gradient cannot explain the observed transition width). Interestingly, we see that it continues to increase up to the highest pressure, meaning that the optimal structural arrangement has not been reached, and that the sample does not degrade under pressure. One can distinguish two regions, one below 15 GPa in which a large increase rate of ~1 K/GPa is observed and one above with a smaller rate of ~0.5 K/GPa.

Furthermore, if the increase of the resistance with decreasing temperature (see Fig. 2), is attributed to Hg-1223, it would imply that the sample is underdoped. One explanation would be that samples obtained by synthesis carried out under high pressure are still underdoped, and that further oxygen doping may increase T_c. A second explanation would be that interatomic distances might play an important role. The strategy in this case would be to introduce a chemical pressure into the compound.

After completion of this work we heard that similar data had been obtained by Chu *et al.* (*10*). The two sets of data are in good agreement except that Chu *et al.* observe a saturation at 153 K ($T_{c\ onset}$) around the maximum pressure attained, 15 GPa. In our case, no saturation of T_c is observed up to 23.5 GPa. Since we measure the pressure, in situ, from the lead superconducting transition, we are sure that the increase of T_c at such high temperatures is real.

REFERENCES

1. S. N. Putilin *et al.*, *Nature* **362**, 226 (1993).
2. A. Schilling *et al.*, *ibid.* **363**, 56 (1993).
3. E. V. Antipov *et al.*, *Physica C* **214** (1993).
4. N. Mori *et al.*, *ibid.* **185–189**, 40 (1991).
5. J. G. Lin *et al.*, *ibid.* **175**, 627 (1991).
6. D. D. Berkley *et al.*, *Phys. Rev. B* **47**, 5524 (1993).
7. O. Chmaissem *et al.*, in preparation.
8. M. Núñez-Regueiro, P. Monceau, A. Rassat, P. Bernier, A. Zahab, *Nature* **354**, 289 (1991).
9. L. Gao *et al.*, *Physica C* **213**, 261 (1993).
10. C. W. Chu *et al.*, *Nature*, in press.

7 September 1993; accepted 9 September 1993

Protein Enhancement of Hammerhead Ribozyme Catalysis

Zenta Tsuchihashi,* Mala Khosla, Daniel Herschlag†

When the recognition sequence of a ribozyme is extended beyond a certain length, turnover is slowed and specificity is decreased. Here, it is shown that a protein can help a ribozyme overcome these general limitations on ribozyme activity. Cleavage of an RNA oligonucleotide by a hammerhead ribozyme is enhanced 10- to 20-fold upon addition of a protein derived from the p7 nucleocapsid (NC) protein of human immunodeficiency virus–type 1. The NC protein also enhances the ability of the ribozyme to discriminate between cleavage of RNA oligonucleotides with differing sequences. These catalytic improvements can be attributed to the strand exchange activity of this RNA binding protein. It is conceivable that endogenous or added proteins may provide analogous increases in ribozyme activity and specificity in vivo.

The discovery of RNA-mediated cleavage of RNA has led to interest in the molecular and cellular function of RNA enzymes or ribozymes. The use of ribozymes to target the destruction of specific RNAs in vivo has been proposed. This approach could greatly simplify drug design because ribozymes can recognize specific RNA sequences by base pairing (*1*). However, intrinsic kinetic and thermodynamic features of ribozyme activity limit turnover and specificity. Problems arise because a recognition sequence of ~15 bases is necessary to form a fully base paired duplex uniquely with a single cellular RNA (*2*). Long recognition sequences result in strong binding and slow product release for several ribozymes, thus limiting the maximal rate of turnover (*3*, *4*). Experimental work and theoretical analysis have demonstrated that strong binding also limits specificity (*5–7*). Binding becomes so strong that the ribozyme cleaves both a matched RNA substrate and some mismatched RNA substrates each time they bind to the ribozyme, which results in similar rates of cleavage for both matched and mismatched RNA substrates; these similar rates mean that specificity is low (6).

