The EMBO Journal vol.13 no.12 pp.2913–2924, 1994

An RNA chaperone activity of non-specific RNA binding proteins in hammerhead ribozyme catalysis

Daniel Herschlag, Mala Khosla, Zenta Tsuchihashi[1,2] and Richard L.Karpel[3]

Department of Biochemistry, B400 Beckman Center, and [1]Howard Hughes Medical Institute, Stanford University, Stanford, CA 94305-5307 and [3]Department of Chemistry and Biochemistry, University of Maryland, Baltimore County, Baltimore, MD 21228-5398, USA
[2]Present address: Somatix Therapy Corporation, 850 Marina Village Parkway, Alameda, CA 94501, USA

Communicated by T.R.Cech

We have previously shown that a protein derived from the p7 nucleocapsid (NC) protein of HIV type-1 increases k_{cat}/K_m and k_{cat} for cleavage of a cognate substrate by a hammerhead ribozyme. Here we show directly that the increase in k_{cat}/K_m arises from catalysis of the annealing of the RNA substrate to the ribozyme and the increase in k_{cat} arises from catalysis of dissociation of the RNA products from the ribozyme. A peptide polymer derived from the consensus sequence of the C-terminal domain of the hnRNP A1 protein (A1 CTD) provides similar enhancements. Although these effects apparently arise from non-specific interactions, not all non-specific binding interactions led to these enhancements. NC and A1 CTD exert their effects by accelerating attainment of the thermodynamically most stable species throughout the ribozyme catalytic cycle. In addition, NC protein is shown to resolve a misfolded ribozyme–RNA complex that is otherwise long lived. These *in vitro* results suggest that non-specific RNA binding proteins such as NC and hnRNP proteins may have a biological role as RNA chaperones that prevent misfolding of RNAs and resolve RNAs that have misfolded, thereby ensuring that RNA is accessible for its biological functions.

Key words: chaperone/HIV nucleocapsid/hnRNP/ribozyme

Introduction

Specific interactions between proteins and RNA are important in many biological processes including protein translation, pre-mRNA splicing, and translational and transcriptional control. Indeed, even though group I and group II introns can be self-splicing *in vitro*, there is evidence for assistance by specific proteins *in vivo* (Lambowitz and Perlman, 1990; Guo and Lambowitz, 1992). Similarly, the RNA component of RNase P, which alone is catalytic, is found complexed with a specific protein *in vivo* and this protein provides catalytic enhancement *in vitro* (Reich *et al.*, 1988; Altman, 1989).

Proteins designed to recognize a wide range of RNAs may be structurally, mechanistically and functionally distinct from proteins that recognize a specific RNA or group of RNAs highly related in sequence or structure. The hnRNP proteins, which coat nascent RNA concomitant with transcription, are the most abundant class of non-specific RNA binding proteins (Dreyfuss *et al.*, 1993). The term 'non-specific' is used throughout the text for simplicity. However, it should be pointed out that proteins that recognize RNA with broad specificity nevertheless can exhibit distinct sequence preferences and that these preferences may be involved in important biological processes such as choosing between alternative splice sites (McPheeters *et al.*, 1988; Swanson and Dreyfuss, 1988a,b; Buvoli *et al.*, 1990, 1992; Bennett *et al.*, 1992; Mayeda and Krainer, 1992; Cobianchi *et al.*, 1993).

The hammerhead ribozyme reactions cycle (Figure 1) can be used as a model system for investigation of the effects of non-specific RNA binding proteins on RNA-mediated processes. This reaction includes many of the features typical of RNA-mediated processes, such as the formation and dissociation of base pairs and the adoption of a functional tertiary structure. In addition, the previous determination of individual rate and equilibrium constants for the reaction of hammerhead ribozyme HH16 provides a framework for interpretation of the effects of added protein (Figure 1B; Hertel *et al.*, 1994). We have therefore explored mechanistic and functional features of non-specific RNA binding proteins through their effects on this hammerhead ribozyme reaction.

We recently showed that a retroviral nucleocapsid (NC) protein, which binds RNA with broad specificity (Leis *et al.*, 1978; Smith and Bailey, 1979; Nissen-Meyer and Abraham, 1980; Meric *et al.*, 1984; Karpel *et al.*, 1987; Khan and Giedroc, 1992; Dib-Hajj *et al.*, 1993; Lapadat-Tapolsky *et al.*, 1993; Surovoy *et al.*, 1993; You and McHenry, 1993), can overcome general limitations to ribozyme turnover and specificity (Tsuchihashi *et al.*, 1993). NC enhancement of the RNA cleavage by hammerhead ribozyme HH16 (Figure 1A) occurs even though there is no reason to believe that the hammerhead ribozyme and NC protein ever act in concert in nature. Based on the detailed kinetic and thermodynamic framework for the HH16 reaction (Figure 1B; Hertel *et al.*, 1994), the increases in turnover and specificity caused by this protein were attributed to effects from its strand annealing and strand dissociation activities (Tsuchihashi *et al.*, 1993), activities that are also exhibited by A1 and other hnRNP proteins (Kumar and Wilson, 1990; Pontius and Berg, 1990, 1992; Munroe and Dong, 1992; Portman and Dreyfuss, 1994). In addition, non-specific binding interactions of the *Escherichia coli* S12 ribosomal protein appear to aid other functional RNAs, the group I self-splicing introns and the hammerhead ribozyme (Coetzee *et al.*, 1994).

Here we investigate further the basis of the enhancement of ribozyme catalysis by NC protein. Individual steps for the reaction in the presence and absence of NC protein are isolated to determine directly the role of the protein in

© Oxford University Press

catalysis; these results confirm and extend our previous model. The effects of NC protein are compared with those of a peptide polymer derived from the repetitive sequence of the C-terminal domain of the hnRNP A1 protein, a series of highly charged peptides and a cationic detergent (CTAB). The results suggest that some non-specific RNA binding proteins can facilitate thermodynamic equilibration between RNA structures without shutting down the RNA's ability to function. These and other results suggest that non-specific RNA binding proteins may have a biological role as RNA chaperones, to prevent and resolve the misfolding of RNA.

Results

NC facilitates dissociation of the oligonucleotide products from HH16

When the RNA substrate (S) is present in excess of ribozyme HH16, there is an initial burst of product formation which is stoichiometric with ribozyme, followed by a slower subsequent cleavage of S (Figure 2). This arises because product release is slower than the cleavage step in the HH16 reaction (Figure 1B; Hertel *et al.*, 1994). Addition of the p7 NC protein overcame the slow phase subsequent to the burst, leading to the conclusion that NC facilitates product release, the rate-limiting step in the absence of NC (Figure 2; Tsuchihashi *et al.*, 1993).

Although partial degradation of the ribozyme by a nuclease contaminant could give shorter recognition arms and thus could accelerate rate-limiting product dissociation and multiple turnover, there was no detectable degradation (<5%) of 5′-end-labeled ribozyme (HH16*) in reaction mixtures containing concentrations of NC that stimulated the reaction, and 5′-end labeled substrate (S*) was cleaved to only the expected product (P*; Figure 2A; data not shown). In addition, NC proteins prepared by different procedures in different laboratories both gave stimulation of the HH16 reaction (Tsuchihashi *et al.*, 1993). Furthermore, the results in Figure 2B show that single-stranded (ss) DNA shuts down the enhancement from NC even after the NC stimulation had commenced. This shut-down is expected for enhancement from NC exerting its effect in each round of catalysis. In contrast, if nuclease were responsible for the enhanced turnover, then quenching the nuclease activity by addition of ssDNA after the enhanced turnover had already begun would not have shut down the rate enhancement.

