to formation, is fully consistent with our data. First, whereas only a fraction of the group I precursor molecules splice readily, with the remainder being kinetically trapped in an unfavorable conformation (e.g., Walstrum and Uhlenbeck 1990), splicing goes virtually to completion in the presence of S12 (Figs. 2B and 7). Second, the ability of S12 to facilitate both helix formation and destabilization in the hammerhead ribozyme system argues for a role of the protein in preventing misfolding and resolving misfolded RNAs (Fig. 8; Herschlag et al. 1994). Third, the ability of S12 to exert its effect during an incubation period prior to initiation of the splicing reaction (Table 1) is the hallmark of a protein that assists in folding. Finally, the ability of S12 to reverse the inhibitory effect on splicing of the *sun*Y intron ORF (Fig. 5) suggests that the long stretches of ORF RNA engage in inhibitory interactions with the intron core and that S12 and related proteins promote splicing by preventing or resolving these unproductive interactions.

The inhibitory nature of extraneous RNA sequences is underscored not only by the inverse relationship between ORF length and self-splicing ability (Fig. 5) but also by our observation that removal of exon sequences significantly increases the efficiency of self-splicing in *trans* (Coetzee 1993). Our finding that S12 may exhibit a slight binding preference for exon and intron ORF RNA over intron core RNA suggests that the protein may thereby prevent spurious interactions of these relatively unstructured RNAs with the intron core without occluding the intron, thus driving the formation of a catalytically active complex (Fig. 9).

Some of our data are also consistent with the ability of S12 and related proteins to facilitate association of spatially separated precursor RNAs (Fig. 9), as, for example, through charge buffering by interaction with the RNA or protein–protein interactions (Portman and Dreyfuss 1994) (Fig. 9). First, as noted above, S12 promotes the association of a hammerhead ribozyme with its RNA substrate (Fig. 7B). Second, we have noted a larger increase in protein-enhanced splicing at low precursor concentrations than at higher concentrations, with a 20-fold greater enhancement with 15 nM precursors than with 250 nM precursors at 37°C (T. Coetzee and M. Belfort, unpubl.), suggesting stimulation of an association step. Third, in the case of *cis*-splicing, where the intron ORF separates critical elements of the ribozyme (much like the *trans* intron), the direct relationship between splicing facilitation and ORF length might be explained in
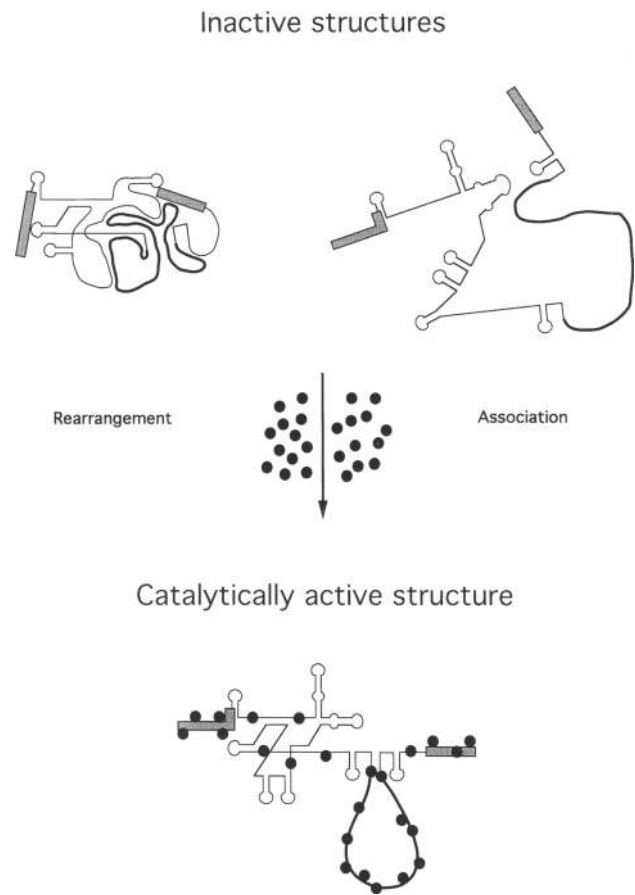
part by the ability of these proteins to bring remote pairing elements into proximity (Fig. 9).