The hammerhead ribozyme HH16 (Fig. 1) provides an example of a ribozyme with slow turnover and low specificity because of strong binding (*4*, *7*). We reasoned that a protein capable of increasing the rates of helix association and dissociation could potentially enhance HH16 activity and specificity (6). Specifically, because binding of the RNA substrate (S) (Fig. 1) is rate-limiting for HH16 under subsaturating conditions and product dissociation is rate-limiting with saturating concentrations of S (*4*), increases in the rate of helix association and helix dissociation could increase the reaction rate under subsaturating and saturating conditions, respectively. We used the p7 nucleocapsid (NC) protein from human immunodeficiency virus (HIV) in our initial search for protein enhancement of ribozyme activity (*8*). Retroviral NC proteins are required for viral packaging and its specificity and presumably for reverse transcription, essential steps in the viral life cycle (*9*). In vitro, they can bind RNA without high specificity (*10–12*), mediate dimerization of the viral RNA (*13*, *14*), catalyze annealing of a transfer RNA (tRNA) primer to the viral RNA genome (*13*, *14*), and catalyze strand annealing and strand exchange with DNA oligonucleotides (*15*).

In multiple turnover reactions catalyzed by the HH16 ribozyme, there is an initial burst of product formation followed by slower product formation. The magnitude of the burst is stoichiometric with the amount of ribozyme. For example, without NC protein ~10% of the 30 nM RNA substrate is rapidly converted to product by 3 nM HH16 (Fig. 2A). A detailed investigation of the rate constants of individual steps in the HH16 reaction showed that this burst corresponds to the chemical step and that the slow phase is limited by the dissociation of products, which is slower than the chemical cleavage step (*4*). However, addition of NC protein overcomes the slow phase, with the result that the entire reaction can proceed at a rate similar to that for the cleavage step (Fig. 2A) (*16*). The rate constant for the slow multiple turnover phase, k_{cat}, increases from 0.01 min^{-1} to 0.2 min^{-1} upon addition of the NC, which represents a rate enhancement of ~20-fold (Fig. 2A).

Control experiments (Fig. 2B) strongly suggest that the rate enhancement arises as a result of NC protein. Only a small amount of cleavage of 5′ end-labeled substrate (S*) occurred with added ribozyme in the absence of the NC (Fig. 2B, lane 3). However, the reaction was nearly complete with added NC, and only the normal reaction product was observed (Fig. 2B, lane 4). Furthermore, incubation of S* with the NC in the absence of ribozyme gave no detectable hammerhead reaction product or degradation product (lane 2), and protease treatment abolished the enhancement of activity, as expected for a protein effector (lane 6). The enhancement was also abolished upon addition of a single-stranded DNA 28-mer (ssDNA; lanes 7 and 8); NC protein binds tightly to ssDNA (*12*, *15*). Other single-strand nucleic acid binding

Z. Tsuchihashi, Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305.
M. Khosla and D. Herschlag, Department of Biochemistry, Stanford University, Stanford, CA 94305.
*Present address: Somatix Therapy Corporation, 850 Marina Village Parkway, Alameda, CA 94501.
†To whom correspondence should be addressed.

proteins, T4 phage gene 32 protein and *Escherichia coli* single-strand binding protein (SSB), could not substitute for the NC in multiple turnover or single turnover reactions, even at concentrations up to ~5 to 10 μM (monomer concentration). In contrast, an independent preparation of NC protein was capable of enhancing both multiple turnover and single turnover reactions, in spite of sequence differences between the two NC proteins (*17, 18*).

Essentially every molecule of S that binds HH16 is cleaved before it has a chance to dissociate ($k_2 >> k_{-1}$) (Fig. 1B) (*4*). Thus k_{cat}/K_m, the observed second-order rate constant for the reaction with subsaturating ribozyme and substrate (HH16 + S → products) with K_m as the Michaelis constant, is limited by the rate of binding. This reaction is easiest to follow under single turnover conditions, with ribozyme in excess of substrate. Figure 3A shows the reaction time course for RNA cleavage under single turnover conditions in the presence and absence of the NC. There was a large increase in the rate of product formation by the NC, which was lost upon addition of ssDNA or prior treatment of the NC with protease. The observed first-order rate constants for cleavage of S were determined to be ~10-fold larger in the presence of the NC (Fig. 3A). Because binding is rate-limiting in the absence of the NC, the protein must speed association of substrate and ribozyme at least 10-fold.