As described above, the kinetic and thermodynamic

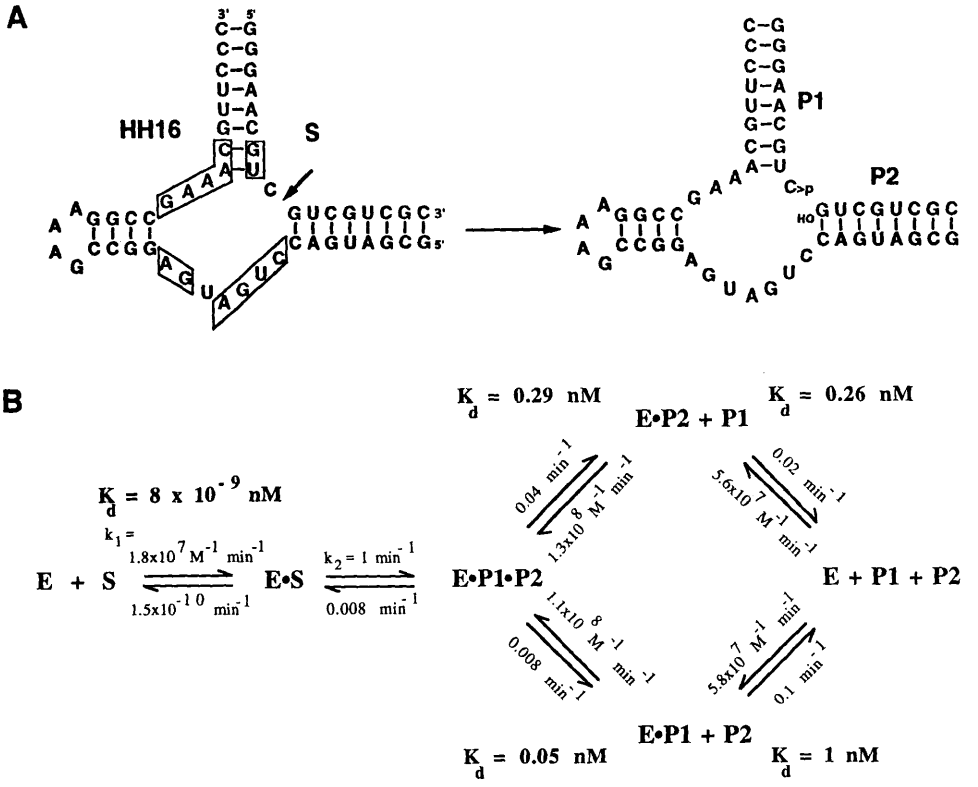

Fig. 1. The hammerhead ribozyme reaction. (**A**) The ribozyme HH16 complexed with its oligonucleotide substrate (S). The boxed residues are conserved and are thus presumed to be responsible for the architecture of the active site (Symons, 1992). There are eight base pairs on either side of the cleavage site for HH16, giving a recognition sequence of 16. The cleavage reaction gives a 5′ product, P1, with a 2′,3′-cyclic phosphate and a 3′ product, P2, with a 5′ hydroxyl group. (**B**) Kinetic and thermodynamic framework for the HH16 reaction. All parameters were determined in a detailed kinetic and thermodynamic analysis of RNA cleavage by HH16 (25°C; 50 mM Tris, pH 7.5 and 10 mM $MgCl_2$; Hertel *et al.*, 1994). At low concentrations of the HH16 and the RNA substrate (subsaturating or 'k_{cat}/K_m' conditions), the rate-limiting step is binding of the substrate to the ribozyme to form the base paired ribozyme–substrate complex (k_1). In single turnover reactions of the preformed ribozyme–substrate complex, the chemical cleavage step (k_2) is followed, occurring with a rate constant of $\sim 1\ min^{-1}$. However, with saturating RNA substrate, multiple turnover is considerably slower than the chemical cleavage step ($k_{cat} \approx 0.01\ min^{-1}$), as it is limited by the slow release of the cleaved RNA products. The values in the scheme are for reaction of a substrate with a 3′-terminal C residue added to S; this addition has no effect on the cleavage step and only small effects on the binding of the oligonucleotide substrate and 3′ product (Hertel *et al.*, 1994; K.J.Hertel and D.Herschlag, unpublished results). The individual rate constants obtained for the comparisons herein of $k_2 = 0.6\ min^{-1}$, $k_1 = 1 \times 10^7\ M^{-1}\ min^{-1}$ and $k_{cat} = 0.01\ min^{-1}$ agreed reasonably well with those shown, but were slightly smaller than the values previously obtained.

framework for the HH16 reaction in Figure 1B, combined with the data in Figure 2, strongly suggest that NC facilitates dissociation of the oligonucleotide products. This was tested directly by 'pulse−chase native gel electrophoresis' (Hertel *et al.*, 1994). This technique uses a native gel to monitor the amount of dissociation of labeled oligonucleotide that has occurred following a 'chase' of unlabeled oligonucleotide and prior to loading the sample onto the gel. NC enhances the dissociation of P1, the 5′ product (Figure 3A, t_2 = 20 min). ssDNA abolished this effect (Figure 3A), mirroring its ability to abolish NC enhancement in multiple turnover reactions (Tsuchihashi *et al.*, 1993). The dissociation of P2, the 3′ product, is also enhanced by NC, as determined from analogous pulse−chase native gel experiments (Figure 3B). Quantification of data analogous to those in Figure 3 at several time points gave rate constants for dissociation of P1 and P2 of ~0.2−0.3 min^{-1} in the presence of 400−1600 nM NC, similar to the rate of multiple turnover in the presence of NC of ~0.3 min^{-1} that was observed in side-by-side experiments but 20- to 30-fold faster than the rate of multiple turnover in the absence of NC (data not shown). The NC protein also affected the fraction of

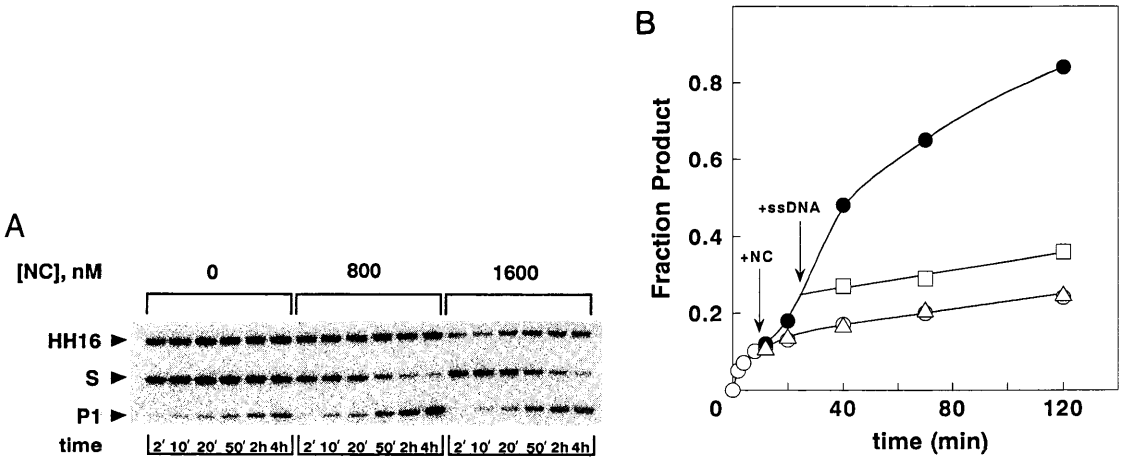

Fig. 2. Effect of NC on multiple turnover reactions of HH16. (**A**) The cleavage of 30 nM S by 3 nM HH16 in the absence of NC and in the presence of 800 or 1600 nM NC protein. Both HH16 and S are 5′-end-labeled. A PhosphorImager printout is shown following separation by electrophoresis on a 20% polyacrylamide−7 M urea denaturing gel. (**B**) The NC enhancement is shut down by subsequent addition of ssDNA. Cleavage of 100 nM S* by 10 nM HH16 in the absence of NC or ssDNA (○) or with the addition of protease-treated NC (△). At time t = 10 min, NC was added to an aliquot from the reaction mixture of 100 nM S* and 10 nM HH16 alone to a final concentration of 400 nM (●). After an additional 15 min, at t = 25 min, ssDNA was added (final concentration 2 μM) to an aliquot from the reaction with NC (□). Addition of ssDNA in the absence of NC had no effect on the reaction (not shown).

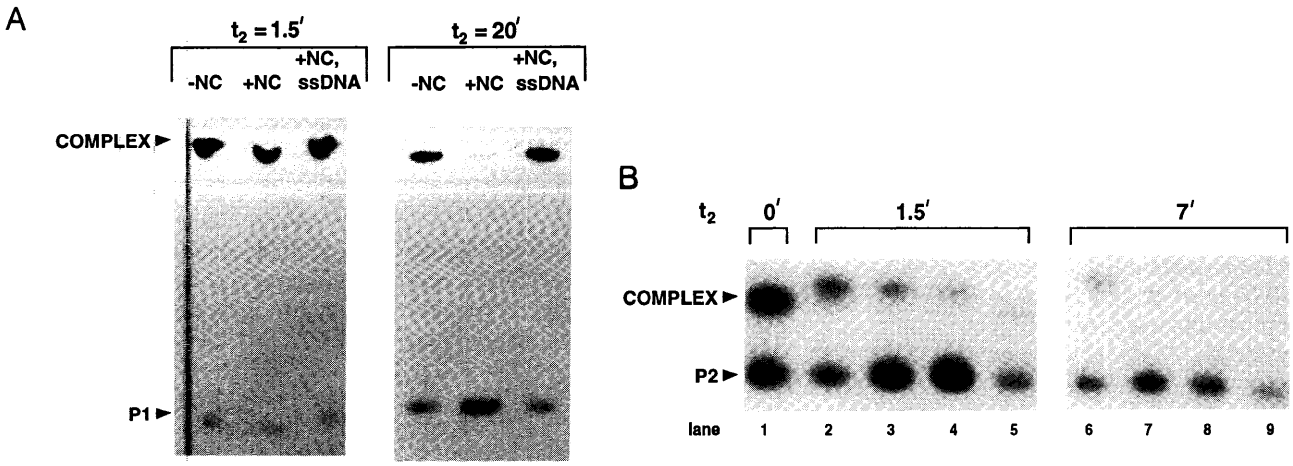

Fig. 3. NC enhances the rate of product dissociation with HH16. (**A**) Dissociation of product P1. Separation by native gel electrophoresis of 5′-labeled P1 (P1*) from P1* complexed with HH16 ('COMPLEX') as a function of time subsequent to the addition of a 'chase' of unlabeled S. The role of the chase is to prevent rebinding of molecules of P1* that have dissociated. Concentrations following the chase: 10 nM HH16, ~0.5 nM P1*, 100 nM unlabeled S, with or without 400 nM NC and 1 μM ssDNA as noted in the figure. (**B**) Dissociation of product P2. Separation by native gel electrophoresis of labeled P2* from P2* complexed with HH16 ('COMPLEX') as a function of time subsequent to the addition of a 'chase' of unlabeled S, except for lanes 5 and 9, for which unlabeled S was omitted from the chase. Lane 1 shows the extent of complex formation observed in the native gel just prior to addition of the chase. Concentrations following the chase: 2 nM HH16, ~0.5 nM P1* and 70 nM unlabeled S (except lanes 5 and 9), and no NC (lanes 2 and 6), 800 nM NC (lanes 3 and 7) or 1600 nM NC (lanes 4 and 8 and 5 and 9). Lanes 5 and 9 contained 1600 nM NC, as did lanes 4 and 8, but unlabeled S was omitted in the chase and P1* rebinding was instead prevented by an additional 2-fold dilution to give 1 nM HH16 and ~0.25 nM P1* during the chase. (Thus the bands in lanes 5 and 9 are less intense.) Note that in the absence of unlabeled S, the complex migrates more quickly (lanes 1 and 5 versus lanes 2−4). This suggests that a ternary complex of HH16−S−P2* forms, in which S and HH16 interact via the base pairs that are usually formed between P1 and HH16 (Figure 1). Analogous data (not shown) suggest the occurrence of an E−S−P1* complex.