The extent to which splicing enhancement is attributable to the protein preventing or resolving misfolding and/or to an intrinsic function of the protein that promotes association cannot yet be resolved. However, the effect cannot simply be the result of macromolecular crowding, as neither BSA nor polyethylene glycol can mimic the effect of S12. Furthermore, our finding that other basic RNA-binding proteins (*E. coli* IHF, HU, L14; phage T4 gp32) cannot stimulate splicing suggests that electrostatic shielding alone cannot promote splicing and that specific properties of RNA-binding proteins are required to promote the correct RNA conformation (Coetzee 1993; T. Coetzee and M. Belfort, unpubl.).

Figure 9. Model for the action of EXT proteins. The schematic represents an idealized model, wherein two extreme forms of inactive structures adopted in the absence of proteins are presented. (*Top left*) The intron ORF (bold line) and exon sequences (shaded boxes) are shown making spurious interactions with the intron core (thin lines); (*top right*) the large unstructured intron ORF is depicted as separating the catalytic components of the intron. Proteins like S12 (●) then interact preferentially with ORF and exon sequences to promote rearrangement and/or association of pairing elements, thereby facilitating formation of the catalytically active tertiary structure. Proteins are then dispensable for the catalytic steps.

A generalized role for polyspecific RNA-binding proteins

Support for a nonspecific role of proteins in preventing misfolding can be found in studies of ribosome assembly. A number of ribosomal proteins, including S4 and S12, have polyspecific effects such that they interact with regions of the RNA outside their defined binding domains (Stern et al. 1986, 1989; Vartikar and Draper 1989). Consistent with our observations with group I introns, such polyspecific effects suggest a mechanism whereby the ribosomal proteins occlude portions of the RNA molecule to prevent spurious interactions that would disrupt the ribosome assembly process (Stern et al. 1989; Powers et al. 1993). Thus, nonspecific RNA–protein interactions involving specific RNA-binding proteins may serve more generally to ensure correct folding of structurally complex RNAs.

The need for molecules that advance the assembly and proper folding of the group I introns is becoming increasingly apparent. Long stretches of "inhibitory" ORF sequences not only interrupt critical catalytic pairings of the three phage T4 introns but also those of the majority of other group I introns. Of the 87 introns used in developing the three-dimensional model for group I intron structure, 57 contain stretches of noncore sequences longer than 500 nucleotides (Michel and Westhof 1990). Although these sequences are usually localized in peripheral sections of the intron core structure, one needs to consider the RNA folding environment within the cell. It is probable that these noncore sequences would tend to form unproductive pairings with the intron core during transcription. We propose that cellular proteins are recruited to prevent formation and/or accumulation of such "incorrect" structures, thereby promoting association of pairing elements that are critical to catalysis. RNA-binding proteins have been demonstrated to resolve misfolded tRNA and 5S RNA (Karpel et al. 1974, 1982) and to aid assembly processes (e.g., Kumar and Wilson 1990; Pontius and Berg 1990, 1992; Munroe and Dong 1992; Fang and Cech 1993; Sundquist and Heaphy 1993; Tsuchihashi et al. 1993; Herschlag et al. 1994; Portman and Dreyfuss 1994). We suspect that other cellular RNAs will have analogous misfolding and assembly problems, necessitating the general involvement of RNA chaperones.