In contrast to the NC enhancement of the single turnover reaction (HH16 + S → products), there is no effect of the NC on single turnover reactions when the ribozyme and substrate have been pre-annealed (Fig. 3B). Under these conditions, HH16·S is the starting species, and the chemical cleavage step limits the rate of reaction (k_2) (Fig. 1B) (*4*). The loss of stimulation upon pre-annealing is consistent with an effect of the NC on the annealing step, as concluded above.

The NC has been shown to bind ssDNA more strongly than double-stranded DNA and is capable of denaturing tRNA (*11, 12*). Thus, even though the NC was known to have the ability to speed oligonucleotide association and dissociation, it was not known if a concentration window would exist with concentrations high enough to allow stimulation of substrate binding and product dissociation without also shutting down the cleavage step because of disruption of the catalytic core of the ribozyme. As expected, high concentrations of the NC did indeed inhibit the cleavage step (*19*). However, a window for stimulation does exist (Figs. 2 and 3). Furthermore, the disappearance of the burst phase in the multiple turnover reactions suggests that the NC completely overcame the rate-limiting product dissociation without significantly affecting the cleavage step (Fig. 2A). Thus, the ~20-fold stimulation of multiple turnover may not represent the maximum possible rate stimulation; greater stimulation may be observed for ribozymes with longer recognition sequences and stronger binding.

The specificity of HH16 is poor under our reaction conditions. Neither removal of six residues from the 3′ end of the 17-mer substrate S to give S_{11mer} nor the single mismatch of substrate S'_{17mer} has an effect on k_{cat}/K_m (*7, 20*); it is the relative values of k_{cat}/K_m for competing substrates that determines specificity (*21*). Indeed, even a substrate with this mismatch and four residues missing from the 3′ end (S'_{13mer}) is reduced only threefold under subsaturating conditions relative to the full-length matched substrate, S_{17mer}, even though these changes are predicted to decrease the overall binding free energy by ~15 kcal/mol (*7*).

Figure 4 compares the cleavage of S_{13mer} and S'_{13mer}, the matched and mismatched substrates, respectively, that lack four residues at the 3′ end. There is only a small difference in their reactivity without the NC, but there is a large difference when the NC is added. These experiments were carried out under subsaturating conditions, so that the relative values of the observed rate constant (Fig. 4) give a direct measure of specificity. Thus, the NC enhances by

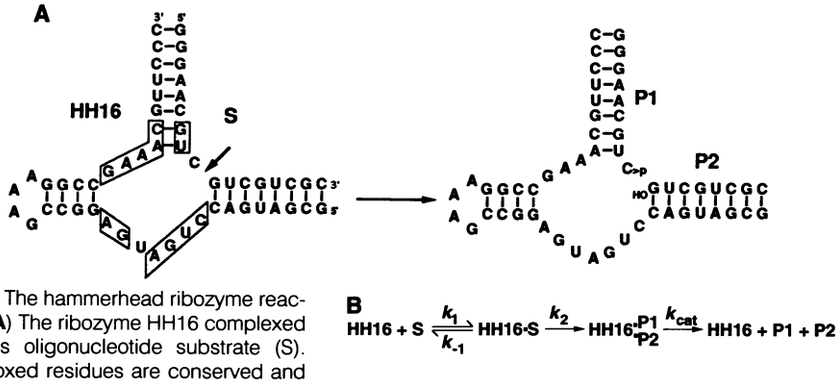

Fig. 1. The hammerhead ribozyme reaction. (**A**) The ribozyme HH16 complexed with its oligonucleotide substrate (S). The boxed residues are conserved and are thus presumed to be responsible for the architecture of the active site (*28*). There are eight base pairs on either side of the cleavage site, which gives a recognition sequence of 16 for this ribozyme. The cleavage reaction gives a 5′ product, P1, with a 2′,3′-cyclic phosphate and a 3′ product, P2, with a 5′ hydroxyl group. For specificity studies, a second substrate, S′, was also investigated (*20*). HH16 was prepared by in vitro transcription with T7 RNA polymerase, and S and S′ were prepared by automated RNA synthesis (*4*). (**B**) An abbreviated reaction scheme. Rate and equilibrium constants for each reaction step, including the individual product association and dissociation steps that are not shown, have been determined (*4*). These determinations provided a framework for understanding the effects of the NC in terms of individual reaction steps. Multiple turnover is limited by the release of the products and is represented by the single rate constant k_{cat}. The helices between HH16 and the reaction products behave largely like simple duplexes (*4*). All experiments herein were carried out under conditions identical to those used in the previous determinations [50 mM tris (pH 7.5) and 10 mM $MgCl_2$ at 25°C].