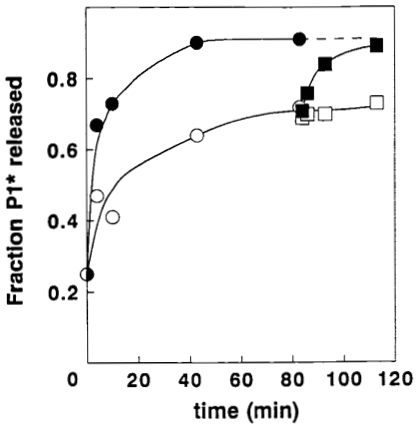

Fig. 4. Resolution of a stable, alternative RNA conformer by NC protein. The dissociation of 5′-labeled product P1* from its complex with HH16 was followed in the absence (○) and presence (●) of 1400 nM NC by native gel electrophoresis (see Figure 3 and Materials and methods). At t = 90 min, NC was added to aliquots from the reaction mixture lacking NC to a final concentration of 800 nM NC (■) or buffer was added in a side-by-side control reaction (□). Concentrations following the chase with unlabeled S at t = 0 (see Materials and methods): 3 nM HH16, ~0.5 nM P1*, 70 nM unlabeled S, with or without 1400 nM NC. The ~10% of P1* that appears to remain bound at later times can be accounted for by a small amount of smearing in the gel in addition to the 4% expected to remain bound, since unlabeled S is in ~25-fold excess of HH16 following the chase.

the product dissociating in a single first-order process, as is described below.

Two general classes of facilitated product release can be envisioned: NC could simply lower the activation barrier for the dissociation process or it could catalyze a strand exchange reaction in which the new substrate oligonucleotide, S, is involved in 'pushing off' the bound product oligonucleotide. Lanes 5 and 9 of Figure 3B show that NC facilitates P2 dissociation in the absence of added S, strongly suggesting that the strand exchange process is not required for NC action. Analogous results were obtained for P1 dissociation (data not shown).

NC can resolve an apparently misfolded RNA species that is kinetically trapped in an alternative conformation

In the absence of NC, 20–30% of P1* remained bound to HH16 even at long times in the pulse–chase native gel experiments described in the previous section (Figure 4, open symbols). A trivial explanation for this would be that insufficient unlabeled S was added to compete fully with binding of P1*. However, unlabeled S was present in 20-fold excess over HH16 and even larger excess over P1*. In addition, varying neither the amount of unlabeled S in the chase by 3-fold nor the amount of HH16 present by 3-fold had a significant effect on the fraction of P1* that remained bound (data not shown). This strongly suggests the model presented in equation 1. The observation that ~70% of the P1* dissociates as a single species in a well-behaved first-order process, whereas ~20–30% of the P1* remains bound suggests that the dissociating and non-dissociating species are distinct and do not interconvert on the time-scale of this experiment. Thus, it is suggested that P1* and the ribozyme are kinetically trapped in a complex [(HH16–P1*–S)$_{trap}$; equation 1], even though they are

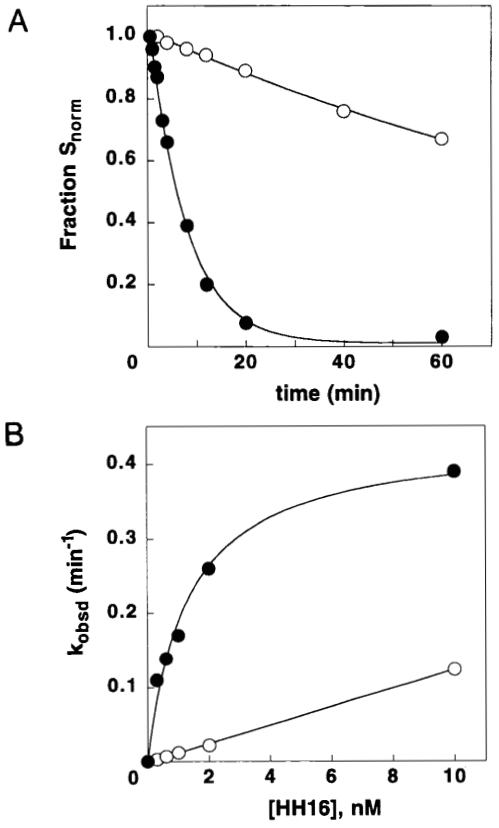

Fig. 5. Effect of NC on single turnover reactions. (**A**) Reaction of 0.6 nM HH16 and 0.3 nM S* in the absence (○) and presence (●) of 300 nM NC. The amount of S* remaining was normalized to account for ~10% unreactive S* at later times: S_{norm} = (Fraction S* − 0.1)/0.9 and the lines are non-linear least squares fits to the data that give k_{obs} = 0.07 min^{-1} and 0.13 min^{-1} in the absence and presence of NC, respectively. (**B**) Rate constants for reactions of varying concentrations of HH16 and 0.2 nM S* in the absence (○) and presence (●) of 300 nM NC. The values of k_{obsd} were obtained from the first-order disappearance of S* as in (A), and non-linear least squares fits give k_{cat}/K_m = 30 × 10^7 M^{-1} min^{-1} and 1 × 10^7 M^{-1} min^{-1} in the presence and absence of NC, respectively. Maximal rate constants of $k_c \approx$ 0.6 min^{-1} with saturating HH16 were obtained in the presence and absence of NC (data not shown).

readily dissociable when bound in the normal complex (HH16–P1*–S). Upon addition of S to HH16–P1*, there is a decrease in native gel mobility of the labeled species (Figure 3B and legend), suggesting that an HH16–P1*–S complex is formed, presumably with S making the base pairing interactions to HH16 that are otherwise made between the P2 product and HH16 (Figure 1A). The (HH16–P1*–S)$_{trap}$ complex comigrates with the HH16–P1*–S complex that dissociates rather than with a HH16–P1 complex (not shown), suggesting that the trapped species also contains unlabeled S from the chase. The nature of this alternative species is not known. It could involve additional fortuitous interactions of the partially base-paired S.

$$\begin{array}{ccc} \text{HH16–P1*–S} & \xrightarrow{k_{off}^{P1}} & \text{HH16 + P1*} \\ (\sim 70\%) & & \\ \not\downarrow & & \\ (\text{HH16–P1*–S})_{trap} & \not\rightarrow & \text{HH16 + P1*} \\ (\sim 30\%) & & \end{array} \qquad (1)$$

In contrast to the results described above, essentially all of the P1* dissociates from the ribozyme complex with NC protein present (Figure 4, closed circles). This suggests that either the NC protein does not allow formation of the alternative complex or it can resolve this species after it is formed. In order to determine if the NC protein can resolve the misfolded complex, the misfolded complex was allowed to form in the absence of NC, and NC was subsequently added. The closed squares in Figure 4 show that addition of NC after the dissociation of P1* had already plateaued caused the remainder of the bound P1* to dissociate. Furthermore, addition of ssDNA abolished the ability of NC to dissociate this complex (not shown). These results suggest that the NC protein can resolve the kinetically trapped alternative conformer, $(HH16-P1^*-S)_{trap}$.

The effect of NC on single turnover reactions: increases in the rate of association of HH16 and S

NC protein increases the rate of single turnover reactions at low concentrations of HH16 by ~20-fold (Figure 5; Tsuchihashi *et al.*, 1993). However, the same maximal rate for single turnover reactions is obtained with saturating concentrations of HH16 in the presence and absence of NC at concentrations that are stimulatory at low [HH16] (data not shown), and preannealing of HH16 and S also eliminates the stimulatory effect of NC (Tsuchihashi *et al.*, 1993). Thus, NC enhances the apparent second-order rate constant, k_{cat}/K_m, for the reaction: HH16 + S → products, but does not enhance the rate of the single turnover reaction of the HH16–S complex. Binding of S is rate-limiting for k_{cat}/K_m in the absence of NC (k_1, Figure 1B; Hertel *et al.*, 1994). Thus, the increase in k_{cat}/K_m with NC present suggests that the protein increases the rate of annealing of the ribozyme and substrate (Tsuchihashi *et al.*, 1993). For single turnover reactions of the HH16–S complex, the cleavage step is rate-limiting (k_2, Figure 1B; Hertel *et al.*, 1994), suggesting that there are concentrations of NC stimulatory for k_{cat}/K_m that do not have a significant effect on the cleavage step (Tsuchihashi *et al.*, 1993).