Materials and methods

Bacterial strains, plasmids, and media

E. coli strain Rue10 [F⁻, *thi*-1, *thyA*, *hsd*S20 (r_B^-, m_B^-), *supE44*, *recA13*, *ara*-14, *leuB6*, *proA2*, *lacY1*, *rpsL20* (str^r), *xyl*-5, *mtl*-1] is a *thyA* derivative (Rubin et al. 1980) of HB101 (Bolivar and Backman 1979) and was used for preparation of EXT used in Figures 1, 2, and 5. *E. coli* strain MRE-600 was used for large-scale preparation of 70S ribosomes and EXT. *E. coli* strain BL21(DE3) is derived from BL21 (F⁻ *hsdS gal* r_S^-, m_S^-) and contains a λ prophage (DE3) with the gene for T7 RNA polymerase under control of the *lac*UV5 promoter (Studier and Moffat 1986). Plasmids were maintained in DH5α (F⁻, Φ80, *lacZ*ΔM15, *recA1*, *endA1*, *gyrA96*, *thi*-1, *hsd*R17, (r_K^-, m_K^+),

supE44, *relA1*, *deoR*, Δ(*lacZYA–argF*) U169 (GIBCO–BRL), C600 (F⁻, *thi*-1, *thr*-1, *leuB6*, *lacY1*, *tonA21*, *supE44*), JM101 [*supE*, *thi*, Δ*lac–proAB*, (F′, *traD36*, *proAB*, *lacIᵍZM15*)] (Jendrisak et al. 1978; Yanisch-Perron et al. 1985), JM109*thy*⁻ [*endA1*, *recA1*, *gyrA96*, *thi*, *hsd*R17, (r_K^-, m_K^+), *relA1*, *supE44*, Δ*lac–proAB* (F′, *traD36*, *proAB*, *lacIᵍZM15*)] (Yanisch-Perron et al. 1985).

Plasmids pTZH1 and pTZH2 were described previously (Galloway-Salvo et al. 1990). Plasmid pTZ*td*CAT was generated by cloning the chloramphenicol transacetylase gene into the *XbaI* site with pTZ*td*ΔP6-1 (Galloway-Salvo et al. 1990). This plasmid was maintained in JM109*thy*⁻. Plasmid pTZ*td*X1 was used for generating wild-type *td* pre-mRNA. This plasmid consists of an *EcoRI* fragment containing the intact exons and intron cloned into pTZ18U (U.S. Biochemical). Expression of the internal ORF was disrupted by insertion of an *XbaI* linker into an internal *DraI* site (Belfort et al. 1987) and the plasmid maintained in DH5α.

Plasmids used to generate *nrdB* mRNA were maintained in JM101 and *sunY* mRNA in C600. All of the constructs are in pBSM13(+) (Stratagene). Plasmid pJSE17, which contains the entire *nrdB* gene and some downstream sequences, was used to generate *nrdB* pre-mRNA (Gott et al. 1986). Plasmids pMPX401 and pMAX1 were used to generate *sunY* mRNA. They differ in that 796 nucleotides upstream from the 5′ splice site have been removed from pMAX1 (Xu and Shub 1989). Plasmids pMAD1, pMAD6, and pMAD17 are derivatives of pMAX1 in which 593, 413, and 290 nucleotides, respectively, have been deleted from the ORF sequence (Xu and Shub 1989).

For purification purposes, the gene encoding ribosomal protein S12 was cloned into the overexpression vector pT7-5 (Tabor and Richardson 1985). Briefly, plasmid pNO1523 (Dean 1981), which contains the *rpsL* gene encoding S12 and its promoter as well as a portion of the *rpsG* gene, was the starting construct. The *SstII–BamHI* fragment of pNO1523, which contains the entire *rpsL* gene, was subcloned into the *SmaI–BamHI* site of plasmid pSU18 (Martinez et al. 1988). Because *SstII* generates 3′ overhangs, these were removed by treatment with the Klenow fragment of DNA polymerase I (GIBCO–BRL) to generate a blunt end (Sambrook et al. 1989). Maintenance of *rpsL* in high copy number was toxic unless the strain carried the *pcnB* mutation, which lowered the copy number of the plasmid. The *EcoRI–BamHI* fragment of pSU18S12 was then subcloned into the *EcoRI–BamHI* site of pT7-5.