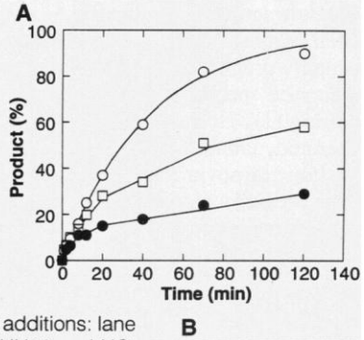

Fig. 2. The NC stimulates multiple turnover reactions of HH16. (**A**) Cleavage of 30 nM 5′ end-labeled substrate (S*) catalyzed by 3 nM HH16 in the presence of 0 (●), 200 (□), or 400 nM (○) NC [10 mM $MgCl_2$ and 50 mM tris (pH 7.5)] (*29, 30*). The slope of the line through the closed circles gives a value of k_{cat} = 0.01 min^{-1} in the absence of the NC. The line through the open circles is a nonlinear least squares fit giving a first-order rate constant of 0.02 min^{-1}, which corresponds to a value of k_{cat} = 0.2 min^{-1} in the presence of the NC. (**B**) Autoradiogram of a denaturing gel (20% polyacrylamide and 7 M urea) used to separate products following reactions [10 mM $MgCl_2$ and 50 mM tris (pH 7.5)] (*29*). Incubation was for 90 min with 100 nM 5′ end-labeled substrate (S*) and the following additions: lane 1, none; lane 2, 400 nM NC; lane 3, 10 nM HH16; lane 4, HH16 and NC; lane 5, HH16 and diluted storage buffer for NC; lane 6, HH16 and protease-digested NC; lane 7, HH16, NC, and 400 nM ssDNA; and lane 8, HH16, NC, and 2000 nM ssDNA. The slow migrating band corresponds to intact S* and the fast migrating band to the labeled 5′ product, P1* (Fig. 1A) (ssDNA is a 28-mer of randomly chosen sequence: ATGCACTGCTAGAGATTTTCCACAAGTC).

~20-fold the ability of HH16 to discriminate between the 13-mer substrates. Furthermore, the NC enhances the discrimination between the matched full-length substrate (S_{17mer}) and the mismatched 13-mer substrate by ~100-fold (Figs. 3A and 4) (*19*). This effect arises from a combination of ~10-fold stimulation of the S_{17mer} reaction (as in Fig. 3A) and ~10-fold inhibition of the S'_{13mer} reaction.

The ability of the NC to speed binding to and dissociation from HH16, as suggested by the enhancement of single turnover and multiple turnover reactions (Figs. 2 and 3A), would be expected to enhance specificity. This is because the NC would promote equilibrium binding between the ribozyme and its potential substrates, thereby allowing the ribozyme to take advantage of the difference in binding affinity between matched and mismatched or longer and shorter substrates. Thus, the ribozyme is provided with a greater ability to choose which substrate to cleave (6). This increase in the rate of equilibration cannot, however, fully account for the increased specificity; it does not describe why the reaction of S'_{13mer} is inhibited by the NC (Fig. 4). The inhibition may arise from an additional effect of the NC to thermodynamically destabilize the ribozyme-substrate complex relative to the free species. Such destabilization is consistent with observations that the NC binds more strongly to single-stranded than to double-stranded nucleic acids and that the NC can denature tRNA (*11, 12*).

Several group I and group II introns and ribonuclease (RNase)P use proteins in reactions that can be catalyzed by the RNA in the absence of these proteins (*22*). The CYT-18 protein may aid group I activity by specifically binding to and stabilizing the catalytically active conformation of the ribozyme (*23*). The protein component of RNase P, which also specifically binds its cognate RNA, has been proposed to enhance product release (*24*). Here, we have shown that a protein presumably not designed for specific interactions with a ribozyme can also have profound effects on ribozyme activity. The NC protein enhances the activity and specificity of a hammerhead ribozyme, and the catalytic improvements appear to originate from the protein's strand exchange, strand annealing, and RNA binding activities.