The pulse–chase experiment outlined in Figure 6A was carried out to provide a direct test of the conclusion that NC protein increases the rate of association of HH16 and S. The results of one such experiment are shown in Figure 6B. In 2 min there is essentially complete binding in the presence of NC, whereas <20% of S* is bound in the control without NC. As expected, ssDNA abolished this enhancement. Furthermore, the rate of binding was shown to increase linearly with HH16 concentration as expected for the second-order binding process: HH16+S* → HH16–S* (data not shown). Quantification of the data of Figure 6B and analogous data gave rate constants for association of $k_1 = 1 \times 10^7\ M^{-1}\ min^{-1}$ in the absence of NC and $k_1 = (10-40) \times 10^7\ M^{-1}\ min^{-1}$ in the presence of 300–1600 nM NC, consistent with the increase of ~20-fold in k_{cat}/K_m observed in Figure 5.

High concentrations of NC inhibit multiple turnover and single turnover by shutting down the cleavage step

The concentration of NC required to stimulate cleavage of S was the same with 3 nM HH16 and 30 nM S as with 10 nM HH16 and 100 nM S (Figure 7A). This indicates that the NC concentration effect does not arise from a simple

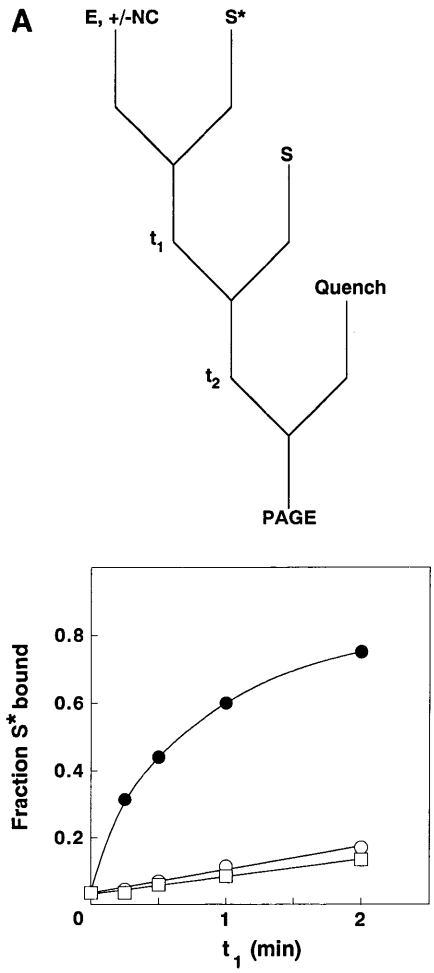

Fig. 6. NC enhances the rate of association of HH16 and S. (**A**) Outline of pulse–chase experiment to measure association rate. In this experiment, HH16 and a trace amount of 5′-end-labeled S (S*) were incubated together for varying times t_1 in the presence or absence of NC. At time t_1 a 'chase' with a large excess of unlabeled S was added to quench further binding of S*. Aliquotes were removed after allowing sufficient time for cleavage of all of the bound S* and were analyzed by denaturing gel electrophoresis. Bound S* is thus detected as the cleavage product P1* (see Materials and methods). (**B**) Results from pulse–chase experiment with 10 nM HH16, 1 nM S* in the absence of NC (○), the presence of 400 nM NC (●), or the presence of 400 nM NC and 3 μM ssDNA (□). The fraction trapped corresponds to the amount of S* bound during time t_1, and the lines are non-linear least squares fits that give second-order rate constants for binding of $k_1 = 1 \times 10^7\ M^{-1}\ min^{-1}$ in the absence of NC, $16 \times 10^7\ M^{-1}\ min^{-1}$ in the presence of NC, and $0.7 \times 10^7\ M^{-1}\ min^{-1}$ in the presence of both NC and ssDNA.

stoichiometric titration. Figure 7A also shows that concentrations of NC protein higher than required for stimulating multiple turnover decrease the rate of reaction. The molecularity of NC action was not further investigated. Similarly, as NC concentration is increased, the observed rate constant for single turnover reactions first increases, then decreases (Figure 7B).

It was predicted that if the NC protein was inhibiting multiple turnover, then ssDNA, which can abolish the stimulatory activity of NC, should also abolish the inhibitory activity of NC. This was indeed observed (Figure 8, triangles). In addition, intermediate concentrations of ssDNA allowed stimulation in the presence of a concentration of NC that alone was inhibitory (Figure 8, squares), as predicted

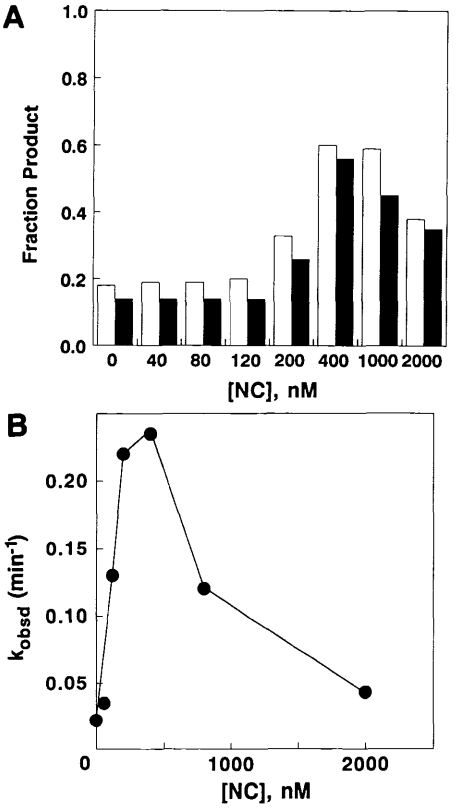

Fig. 7. Concentration dependence of the NC effect on multiple turnover and single turnover reactions. (**A**) Multiple turnover reactions. Extent of reaction after 40 min with 3 nM HH16 and 30 nM S* (open bars) or 10 nM HH16 and 100 nM S* (closed bars). The same trends of reaction extents held throughout the reaction time course (not shown). Though the trends observed in this figure were reproducible, the exact concentration of NC required for stimulation and inhibition varied with the experiment. (**B**) Single turnover reactions. The observed rate constant for cleavage of S* in single turnover reactions of 2 nM HH16 and ~0.2 nM S* with various concentrations of added NC. Reactions were initiated by addition of S*.

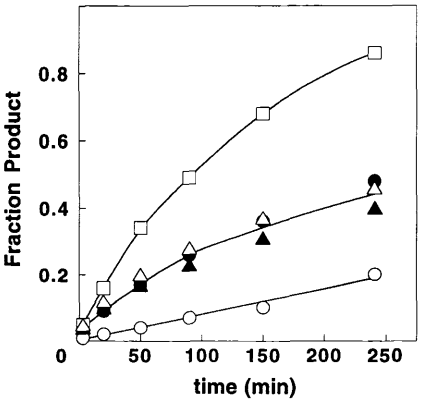

Fig. 8. The inhibitory effect of NC is overcome by ssDNA. Multiple turnover reactions with 3 nM HH16 and 30 nM S* in the presence (open symbols) or absence (closed symbols) of 800 nM NC with varying concentrations of ssDNA: no ssDNA (○,●); 400 nM ssDNA (□); 800 nM ssDNA (△,▲). The ssDNA had no effect in the absence of NC (closed symbols and data not shown). Reactions were carried out in the presence of 3 mM $MgCl_2$ instead of the typical 10 mM concentration because inhibition was more marked at the lower Mg^{2+} concentration.

for an effect arising from the NC that remains unbound to ssDNA.

There are three models that can account for the inhibition by high concentrations of NC: (i) NC prevents annealing to form the HH16–S complex by binding to HH16 and/or S individually; (ii) NC binds to and destabilizes the HH16–S complex, resulting in its disassembly; or (iii) NC binds to the HH16–S complex in such a way as to block the cleavage step without causing disassembly. The following experiments strongly suggest that the inhibition arises from NC binding to the HH16–S complex and blocking the cleavage step [model (iii)].

5′-end-labeled S (S*) was preannealed with an excess of HH16; varying concentrations of NC were added and reactions were initiated by addition of Mg^{2+}, analogous to the protocol used in multiple turnover reactions. As expected, high concentrations of NC (2–10 μM) inhibited the cleavage of S* (~5-fold inhibition). In order to determine whether the HH16–S* complex persisted under these inhibitory conditions, aliquots from these reaction mixtures were diluted 10-fold into a large excess of unlabeled S and ssDNA (1–4 nM HH16; 0.1 nM S*; 50 nM unlabeled S; 1 μM ssDNA; 0.2–1 μM NC). ssDNA was included in the dilution to quench the inhibitory effect of the NC so that if the HH16–S* complex were present, it would be able to react; the large excess of unlabeled S ensured that, if the HH16–S* complex had dissociated, S* would not be able to react because essentially all of the HH16 ribozyme would be sequestered by the excess unlabeled S. However, essentially all of the S* present was cleaved at the rate expected for reaction of the HH16–S* complex (data not shown). This strongly suggests that the HH16–S* complex remained intact and that the inhibitory concentrations of NC did not cause its disassembly [model (ii)], but rather blocked its reaction [model (iii)].