Plasmids pTZ*td*ΔES, pTZEXII, pAII17SunΔBB, and p67YF0 were used to generate substrate RNAs for filter-binding experiments. Plasmid pTZ*td*ΔES, a derivative pTZΔP6-2, yields pure *td* intron transcripts that span the 3′ half of the P1 stem through the P9 stem of the intron when digested with *PvuII* (Heuer et al. 1991). Plasmid pTZEXII contains a fragment of *td* exon 2 cloned into the *SmaI* site of pTZ18U. Plasmid pAII17SunΔBB contains pure ORF sequences derived from the *sunY* intron ORF (Quirk et al. 1989), cloned into pAII17, a derivative of plasmid pET11c (Kong et al. 1993). Plasmid p67YF0 contains the yeast tRNA^Phe gene under control of a T7 promoter (Sampson and Uhlenbeck 1988).

Growth media (TBYE) consisted of tryptone broth [1% Bacto-tryptone (Difco) and 0.5% NaCl] supplemented with 0.5% yeast extract (Difco) and 50 μg/ml of thymine for *thy*⁻ strains. When required for selection of plasmids, ampicillin was added at 200 μg/ml.

In vitro transcription

DNA was prepared by alkaline lysis (Sambrook et al. 1989). Plasmids pTZ*td*X1 and pTZ*td*CAT were linearized with

*Eco*RV, whereas pTZH1 and pTZH2 were linearized with *Sal*I and *Hind*III, respectively. Plasmid pJSE17 was linearized with *Hpa*I, and pMPX401, pMAX1, pMAD1, pMAD6, and pMAD17 were linearized with *Xba*I. Templates were prepared for transcription as described previously by Galloway-Salvo et al. (1990).

H1 and H2 RNAs are *trans*-splicing precursors that represent the *td* intron split in the loop emanating from P6a (Fig. 5B; Galloway-Salvo et al. 1990). These were prepared from pTZH1 and pTZH2 following the directions of the manufacturer (U.S. Biochemical). For all other RNAs used for splicing experiments, transcription was carried out under low magnesium conditions described by Galloway-Salvo et al. (1990) except that transcription was performed at 30°C for 60 min. T3 polymerase was used for all *nrd*B and *sun*Y RNAs, and T7 RNA polymerase for all *td* RNAs. RNA was internally labeled by adding 10 μCi [α-^{35}S]CTP (800 Ci/mmole; Amersham) or 10 μCi [α-^{32}P]CTP (800 Ci/mmole; New England Nuclear) to the transcription reaction mixture.

Plasmid pTZ*td*EXII was linearized with *Eco*RV, pAII17Sun∆BB with *Ssp*I, pTZ*td*∆ES with *Pvu*II, and p67YF0 with *Bst*NI to yield transcripts of 245 nucleotides (exon), 348 nucleotides (ORF), 210 nucleotides (intron), and 72 nucleotides (tRNA), respectively. High specific activity substrates were prepared for filter-binding experiments by in vitro transcription using the same protocol for the *trans*-splicing RNAs, except that nonradioactive CTP concentrations were reduced to 40 μM and 100 μCi of [α-^{32}P]CTP (800 Ci/mmole; New England Nuclear) was added. Following transcription for 2 hr at 37°C, RNAs were ethanol-precipitated, pelleted, and separated on a 6% polyacrylamide/8 M urea gel. RNAs were purified from the gel using the protocol of Milligan and Uhlenbeck (1989). Prior to use, the RNAs were renatured using the following protocol, which was adapted from Heuer et al. (1991) and Michel et al. (1992). RNAs were heated to 95°C for 3 min. Then, 1 part 10× reaction buffer and 9 parts RNA were mixed and cooled to room temperature for 5 min.