Our experiments thus provide information about the types of effects proteins can exert on RNA-catalyzed reactions beyond structural stabilization. The catalytic enhancement by another nonspecific nucleic acid binding protein, *E. coli* SSB, has recently been demonstrated in the cleavage of DNA by the *Tetrahymena* group I ribozyme, though the reason for this effect is not yet understood (*25*).

The ability of a nonspecific RNA binding protein to enhance ribozyme activity also suggests a possible mechanism for the introduction of peptides and proteins in an RNA world early in evolution (*26*) because there may be a larger number of peptides that can act nonspecifically than can act specifically and because a nonspecific peptide might be able to interact with multiple RNAs.

Preliminary results suggest that the heterogeneous ribonucleoprotein (hnRNP) A1 can also enhance the activity of HH16 (*27*). It is possible that several proteins will provide analogous catalytic enhancement. The role of such proteins in vivo may be to facilitate the attainment of the active RNA conformation. For RNAs that function with distinct secondary and tertiary structures, these proteins may act as intramolecular "RNA chaperones," helping to prevent and resolve misfolded species. For RNAs such as some mRNAs that function without a distinct structure, the RNA chaperones may prevent misfolding of the RNA into inaccessible secondary and tertiary structures and resolve such structures if they do form (*19*).

Our results suggest that the efficacy of ribozymes for the rapid and specific cleavage of RNA in vivo might be enhanced by endogenous proteins or by addition of an NC or a related protein, peptide, or small molecule. An NC or a related protein could be introduced along with a ribozyme by means of a gene therapy approach.

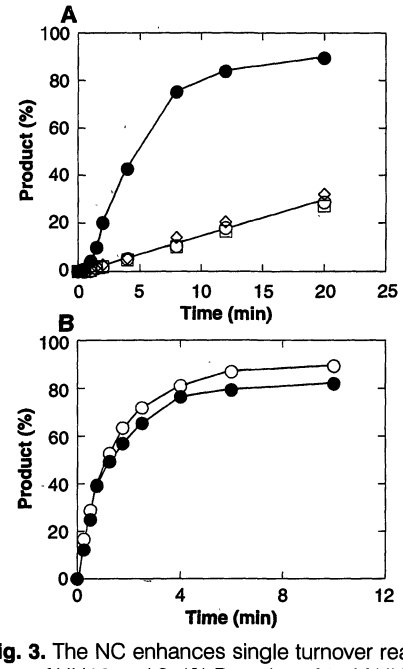

Fig. 3. The NC enhances single turnover reactions of HH16 and S. (**A**) Reaction of 2 nM HH16 and 0.2 nM 5′ end-labeled S (S*) in the absence (○) or presence (●) of 400 nM NC, with 400 nM NC and 2000 nM ssDNA (□) (Fig. 2) or with 400 nM NC digested with protease K (◇) (*31*). The small lag is consistent with that expected for a two-step reaction before the steady state, because the product-producing cleavage step (k_2) (Fig. 1B) cannot occur until after formation of the HH16·S* complex (k_1). (**B**) Reaction of the HH16·S* complex in the absence (○) or presence (●) of 400 nM NC. The S* (1 nM) was pre-annealed with HH16 (40 nM) before reaction (*29, 30*). Changing the concentration of HH16 or S* had no effect, which demonstrates that the annealing was complete.

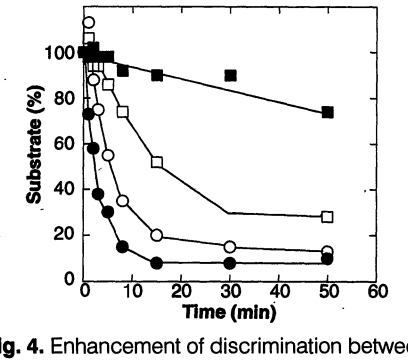

Fig. 4. Enhancement of discrimination between matched and mismatched RNAs by the NC. Reaction of 8 nM HH16 and a trace amount of 5′ end-labeled S_{13mer} (circles) or S'_{13mer} (squares) in the absence of the NC (open symbols) and in the presence of 400 nM NC (closed symbols). S_{13mer} and S'_{13mer} form matched and mismatched duplexes, respectively, with HH16 (Fig. 1). HH16 and the RNA substrates were not pre-annealed (*30–32*). HH16 was in large excess over the total concentration of 5′ end-labeled RNA substrate (<1 nM), which is important in this experiment (*32*).