The origin of the NC inhibition was tested in another way by varying the concentration of HH16 present in reactions with inhibitory concentrations of NC. The reactions were again initiated by Mg^{2+} addition subsequent to annealing S* with an excess of HH16. If NC caused dissociation of the HH16–S* complex [model (ii)], then the higher concentrations of HH16 would be predicted to speed the second order reaction of HH16 + S* → products, whereas if there were no dissociation then the higher concentrations of HH16 would have no effect on the first order reaction of HH16–S* → products [model (iii)]. Varying the HH16 concentration from 5 to 20 nM did not affect the rate of S* cleavage either in the absence or presence of inhibitory concentrations of NC of 2–5 μM, suggesting that the HH16–S complex remains during inhibition.

Finally, the pulse–chase experiments outlined in Figure 6A were used to measure the rate of annealing of HH16 and S in the presence of NC concentrations that inhibit the single turnover reactions in order to determine if annealing is inhibited [model (i)]. However, concentrations of NC up to at least 5 μM still stimulated annealing. Thus, the inhibition of multiple turnover reactions is attributed to inhibition of the cleavage step from binding of NC to the HH16–S complex [model (iii)]. The molecularity of this inhibitory effect was not determined.

A peptide polymer derived from the hnRNP A1 protein (A1 CTD) also enhances HH16 catalysis

It was previously demonstrated that the hnRNP A1 protein enhances the rate of oligonucleotide annealing for both RNA and DNA (Kumar and Wilson, 1990; Pontius and Berg,

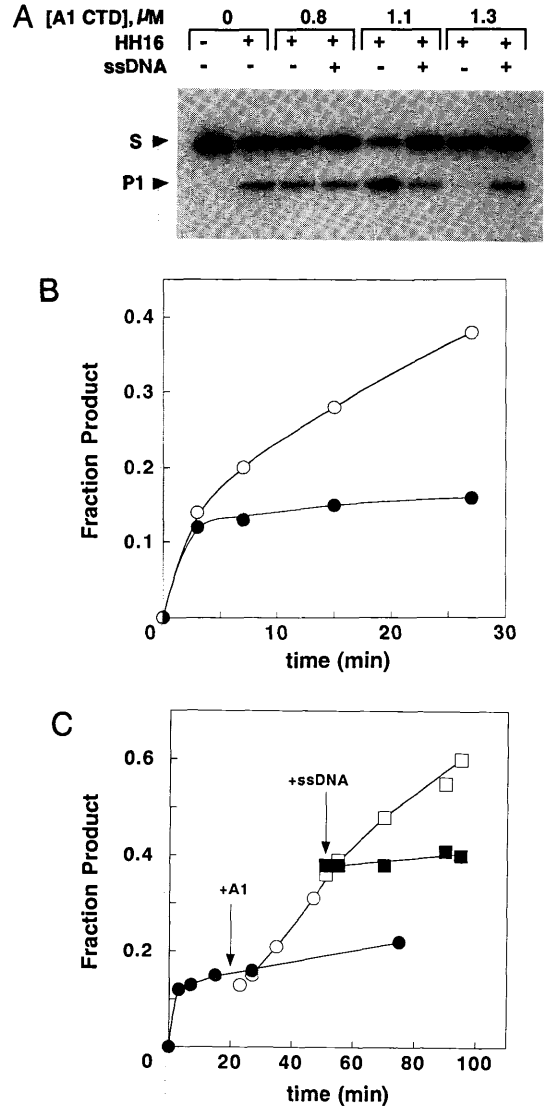

Fig. 9. A1 CTD enhances HH16 multiple turnover. (**A**) Autoradiogram of reactions with 3 nM HH16, 30 nM S* with 3 mM $MgCl_2$ in the absence of A1 CTD and in the presence of 0.8, 1.1 or 1.3 μM A1 CTD with or without 5 μM ssDNA at t = 240 min. (**B**) Timecourse for reactions of 3 nM HH16, 30 nM S* with 3 mM $MgCl_2$ in the absence (●) or presence (○) of 0.6 μM A1 CTD. (The concentration of A1 CTD required for activation varied by ~50% in independent experiments.) (**C**) Cleavage of 30 nM S* by 3 nM HH16 in the presence of 3 mM $MgCl_2$ (●); with A1 CTD (final concentration, 0.6 μM) added to aliquots from this reaction at t = 20 min (○); and with ssDNA (final concentration, 1 μM; ■) or buffer (□) added to aliquots from the reactions with A1 CTD at t = 50 min.

1990; Munroe and Dong, 1992; Casas-Finet *et al.*, 1993). Furthermore, hnRNP proteins coat nascent RNA transcripts *in vivo* (Dreyfuss, 1986), suggesting that these proteins may have non-specific functional interactions with RNA (see Introduction). We therefore tested whether or not the A1 protein could enhance hammerhead ribozyme catalysis. Preliminary experiments demonstrated small enhancements in both single turnover (k_{cat}/K_m) and multiple turnover (k_{cat}) reactions upon addition of A1 protein (data not shown). We chose to investigate this effect further using a peptide polymer with a sequence derived from the glycine-rich C-terminal domain of A1 (A1 CTD) because the strand annealing activity of A1 was previously attributed to this domain. This C-terminal domain contains on average 14 repeats of an eight residue consensus sequence, GN(F/Y)GG(G/S)RG (Casas-Finet *et al.*, 1993).

Figure 9 shows that A1 CTD (1.1 μM) enhances multiple turnover reactions of HH16. All of the reactions with A1 CTD were carried out with 3 mM $MgCl_2$ instead of the standard 10 mM in order to minimize the required concentration of A1 CTD; it is interesting to note that A1 CTD has a lower density of positive charge than many RNA binding proteins (see below). The autoradiogram in Figure 9A shows that A1 CTD can both stimulate and, at higher concentrations, inhibit multiple turnover reactions of HH16; both the stimulation and inhibition were abolished by addition of ssDNA, consistent with the ability of A1 CTD to bind ssDNA (Cobianchi *et al.*, 1988; Kumar *et al.*, 1990; Casas-Finet *et al.*, 1993) and analogous to the effects obtained with NC (Tsuchihashi *et al.*, 1993). Quantification of the effect of A1 CTD, an example of which is shown in Figure 9B, revealed maximal rate enhancements of 5- to 10-fold for the multiple turnover. (Note that multiple turnover occurs subsequent to the initial burst of ~10%, which corresponds to the product formed in the first turnover.) A control experiment, analogous to that shown in Figure 2B for NC, showed that addition of ssDNA subsequent to A1 CTD shut down further enhancement by A1 CTD (Figure 9C). This argues strongly against an effect from a contaminating nuclease.

A1 CTD was also shown to provide an enhancement of up to ~20-fold in single turnover reactions (k_{cat}/K_m) at concentrations of ~1 μM; this effect was also quenched by ssDNA (data not shown). In contrast to the effects of A1 CTD, a 16 residue peptide containing just two of the A1 C-terminal domain consensus sequences, GNFGGGRG-GNYGGSRG, did not stimulate or inhibit either single or multiple turnover, even at concentrations of up to 50 μM and 400 μM, respectively. This absence of an effect is consistent with the inability of this peptide to associate significantly with RNA (Casas-Finet *et al.*, 1993).

Can highly charged peptides mimic the NC effects?

The 78 amino acid NC protein used in this work contains 18 Lys and Arg residues, and a 55 amino acid version of this protein containing 15 Lys and Arg residues was also effective at enhancing single and multiple turnover reactions of HH16 (Tsuchihashi *et al.*, 1993). [The 78 amino acid NC protein contained an N-terminal Met-$(His)_6$ tag to aid purification (Tsuchihashi *et al.*, 1993; Z.Tsuchihashi and P.O.Brown, in preparation). His_6 alone had no effect on the HH16 reactions investigated herein, even up to 5 μM, well above the concentrations of NC that give stimulation and inhibition.] Two Lys residues of the murine leukemia virus (MLV) NC protein have been suggested to be important for annealing activity (Prats *et al.*, 1991), and a 27 residue peptide derived from HIV-1 NC, containing seven Lys and Arg residues, maintained annealing activity (DeRocquigny *et al.*, 1992). A peptide comprised of the 19 N-terminal amino acid residues of HIV-1 NC, five of which are Lys or Arg, still binds RNA (Surovoy *et al.*, 1993). Several other proteins that bind RNA strongly, such as tat and rev, have regions of high positive charge, and peptides of the regions of high positive charge from tat and rev also bind strongly to RNA (Frankel, 1994). In order to determine if the catalytic enhancements by NC and A1 CTD result simply from a high density of positively charged groups that appears to be typical

Table I. Summary of peptide effects on the HH16 reaction[a]