Extract preparation

Rue10 cells were grown with vigorous shaking at 37°C to an OD_{650} of 0.2 and harvested. Cells were resuspended in buffer 1 [20 mM Tris-HCl (pH 7.5), 25 mM NaCl, 1 mM DTT, and 1 mM EDTA (pH 8.0)] in a ratio of 1 ml of buffer per gram wet weight of cells and lysed by two passages through a French pressure cell. The crude lysate was clarified by centrifugation at 30,000g in a Sorvall RC5B centrifuge for 30 min at 4°C. The supernatant was then aliquoted and stored at −80°C prior to chromatography. MRE-600 cells were lysed by passage through a Sandsted cell disruptor (Energy Service Systems).

Five milliliters of crude extract was applied to a 6 × 2.5-cm column (30-ml bed volume) consisting of TSK CM-650M (Supelco) at 4°C. The column was washed with 4–5 volumes of buffer 1. Subsequently, bound protein was eluted with buffer 2 [20 mM Tris-Cl (pH 7.5), 1 M NaCl, 1 mM DTT, 1 mM EDTA (pH 8.0)], and 5-ml fractions were collected. Fractions were dialyzed against buffer 1 at 4°C and then assayed for protein using the method of Bradford (Bollag and Edelstein 1991). Fractions containing protein were aliquoted and stored at −80°C.

Splicing assays

Splicing was assayed with internally labeled RNAs or by GTP incorporation using unlabeled RNA substrates. With labeled RNAs, the assay consisted of 36 mM Tris-HCl (pH 7.5), 90 μM GTP, 4.6 mM DTT, 4 mM $MgCl_2$, 16 units of RNasin (Promega)

and 20 nM RNA substrate in a volume of 10 μl. Following the addition of substrates, a master mix was aliquoted into microcentrifuge tubes containing EXT or buffer A. The reaction was mixed, centrifuged, incubated at 37°C for 20 min, and stopped by addition of 35 μl of 2.5 mM EDTA and 50 μg of yeast tRNA. Subsequently, the reaction mixture was extracted once with PCIA and the RNA precipitated as described above. The final pellet was resuspended in 5 μl of water and 5 μl of formamide dye mix (FDM). The entire reaction mixture was then heated to 95°C for 5 min and separated on a 5% polyacrylamide (39:1 acrylamide/bisacrylamide)/8 M urea gel under 15 W constant power.

For the experiment shown in Figure 2B, the reactions were carried out with unlabeled RNA from pTZ*td*CAT using identical conditions to those described above. After phenol extraction, 150 μl of 6.15 M formaldehyde–10× SSC (Sambrook et al. 1989) was added and the sample incubated at 65°C for 15 min. The sample was applied to Hybond-N (Amersham) membranes using a Minifold II slot–blot manifold (Schleicher & Schuell). Each slot was first washed with 200 μl of 10× SSC, followed by application of the sample. Subsequently, each well was washed with 400 μl of 10× SSC. The membrane was then subjected to UV irradiation for 5 min. Membranes were then probed with end-labeled oligonucleotides (Galloway-Salvo et al. 1990), filters autoradiographed, and splicing quantitated by scanning densitometry using a Hoeffer GS300 densitometer.

For reactions using unlabeled substrates and labeled GTP, the assay conditions consisted of 40 mM Tris-Cl (pH 7.5), 3 mM $MgCl_2$, 0.4 mM spermidine, 4 mM DTT, 20 units of RNasin, 10 μCi [^{32}P]GTP (3000 Ci/mmole; Amersham or New England Nuclear), and 20 nM RNA substrate in 10 μl. The reaction was carried out at 37°C for 20 min for *td* (*trans* and *cis*) and room temperature or 30°C for *nrd*B and *sun*Y RNAs. For time courses with pMAX1 and its derivatives, the reaction was performed at 30°C and aliquots withdrawn at 2.5, 5, and 10 min. Reactions were stopped, and samples were worked up as described above. Splicing activity was quantified on a Betascope 603 blot analyzer (Betagen). Experiments with uniformly labeled precursors indicated that neither reverse splicing nor inhibition of cyclization appear to influence GTP incorporation in the presence of EXT or S12.