REFERENCES AND NOTES

1. T. R. Cech, *J. Am. Med. Assoc.* **260**, 3030 (1988); J. Haseloff and W. L. Gerlach, *Nature* **334**, 585 (1988); J. J. Rossi and N. Sarvar, *Trends Biotechnol.* **8**, 179 (1990).
2. P. Miller and P. T'so, *Anti-Cancer Drug Des.* **2**, 117 (1987); C. Helene and J. J. Toulme, *Biochim. Biophys. Acta* **1049**, 99 (1990).
3. D. Herschlag and T. R. Cech, *Biochemistry* **29**, 10159 (1990); A. T. Perrotta and M. D. Been, *ibid.* **31**, 16 (1992); M. J. Fedor and O. C. Uhlenbeck, *ibid.*, p. 12042. Product release has also been shown to be rate-limiting for RNase P, which does not use base pairing for recognition [D. Smith, A. B. Burgin, E. S. Haas, N. R. Pace, *J. Biol. Chem.* **267**, 2429 (1992)].
4. K. Hertel, D. Herschlag, O. C. Uhlenbeck, in preparation.
5. D. Herschlag and T. R. Cech, *Biochemistry* **29**, 10172 (1990); S. Joseph, A. Berzal-Herranz, B. M. Chowrira, S. E. Butcher, J. M. Burke, *Genes Dev.* **7**, 130 (1993).
6. D. Herschlag, *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6921 (1991).
7. K. Hertel, D. Herschlag, O. C. Uhlenbeck, unpublished results.
8. A 71–amino acid version of HIV NC protein was expressed from a bacterial overproducing plasmid (*15*) obtained from a pNL4-3 plasmid clone [A. Adachi *et al.*, *J. Virol.* **59**, 284 (1986)] by polymerase chain reaction. This protein was used in all experiments herein, unless otherwise noted. It was expressed in *E. coli* as a fusion with Met-$(His)_6$ attached at its NH_2-terminus and was purified by nickel–nitrilotriacetic acid column chromatography (Qiagen, Chatsworth, CA). It was