Peptide	Single turnover reaction[b] Increase?	k_{on}^{rel} [c] Increase?	Multiple turnover reaction[d]		k_2^{rel} [e]		
			Increase?	Inhibition?	Inhibition?	$K_{1/2}$ (μg/ml)[f]	μM[f]
Tat47–58	+	++	–	yes	yes	2 [1 μM]	1
Tat38–58	–	+	–	yes	yes	5 [2 μM]	2
YK_9A	+	++	–	no	yes	10 [7 μM]	7
YR_9A	–	+	–	yes	yes	0.5 [0.2 μM]	0.2
$YR_{15}A$	–	+	–	yes	yes	0.2 [0.1 μM]	0.1
Rev34–50	+	++	–	yes	yes	3 [1 μM[	1
CTAB	++	++	–	yes	yes	[20 μM]	20

[a]All peptides were tested from 0 to 10 μg/ml and CTAB over 1–30 μM; effects varied over this range. All values are relative to that obtained in a side-by-side reaction in the absence of added peptide or CTAB. Symbols: –, <1.5-fold effect; +, 1.5 to 3-fold effect; ++, 3- to 10-fold effect.
[b]The single turnover reaction: HH16 + S* → HH16–P1*–P2, with 2–3 nM HH16 and 0.5 nM S*.
[c]Determined in pulse–chase experiments with 7 nM HH16 according to the protocol outlined in Figure 6A and Materials and methods.
[d]The multiple turnover reaction: S* + HH16 → P1* + P2 + HH16, with 3 nM HH16 and 30 nM S*.
[e]The reaction HH16–S* → HH16–P1*–P2, with 0.5 nM S* preannealed with 10 nM HH16. This determination assumes that the ribozyme–substrate complex is not dissociated by the peptides during these reactions. There was no non-linearity in the reaction rates that would suggest such dissociation.
[f]Concentrations that give inhibition to 50% of the reaction rate in the absence of added peptide or CTAB. The values of $K_{1/2}$ are presented for gross comparison and are not equilibrium constants because the inhibition did not follow a simple hyperbolic binding curve expected for binding of a single molecule of peptide or CTAB to the RNA (suggesting that the effect arise from binding of more than one molecule of peptide or CTAB) and because there were complicating time-dependent effects in some cases.

in RNA binding proteins or if other features of the NC protein and A1 CTD are important, several highly charged peptides, including peptides derived from tat and rev (listed below), were screened for their effect on the HH16 reaction;

Tat47–58	YGRKKRRQRRRP
Tat38–58	FITKALGISYGRKKRRQRRRP
YK_9A	YKKKKKKKKKA
YR_9A	YRRRRRRRRA
$YR_{15}A$	YRRRRRRRRRRRRRA
Rev34–50	TRQARRNRRRRWRWEQR

Each peptide was screened for its effect on the following reactions: a single turnover reaction of free HH16 and S; a multiple turnover reaction with S in excess of HH16; the annealing of HH16 and S (k_1, Figure 1B); and a single turnover reaction in which the ribozyme and substrate had been preannealed (k_2, Figure 1B). The results for each of the peptides are summarized in Table I. Although all of the peptides could increase the rate of annealing, analogous to one of the effects of NC and A1 CTD, only some were able to increase the observed rate constant in a single turnover reaction. This limitation appears to arise from the ability of the peptides to shut down the chemical step. For example, concentrations of YR_9A and $YR_{15}A$ that enhance annealing also shut down the chemical step and thus give no enhancement in single turnover reactions of free HH16 and S. In contrast, YK_9A, which is considerably less effectual at shutting down the chemical step, did give an increase in the rate of reaction between free HH16 and S due to the enhanced rate of annealing (Table I).

In multiple turnover reactions, none of the peptides was stimulatory, but each was inhibitory at higher concentrations. This is in contrast to NC and A1 CTD, which gave stimulation and, at higher concentrations, inhibition. The peptides were also not stimulatory for multiple turnover reactions carried out in 3 mM $MgCl_2$, the conditions used in the A1 CTD experiments (data not shown). The inhibitory effect again appears to originate from shutting down the cleavage step (Table I, k_2^{rel}), as was observed for NC.

In summary, the highly charged peptides did not mimic all of the effects of NC and A1 CTD on the HH16 ribozyme reaction. These results suggest that there are features of the NC and A1 CTD important for enhancement of ribozyme catalysis beyond positive charges.

A cationic detergent did not mimic the effects of NC protein

It has previously been shown that cationic detergents such as cetyltrimethylammonium bromide (CTAB) can increase the rate of duplex formation (Pontius and Berg, 1991). In order to determine whether such a detergent could mimic the NC and A1 CTD effects, CTAB was screened for its effect on single turnover, multiple turnover, annealing and the chemical step, exactly analogous to the peptide experiments described in the previous section (Table I). CTAB was able to increase the rate of single turnover reactions of free HH16 and S at concentrations that gave an increased rate of annealing (~10–20 μM). However, higher concentrations of CTAB that also gave enhanced annealing did not stimulate the single turnover reaction (~30 μM) because the chemical step was inhibited by these CTAB concentrations. In addition, CTAB at higher concentrations inhibited multiple turnover reactions, but lower concentrations did not stimulate the reaction (Table I). Thus, CTAB behaved analogously to the highly charged peptides, but not analogously to NC and A1 CTD.

Discussion

The effects of the non-specific RNA binding proteins and peptides on hammerhead ribozyme catalysis have ramifications in three areas: (i) the reaction steps in the hammerhead catalytic cycle that are affected have been unambiguously identified and some understanding of how the non-specific RNA binding proteins and peptides exert their effects has been obtained; (ii) the results suggest that non-specific RNA

binding proteins may serve *in vivo* as RNA chaperones that could generally prevent and resolve misfolding of RNAs and promote proper RNA complex formation; and (iii) counter-intuitively, non-specific RNA binding proteins may potentiate the ability of ribozymes to cleave specific RNA targets *in vivo*, a potential therapeutic approach which is being actively pursued. Each area is discussed in turn below.

Understanding the effects on individual steps of the HH16 reaction

The effect of the NC protein on individual reaction steps in a hammerhead ribozyme reaction was investigated in order to understand how this protein enhances and inhibits RNA cleavage by the ribozyme. The NC protein increases the rate of reaction of free HH16 and S by increasing the rate of annealing to form the HH16−S complex. The rate of multiple turnover with saturating S increases due to an increase in the rate of dissociation of the product oligonucleotides. Catalysis is enhanced by NC protein because rate-limiting binding of substrate and dissociation of products can be facilitated without inhibiting the cleavage step (k_2, Figure 1B; Tsuchihashi *et al.*, 1993).

The enhanced catalysis is thus consistent with the strand annealing and strand dissociation activities of NC (Prats *et al.*, 1988, 1991; Barat *et al.*, 1989; DeRocquigny *et al.*, 1992; Khan and Giedroc, 1992; Dib-Hajj *et al.*, 1993; Z.Tsuchihashi and P.O.Brown, in preparation). A new strand is not required for NC to facilitate dissociation (Figure 3B) so that the strand dissociation activity arises from increasing the rate of duplex dissociation, which is followed by reassociation with a different strand, rather than from a direct displacement. The simplest model to explain this increase is that NC binds to and destabilizes the duplex, thereby helping to melt it, as is consistent with the greater affinity of NC for single-stranded than for double-stranded nucleic acids (Davis *et al.*, 1976; Smith and Bailey, 1979; Sykora and Moelling, 1981; Khan and Giedroc, 1992; Lapadat-Tapolsky *et al.*, 1993; Surovoy *et al.*, 1993).

The increase in the rate of duplex association caused by NC, A1 CTD, the positively charged peptides and the cationic detergent CTAB could arise from one or a combination of several mechanisms. (i) These effectors could disrupt an intramolecular structure that inhibits duplex formation, analogous to the proposed action of bacteriophage T4 gene 32 protein or *E.coli* SSB in DNA annealing (Alberts and Frey, 1970; Sigal *et al.*, 1972). The rate constant for binding of S to HH16 of $\sim 1 \times 10^7$ M^{-1} min^{-1} is at the lower extreme for duplex formation between oligonucleotides and is 10-fold slower than the rate constant for binding of the product oligonucleotides P1 and P2 to the ribozyme (Hertel *et al.*, 1994). Thus, the increased rate of formation of the HH16−S complex could arise from disruption of intramolecular structure in S. RNA oligonucleotides that enhanced hammerhead ribozyme turnover by base pairing to substrate oligonucleotides outside of the region recognized by a ribozyme presumably also disrupt inhibitory intramolecular structures (Goodchild, 1992). (ii) The positively charged effectors could help overcome electrostatic repulsion and/or these or other effectors could bridge together the annealing strands to increase their effective concentration (Page and Jencks, 1971). Evidence for an increased effective concentration has been presented for the hnRNP A1 protein (Pontius and Berg, 1990; Pontius, 1993). (iii) An effect from aligning the bases to maximize the probability of duplex formation following collisional encounter is also conceivable.