Two-dimensional gel electrophoresis

Two-dimensional gel electrophoresis was performed as described by Datta et al. (1988) with the following modifications. Micropipettes [0.1 cm inner diameter (i.d.) × 12.7 cm] were used to separate proteins in the first dimension. Approximately 4 μg of EXT and 15 μg of ribosomal proteins were separated. Electrophoresis in the second dimension was performed using a 10% SDS–polyacrylamide resolving gel (30:0.8 acrylamide/bisacrylamide). The gel dimensions were 0.75 × 15 × 17 cm. The gel was then stained for 1 hr with Coomassie blue, destained with a 10% methanol/10% acetic acid solution, and dried. The gels were analyzed by visual inspection and by scanning densitometry using a Howtek scanner and Masterscan software (CSPI).

Purification of EXT proteins

EXT proteins were purified by reverse-phase HPLC using methods described by Cooperman et al. (1988) and Ferris et al. (1984). HPLC was performed on a system consisting of two Isco 2350 pumps and a V^4 UV detector, which was controlled by a PC computer running Chemsearch Software (Isco). The column consisted of a SynChropak RP-P C_{18}-silica column (6.5 μm silica, 300 Å pore, 250 × 4.1 mm i.d.; Synchrom). HPLC was

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performed at room temperature, and peaks detected by setting the detector at 220 nm. Solvents were (A) 0.1% (wt/vol) F_3CCOOH in water; (B) 0.1% (wt/vol) F_3CCOOH in acetonitrile. Solvent A was prepared from water prepared with a Milli-Q system (Millipore) and filtered further through a 0.45-μm nylon filter. Acetonitrile was also filtered through a 0.45-μm filter. F_3CCOOH was added after filtration to both solutions, which were degassed prior to use.

Approximately 112 μg of lyophilized EXT was dissolved in 100 μl of 60% acetic acid and loaded onto the column, which was equilibrated at 15% solvent B with a flow rate of 0.25 ml/min. A separation program spanning 435 min was used with a linear gradient of 25–40% B to resolve the proteins. The program was separated into nine segments. Segment 1 consisted of an isocratic elution at 15% solvent B for 5 min. Segment 2 raised the percent of solvent B to 25% over 30 min. Segments 3 and 4 consisted of the linear gradient of 25–40% solvent B and a 30 min isocratic elution at 40% solvent B, respectively. The majority of proteins eluted in these two segments. Additionally, the flow rate for segments 1–4 was 0.25 ml/min. The remainder of the program was used to clean the column with two linear gradients (40–100% solvent B, and 100–15% solvent B) separated by an isocratic elution at 100% B. Fractions were collected at 5-min intervals using an Isco Retriever II fraction collector. Fractions eluting with protein peaks were identified and dried under vacuum, and pellets were suspended in 50 μl of EXT buffer 1.

Protein concentrations were determined by separating aliquots of protein as well as known quantities of lysozyme on a 15% SDS–polyacrylamide gel (SDS-PAGE). Following staining with Coomassie blue, the protein bands were analyzed by scanning densitometry with a Hoeffer GS300 densitometer and analyzed with GS365 software. The absorbances from the lysozyme standards were used to establish a standard curve from which concentrations of the purified proteins were determined.

Protein sequencing and identification

Proteins were amino-terminally sequenced by Edman degradation at the Albany Medical College protein core facility using a Porton systems sequencer. Amino-terminal sequences were then used for searches of the Swiss Protein database using the FASTA program of the Genetics Computer Group (Devereux et al. 1984).