>95% pure, as judged from Coomassie brilliant blue staining of SDS gels, and was free of detectable deoxyribonuclease and RNase activity. Smearing of the major band suggests that there was some degree of proteolytic degradation. This preparation (1 mg/ml) was stored at −80°C in 50 mM tris-Cl (pH 7.5), 0.1% Triton X-100, 50 mM NaCl, 100 mM imidazole, 10 mM β-mercaptoethanol, and 20% glycerol (*15*). The concentration of protein required to enhance HH16 activity increased slowly with storage, which suggests that there was a loss of activity with time. Thus, the reported concentrations may overestimate the amount of fully active NC required for stimulation.
9. C. Méric and S. P. Goff, *J. Virol.* **63**, 1558 (1989); S. Oertle and P.-F. Spahr, *ibid.* **64**, 5757 (1990); A. Aldovini and R. A. Young, *ibid.*, p. 1920.
10. B. J. Smith and J. M. Bailey, *Nucleic Acids Res.* **7**, 2055 (1979); C. Méric, J.-L. Darlix, P.-F. Spahr, *J. Mol. Biol.* **173**, 531 (1984); J. Leis, J. McGinnis, R. W. Green, *Virology* **84**, 87 (1978); J. Nissen-Meyer and A. K. Abraham, *J. Mol. Biol.* **142**, 19 (1980); R. L. Karpel, L. E. Henderson, S. Oroszlan, *J. Biol. Chem.* **262**, 4961 (1987).
11. R. Khan and D. P. Giedroc, *J. Biol. Chem.* **267**, 6689 (1992).
12. A. Surovoy, J. Dannull, K. Moelling, G. Jung, *J. Mol. Biol.* **229**, 94 (1993).
13. A. C. Prats *et al.*, *EMBO J.* **7**, 1777 (1988); C. Barat *et al.*, *ibid.* **8**, 3279 (1989).
14. A.-C. Prats *et al.*, *Nucleic Acids Res.* **19**, 3533 (1991); H. DeRocquigny *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 6472 (1992).
15. Z. Tsuchihashi and P. O. Brown, in preparation.
16. In the absence of the NC, the rate-limiting step for the burst phase is the cleavage step (*4*). The cleavage step proceeds at the same rate in the presence and in the absence of the NC (Fig. 3B).
17. This NC protein was prepared from an *E. coli* overproducer in the laboratory of L. Henderson (National Cancer Institute, Frederick, MD). This 55–amino acid residue protein lacks 16 COOH-terminal residues as well as the NH_2-terminal $(His)_6$ tag present in our preparation (*8*) and is thought to correspond to the form found in the HIV virion [L. E. Henderson *et al.*, *J. Virol.* **66**, 1856 (1992)]. This preparation contained Zn^{2+} bound to the zinc fingers, whereas it was not known if Zn^{2+} was bound to the NC of the other preparation (*8*). Previous results have shown that binding and annealing activities occur in the absence of Zn^{2+}, with mutations in the zinc fingers and with a variety of shortened versions of the protein (*14*) [H. DeRocquigny *et al.*, *Nucleic Acids Res.* **21**, 823 (1993)]. Stimulation of both single turnover and multiple turnover reactions of HH16 was observed whether the 55–amino acid protein was incubated before reaction with EDTA, which is expected to remove the bound Zn^{2+} (*12*).
18. Additional control experiments are presented elsewhere (*19*).
19. D. Herschlag, M. Khosla, Z. Tsuchihashi, in preparation.
20. S (Fig. 1) is a 17-mer and is referred to as S_{17mer} for clarity; S'_{17mer} has the same sequence as S except for a change of G → U three residues from the 5′ end to give a mismatched duplex with HH16 (Fig. 1). In all cases, residues were removed from the 3′ end of S or S′ to give the shorter substrates.
21. A. Fersht, *Enzyme Structure and Mechanism* (Freeman, New York, ed. 2, 1985), pp. 111–112.
22. C. Guerrier-Takada, K. Gardiner, T. Marsh, N. Pace, S. Altman, *Cell* **35**, 849 (1983); A. M. Lambowitz and P. S. Perlman, *Trends Biochem. Sci.* **15**, 440 (1990); R. Saldanha, G. Mohr, M. Belfort, A. M. Lambowitz, *FASEB J.* **7**, 15 (1993).
23. G. Mohr *et al.*, *Cell* **69**, 483 (1992); Q. Guo and A. M. Lambowitz, *Genes Dev.* **6**, 1357 (1992).
24. C. Reich, G. J. Olsen, B. Pace, N. R. Pace, *Science* **239**, 178 (1988); A. Tallsjo and L. A. Kirsebom, *Nucleic Acids Res.* **21**, 51 (1993).
25. G. F. Joyce and D. L. Robertson, personal communication.
26. G. F. Joyce, *Nature* **338**, 217 (1989).
27. Z. Tsuchihashi, M. Khosla, D. Herschlag, unpublished results.
28. J. M. Buzayan, W. L. Gerlach, G. Bruening, *Nature* **323**, 349 (1986); A. C. Forster and R. H. Symons, *Cell* **49**, 211 (1987).
29. Before reaction, HH16 and S* were mixed and heated to 95°C for 2 min in the presence of 50 mM tris (pH 7.5) and 1 mM EDTA and cooled at room temperature to promote renaturation and annealing. Reactions were initiated by the addition of $MgCl_2$ directly after the addition of the NC. The order of addition had no effect.
30. All results were quantitated with a Phosphorimager (Molecular Dynamics). Rate constants were obtained for all reactions, from the initial rate formation of the 5′ end-labeled product (P*) (after the burst) in multiple turnover reactions and from the first-order disappearance of S* in single turnover reactions.
31. Before reaction, HH16 and S* were separately heated to 95°C for 2 min in the presence of 50 mM tris (pH 7.5) and 1 mM EDTA and cooled at room temperature. After cooling, $MgCl_2$ was added to HH16 and S* and we initiated the reaction by mixing together HH16 and S*. The NC or buffer was added 15 s after initiation. The order of addition had no effect.
32. The reactions whose results are shown in Fig. 4 were followed with a "kinetic ladder" (*7*). An alkaline ladder was generated with 5′ end-labeled S or S′. After this mixture was neutralized, reactions of all of the substrates in the ladder were followed simultaneously. It was important to maintain ribozyme in large excess of the RNA substrates so that the presence of one substrate did not affect the reaction of another substrate—for example, by competing for a limited number of binding sites. The rate constants obtained for several oligonucleotides were compared to those obtained in standard assays with the single purified oligonucleotide; these values were the same, which demonstrates the validity of this approach (*7*). Because we did not have a strong prediction for the length of oligonucleotide substrate that would be required to obtain NC-enhanced specificity and we wished to gather the most information about these reactions, we used the kinetic ladder approach to investigate the effect of the NC on specificity. Data for only S_{13mer} and S'_{13mer} are presented herein.
33. We thank K. Hertel, L. Henderson, and B. Pontius for generous gifts of materials, S. Muthani and M. Chada of CloneTec for oligonucleotides made by automated RNA synthesis, P. Brown for encouragement, and P. Brown, T. Cech, and I. R. Lehman for helpful comments on the manuscript. Supported by a grant from the Lucille P. Markey Charitable Trust to D.H. D.H. is a Lucille P. Markey Scholar in Biomedical Sciences. Z.T. was supported by the Howard Hughes Medical Institute.