There are windows of NC and A1 CTD concentration that give enhanced multiple turnover by the HH16 ribozyme (Figures 7−9). The inhibition by NC at higher concentrations was shown to arise from shutting down the cleavage step (k_2, Figure 1B), suggesting that NC binds more strongly to a denatured or inactive conformation of the hammerhead catalytic core than to the active conformation. This emphasizes that the catalytic enhancements arise from widely specific RNA binding proteins that have not evolved to interact specifically with the ribozyme. There is no window of enhancement for multiple turnover with the highly charged peptides or with CTAB (Table I), suggesting that the enhancement of multiple turnover by NC and A1 CTD is not a generic effect of highly charged species. However, the possibility cannot be ruled out that the distinction between NC and A1 CTD and the highly charged peptides is a quantitative rather than a qualitative distinction, with the peptides providing inhibition that is too strong to allow for an enhancement to be observed.

The peptides rich in arginine residues were especially effective inhibitors of the cleavage step (Table I). It has been demonstrated that several RNA motifs, the TAR sequence, the guanosine binding site of group I introns, and three motifs obtained by *in vitro* selection, have significant affinity for arginine and for other compounds carrying the guanidino functional group (Yarus, 1988, 1989; Weeks *et al.*, 1990; Calnan *et al.*, 1991; Puglisi *et al.*, 1992; Connell *et al.*, 1993). Multiple simultaneous interactions of the guanidino functional group of arginine with functional groups of an RNA could be responsible for the increased binding affinity, as there is an entropic advantage to the formation of multiple interactions from the 'chelate effect' (Page, 1977; Jencks, 1981). With an Arg-rich peptide bound to the ribozyme, it would be unlikely, based on simple probability considerations, that the catalytic hammerhead conformation would be the favored conformation. In addition, recent data indirectly suggest that the catalytic core of the hammerhead ribozyme may not be highly ordered (Hertel *et al.*, 1994), so that it may be relatively easy for a bound peptide or other effector to alter this conformation.

Molecules with hydrogen bonding groups that are somewhat fixed with respect to one another, and especially such molecules that are positively charged, may often bind RNA with considerable affinity, because of the large number of potential hydrogen bonding groups on RNA, because of the large number of degrees of freedom in the RNA backbone that allow some structural rearrangement with relatively small energetic consequences, and because of the negative charges of the phosphodiester backbone. Corollaries of this view are: the more fixed an RNA structure, the less likely such fortuitous interactions become; and the more fixed the peptide or other effector, the less likely it is to complex preferentially with the active RNA conformation.

Non-specific RNA binding proteins as RNA chaperones

It has been suggested that non-specific RNA binding proteins such as NC and hnRNP proteins act as RNA chaperones (Karpel *et al.*, 1974, 1982; Munroe and Dong, 1992; Pontius and Berg, 1992; Sundquist and Heaphy, 1993; Tsuchihashi

et al., 1993; Coetzee *et al.*, 1994; Z.Tsuchihashi and P.O.Brown, in preparation; Portman and Dreyfuss, 1994; see also Fang and Cech, 1993). The results reported herein support and extend this view.

NC and A1 CTD enhance hammerhead ribozyme activity by facilitating physical steps that otherwise present kinetic blocks to the catalytic cycle. These effectors 'coax' the ribozyme complexes through the catalytic cycle, lowering free energy barriers between steps so that the ribozyme does not get trapped in forms such as the ribozyme–product complexes; these complexes have high kinetic barriers to disassembly but are not the most thermodynamically stable species. The physical steps along the hammerhead ribozyme's catalytic cycle might then be viewed as intermolecular cases of kinetic traps. Non-specific RNA binding proteins, as well as specific RNA binding proteins, may act to prevent such kinetic traps in processes that involve RNA–RNA interactions such as spliceosomal assembly and pre-mRNA splicing.

The ribozyme reaction can also be considered an intermolecular model for RNA folding. Analogous to the kinetic traps throughout the ribozyme reaction, large RNAs are often isolated in alternative intramolecular conformations that are inactive. For example, several tRNAs must be heated or acid denatured and renatured in order to be amino acylated *in vitro* (Gartland and Sueoka, 1966; Lindahl *et al.*, 1966; Adams *et al.*, 1967; Ishida and Sueoka, 1968; see also Cole *et al.*, 1972; Walstrum and Uhlenbeck, 1990 and references therein). The misfolded tRNA can be refolded by treatment with UP1, the N-terminal fragment of the hnRNP A1 protein (Karpel *et al.*, 1974, 1982). Similarly, the results herein suggest that NC can resolve a misfolded hammerhead ribozyme complex that is otherwise long-lived. In addition, NC increases the specificity of oligonucleotide cleavage by HH16, allowing the ribozyme to distinguish better between substrates that form matched or mismatched duplexes with its recognition sequence (Tsuchihashi *et al.*, 1993). The increased specificity apparently arises because the NC protein helps the ribozyme sample different potential substrates prior to cleavage. Again, this provides an intermolecular model for RNA folding, suggesting that non-specific RNA binding proteins can help prevent misfolding by resolving misfolded species.

Because RNA misfolding is so common *in vitro*, it seems reasonable that misfolding is a problem that must also be addressed *in vivo*. Indeed, folding of RNA into kinetically trapped alternative conformations may be an inherent property of RNAs (D.Herschlag, in preparation). The ability of non-specific RNA binding proteins to aid RNA folding processes *in vitro* then suggests that these proteins may act as cellular RNA chaperones. The RNA chaperones could prevent misfolded RNAs from forming, analogous to the effect of chaperones in preventing aggregation during protein folding (Fischer and Schmid, 1990; Buchner *et al.*, 1991; Hardy and Randall, 1991). Alternatively, or in addition, the RNA chaperones could resolve RNAs that have misfolded, allowing RNAs that function via an intra- or intermolecular structure to refold into their active conformation (see Ellis, 1991) and RNAs that function without structure to be accessed by cellular components.

NC and A1 CTD, when present at intermediate concentrations, facilitate the physical steps of substrate association and product dissociation in the HH16 reaction while not affecting the functional step of chemical cleavage (Tsuchihashi *et al.*, 1993; data not shown). This is consistent with an RNA chaperone activity that is analogous to chaperones that aid in protein folding, as physical and folding steps but not functional steps are affected. However, unlike chaperones that act in protein folding and do not bind the folded protein, RNA chaperones may stay associated with the properly folded RNA after exerting their RNA chaperone activity due to the strength of non-specific RNA–protein interactions. Furthermore, a protein that acts as an RNA chaperone during folding may stay bound and serve additional specific roles after the RNA correctly folds.

Implications for ribozymes as therapeutics

There is currently interest in the use of ribozymes to target the destruction of specific RNAs *in vivo*. This approach has the potential to simplify drug design greatly as ribozymes can recognize specific RNA sequences by base pairing (Cech, 1988; Haseloff and Gerlach, 1988). Drug design based on base pairing would obviate the requirement to design an inhibitor of protein action for each protein target with unique steric and electrostatic properties. However, there are three types of potential problems associated with this approach: (i) mechanistic problems of turnover and specificity; (ii) problems of ribozyme access to the target RNA *in vivo*; and (iii) the typical problems of pharmaceuticals related to drug delivery, stability, resistance, side effects and cost. The results described herein impact on the first two problems, which are discussed further below.

(i) Turnover and specificity present two fundamental problems for ribozyme catalysis that have been observed *in vitro*. These problems arise because the sequence complexity of cellular RNA necessitates a ribozyme recognition sequence of ~15 nucleotides in length in order to form a perfect Watson–Crick duplex with a unique RNA target *in vivo*: the long duplexes between the ribozyme and the cleavage products are then slow to dissociate, limiting the maximal rate of turnover *in vitro*; in addition, binding to RNA substrates becomes so strong that even non-target RNAs are efficiently cleaved so that specificity is also compromised *in vitro* (Herschlag, 1991). As described previously, the strand annealing and strand dissociation activities of the NC protein from HIV-1 can at least partially overcome these problems (Tsuchihashi *et al.*, 1993): turnover can be enhanced by facilitating the release of the RNA products from the ribozyme; specificity can be enhanced by speeding duplex dissociation, which facilitates equilibration of RNA binding, thereby allowing the preferred binding of the correct RNA to be established prior to cleavage by the ribozyme (see Herschlag, 1991 for a more complete description). The results herein indicate that an effector derived from the hnRNP A1 protein can act analogously to NC. There are presumably other endogenous proteins that can similarly enhance ribozyme function *in vivo*. Recent results suggest that the S12 ribosomal protein from *E.coli* can enhance multiple turnover of HH16 (Coetzee *et al.*, 1994).

(ii) A second class of problems for ribozyme therapeutics arises as a result of the cellular milieu: the target RNA must be accessible to the ribozyme in order for the ribozyme to be effective. Our previous view was that RNA binding proteins might render RNA cleavage very difficult *in vivo* by binding and sequestering target RNAs. However, the

present work suggests a different and more optimistic view. Effectors derived from the nucleocapsid protein of HIV-1 and from the mammalian hnRNP A1 protein can actually enhance, rather than inhibit, hammerhead ribozyme catalysis. More generally, non-specific RNA binding proteins may allow RNAs to explore a variety of intra- and intermolecular interactions, including annealing with exogenously introduced ribozymes, which could then lead to cleavage of the target RNA. Thus, the ribozymes might co-opt the RNA chaperones to help provide access to the target RNA. An additional *in vivo* problem, the need to co-localize the ribozyme and target RNAs within the cell has recently been discussed and, at least in one instance, successfully addressed (Sullenger and Cech, 1993).