Large-scale S12 purification

Plasmid pT7-5S12 was transformed into strain BL21(DE3) (Studier and Moffat 1986) for overexpression purposes. One and one-half liters of TBYE inoculated with 30 ml of a fresh overnight culture were grown up to an OD_{650} of 0.6 at 37°C and induced with 1 M IPTG for 4 hr. Cells were pelleted, resuspended in EXT buffer 1 in a ratio of 10 ml of buffer per gram wet weight of cells, and lysed by sonication. Crude lysate was clarified by centrifugation at 30,000g for 30 min at 4°C. Ammonium sulfate (GIBCO–BRL) was added to 40%, and the precipitate was collected and resuspended in EXT buffer 1 plus 6 M urea. The precipitate was fractionated as described above for EXT except that all buffers contained 6 M urea. Following fractionation over carboxymethylcellulose (CM), the extract was dialyzed against 6% acetic acid, lyophilized, and S12-purified by reverse-phase HPLC as described above except that an 8.2 × 250 mm semipreparative column (Synchrom) was used.

Nitrocellulose filter-binding assays

RNA labeled with ^{32}P (~20 pM; 6 nM in nucleotide) was mixed with 75 nM S12 in 50 μl of TMN buffer [40 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$, 150 mM NaCl, 0.4 mM spermidine] with the indicated amount of competitor RNA at 23°C in a total volume of 50 μl. Following incubation at 37°C for 5 min, 23°C for 5 min, and on ice for 10–20 min, samples were filtered under gentle vacuum through a nitrocellulose filter (BA-85, Schleicher & Schuell) that had been prewet with TMN plus 40 μg/ml of salmon sperm DNA (Sigma). After filtration, filters were washed twice with 200μl of the prewetting buffer and dried at 80°C under vacuum. Radioactivity retained on the filters was measured by liquid scintillation counting in Aquasol scintillant (New England Nuclear). For each data point, duplicate samples were assayed, and each point was corrected for the amount of binding in the absence of S12 at each competitor concentration.

Proteinase K treatment of S12-assembled precursors

Unlabeled *trans*-splicing precursors H1 and H2 (0.2 pmole of each) were preassembled in the presence or absence of 10 pmoles of S12 in a 10 μl volume for 30 min at 37°C. Incubation was either continued for an additional 15 min or 1 μg of proteinase K (Boehringer Mannheim) [1 μg/μl in proteinase K buffer (PKB = 10 mM Tris at pH 7.5)], or PKB was added and the reaction mixtures held at 37°C for 15 min. Finally, 10 μCi of GTP (3000 Ci/mmole; New England Nuclear) was added. Following incubation for 20 min at 37°C, reactions were halted by addition of an equal volume of FDM. Reaction mixtures were separated on 5% acrylamide (39:1)/8 M urea gels, and the GTP-labeled intron band was quantitated on a Betascope 603 blot analyzer. All experiments were performed at least twice.

To verify that no S12 remained after proteinase K treatment, the above reactions were set up in duplicate. Prior to addition of GTP, 6× SDS–loading dye (Bollag and Edelstein 1991) was added to one set of reactions, which were separated on a 15% SDS–polyacrylamide gel and transferred to nitrocellulose. Membranes were probed with rabbit polyclonal anti-S12 antibody. Antibody binding was visualized using horseradish peroxidase (ECL kit, Amersham).

Hammerhead ribozyme and substrate preparations

The hammerhead ribozyme HH16 was synthesized by in vitro transcription with T7 RNA polymerase using a synthetic DNA template (Milligan and Uhlenbeck 1989). The oligonucleotide S, made by solid-phase chemical synthesis and 5'-end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, was purified by denaturing polyacrylamide gel electrophoresis, as described previously (Hertel et al. 1994). The nonspecific ssDNA was a 28-mer: ATG CAC TGC TAG AGA TTT TCC ACA AGT. Reactions were carried out as indicated in the legend to Figure 8.

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