27 May 1993; accepted 2 August 1993

Interaction of Mammalian Splicing Factor SF3a with U2 snRNP and Relation of Its 60-kD Subunit to Yeast PRP9

Reto Brosi, Karsten Gröning, Sven-Erik Behrens, Reinhard Lührmann, Angela Krämer*

In the assembly of a prespliceosome, U2 small nuclear ribonucleoprotein (snRNP) functions in pre–messenger RNA (mRNA) splicing together with splicing factors (SFs) 3a, SF3b, and several other proteins. The 17*S* but not the 12*S* form of U2 snRNP is active in splicing-complex formation. Here it is shown that the SF3a subunits correspond to three of the 17*S* U2 snRNP–specific polypeptides. SF3a interacts with U2 snRNP in the presence of SF3b to generate a structure similar to 17*S* U2 snRNP, which suggests a function for SF3a and SF3b in the incorporation of U2 snRNP into the spliceosome. Furthermore, the 60-kilodalton subunit of SF3a is related to the yeast splicing protein PRP9.

The active spliceosome, which is the site of two transesterification reactions that generate spliced mRNA, is assembled in a stepwise fashion by interactions between pre-mRNA, snRNPs, and a number of proteins (*1*). Components in five chromatographic fractions (SF1, SF3, U2AF, and U1 and U2 snRNPs) obtained from HeLa cell nuclear extracts function in the assembly of the adenosine triphosphate (ATP)–dependent presplicing A complex (*2*). SF3 has been separated into two components (SF3a and SF3b), both of which are required for A-complex formation (*3*). The component SF3a has been purified to homogeneity and consists of three subunits of 60, 66, and 120 kD ($SF3a^{60}$, $SF3a^{66}$, and $SF3a^{120}$, respectively); SF3b has been only partially purified.

As for U2 snRNP, which interacts with the pre-mRNA to form the presplicing complex (*4*), it exists in two forms, 12*S* or 17*S* (*5*, *6*). The 12*S* U2 snRNP contains the common snRNP proteins and the U2 snRNP–specific proteins A′ and B″ (*7*). The 17*S* U2 snRNP contains an additional set of polypeptides of 35, 53, 60, 66, 92, 110, 120, 150, and 160 kD (*6*). This 17*S* U2 particle unlike its 12*S* counterpart is active in spliceosome assembly and splicing

R. Brosi, K. Gröning, A. Krämer, Département de Biologie Cellulaire, Sciences III, Université de Genève, 30, quai Ernest-Ansermet, CH-1211 Genève, Switzerland.
S.-E. Behrens and R. Lührmann, Institut für Molekularbiologie und Tumorforschung, Philipps-Universität Marburg, Emil-Mannkopff-Strasse 2, D-35037 Marburg, Germany.
*To whom correspondence should be addressed.