Materials and methods

Materials

The hammerhead ribozyme HH16 was synthesized by *in vitro* transcription with T7 RNA polymerase using a synthetic DNA template (Milligan and Uhlenbeck, 1989) and was a gift from K.Hertel. The oligonucleotide S was made by *in vitro* transcription or solid phase chemical synthesis and the oligonucleotide P2 was made by chemical synthesis and both were purified by denaturing polyacrylamide gel electrophoresis, as described previously (Hertel *et al.*, 1994). The non-specific single stranded DNA (ssDNA) was a 28mer: ATG CAC TGC TAG AGA TTT TCC ACA AGT C. Peptides were made by automated solid phase synthesis and were a gift from A.Frankel.

A 78 amino acid version of the NC protein was overexpressed as a Met-$(His)_6$ fusion protein and purified as described elsewhere (Tsuchihashi *et al.*, 1993; Z.Tsuchihashi and P.O.Brown, in preparation). NC was stored at −80°C in 50 mM Tris, pH 7.5, 0.1% Triton X-100, 50 mM NaCl, 100 mM imidazole, 10 mM β-mercaptoethanol and 20% glycerol. The storage buffer had no effect on any of the reactions followed herein (data not shown). The concentration of NC required for activation was very consistent within an experiment, but varied significantly between experiments. These observations suggest that there may be a loss of activity of NC during storage. A similar observation has been reported for the DNA strand annealing activity exhibited by a different NC preparation (Dib-Hajj *et al.*, 1993). Thus, all comparisons of concentration effects were performed in side-by-side reactions.

A1 CTD is a polymer containing six to eight repeats of two hnRNP A1 C-terminal consensus sequences: GNFGGGRGGNYGGSRG. The peptide was synthesized and polymerized via a Cys-S-CH_2 linkage between added $ClCH_2$COGlyGly N-terminal and CysSH C-terminal groups, as described previously (Casas-Finet *et al.*, 1993).

Oligonucleotides were 5′-end labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase, as described previously (Zaug *et al.*, 1988), and were purified by electrophoresis on non-denaturing 24% polyacrylamide gels. Note that 5′-end-labeled P2 (P2*) does not correspond exactly to the 3′ reaction product of HH16 cleavage as the actual product contains a 5′-hydroxyl group (Figure 1).

General kinetic procedures

Procedures were essentially as described previously (Tsuchihashi *et al.*, 1993; Hertel *et al.*, 1994) and are described briefly below. All reactions were carried out in 50 mM Tris, pH 7.5 and 10 mM $MgCl_2$ unless otherwise noted. Siliconized Eppendorf tubes were typically used to minimize sticking to tube walls. Reaction mixtures were typically 5−10 μl, and reactions were followed by removing ~1 μl aliquots at specified times and quenching further reaction by addition of ~4 μl of 20 mM EDTA in 90% formamide with 0.005% xylene cyanol, 0.01% bromophenol blue, and 1 mM Tris, pH 7.5. Labeled reaction products were separated from substrates by denaturing gel electrophoresis on 20% polyacrylamide−7 M urea gels, and their ratio at each time-point determined by quantification using a PhosphorImager (Molecular Dynamics). Observed first order rate constants were typically obtained by following reactions for $>2t_{1/2}$, with endpoints determined from the fraction of substrate reacting after $>5t_{1/2}$.

Multiple turnover reactions

Reactions with S in excess of HH16 were typically performed with 3 nM HH16 and 30 nM 5′-end-labeled S (S*). HH16 and S* were preannealed by heating together at 95°C for 2.5 min in the presence of 50 mM Tris, pH 7.5 and a trace amount of EDTA (<1 mM). The mixture was cooled to 25°C (≥5 min) protein, peptide or other effectors were added, and reaction was initiated by addition of $MgCl_2$ typically within ~1 min of effector addition.

Control reactions indicated that gene 32 protein and *E.coli* SSB could not substitute for NC or A1 CTD (Tsuchihashi *et al.*, 1993). Both proteins bind ssDNA under the conditions of the HH16 reaction as determined by fluorescence titration (data not shown; Lohman and Mascotti, 1992).

Single turnover reactions

Reactions with HH16 in excess of S* were performed without preannealing unless otherwise stated. HH16 and S* were separately heated to 95°C for 2.5 min in 50 mM Tris, pH 7.5, to disrupt structures that may have formed during storage. After the solutions were cooled at 25°C, $MgCl_2$ was added to both HH16 and S*, potential effector molecules were added to the HH16 solution, and the reaction was initiated ≤1 min later by addition of S*. At intermediate HH16 concentrations reactions did not follow clean first order kinetics; rather, there was an initial lag as expected for presteady reactions with two steps that occur at similar rates, in this case binding of S* to HH16 and cleavage of S* within the HH16−S* complex. Thus, some of the values of k_{obs} in Figure 5B represent approximations to first order behavior.

Determination of the rate of HH16 and S association by pulse−chase

The protocol for this experiment is outlined in Figure 6A. HH16 and S* were treated prior to reaction as described above for single turnover reactions. The association of S* (typically ~0.5 nM) with an excess of HH16 (2−10 nM) was initiated by adding S* to a solution of HH16 with or without added NC or other effector with a final reaction volume of 3 μl. After specified times, t_1, for binding, a chase containing a large excess of unlabeled S was added to prevent further binding of S* and to allow S* that had bound during t_1 to react and form P1*. The chase of 27 μl gave a 10-fold dilution of HH16 and S* to final concentrations of 0.2−1 nM and ~0.05 nM, respectively, much lower than the concentration of unlabeled S of 50 nM. After a time of t_2 = 10−15 min following the chase (Figure 6A), representing $>5t_{1/2}$ for the cleavage reaction, aliquots were removed and the fraction of S* converted to P1* determined.

Rate constant for dissociation of product oligonucleotides from HH16

Dissociation was followed using native gel electrophoresis to separate complexed from free product oligonucleotide subsequent to a chase with unlabeled oligonucleotide used to initiate dissociation (Hertel *et al.*, 1994). To follow dissociation of P1* from HH16, P1* bound to HH16 was generated *in situ* by cleavage of 5′-end-labeled S. In a typical experiment 120 nM HH16 and 20 nM S* were preannealed in 50 mM Tris, pH 7.5, at 95°C for 2.5 min. Formation of P1* was initiated by addition of an equivolume aliquot of 20 mM $MgCl_2$ (10 mM final concentration) in 50 mM Tris, pH 7.5, and the reaction was allowed to proceed for t_1 = 20 min ($>5t_{1/2}$). At the end of this time, at t_2 = 0, an aliquot from the reaction mixture was diluted 10-fold into a chase solution (50 mM Tris, pH 7.5, 10 mM $MgCl_2$) containing a large excess of unlabeled S (typically 75 nM) with or without NC, in order to determine whether NC facilitated product release. The dissociation of P1* was followed by removing aliquots (~2 μl) at specified times, adding 0.5 μl of native gel loading buffer (20% glycerol/0.002% xylene cyanol), and immediately loading the sample onto a running native 15% polyacrylamide gel with 10 mM $MgCl_2$ in the gel and running buffer (Pyle *et al.*, 1990; Fedor and Uhlenbeck, 1992; Hertel *et al.*, 1994). The gel was maintained at ~5°C to slow further dissociation. Examples of the separation obtained are shown in Figure 3. The dissociation of P2* from HH16 was followed according to an analogous protocol, except that 5′-end-labeled P2 was directly annealed with HH16, rather than being generated *in situ* as for P1*.

Acknowledgements

We greatly appreciate gifts of ribozyme from K.Hertel, peptides from A.Frankel and A1 protein from B.Pontius. We thank G.Dreyfuss for discussions during the course of this work and for sharing unpublished results and T.Cech and O.Uhlenbeck for comments on the manuscript. This work was supported by NIH grant GM49243 to D.H. and a Maryland Industrial Partnership Award (with Trevigen, Inc., #1202.12) to R.L.K. D.H. is a Lucille P.Markey Scholar in Biomedical Sciences and a Searle Scholar (Chicago Community Trust).

References

Adams,A., Lindahl,T. and Fresco,J.R. (1967) *Proc. Natl Acad. Sci. USA*, **57**, 1684–1691.

Alberts,B.M. and Frey,L. (1970) *Nature*, **227**, 1313–1318.

Altman,S. (1989) *Adv. Enzymol.*, **62**, 1–36.

Barat,C., Lullien,V., Schatz,O., Keith,G., Nugeyre,M.T., Gruninger-Leitch,F., Barre-Sinoussi,F., LeGrice,S.F. and Darlix,J.L. (1989) *EMBO J.*, **8**, 3279–3285.

Bennett,M., Pinol-Roma,S., Staknis,D., Dreyfuss,G. and Reed,R. (1992) *Mol. Cell. Biol.*, **12**, 3165–3175.

Buchner,J., Schmidt,M., Fuchs,M., Jaenicke,R., Rudolph,R., Schmid,F.X. and Kiefhaber,T. (1991) *Biochemistry*, **30**, 1586–1591.

Buvoli,M., Cobianchi,F., Biamonti,G. and Riva,S. (1990) *Nucleic Acids Res.*, **18**, 6595–6600.

Buvoli,M., Cobianchi,F. and Riva,S. (1992) *Nucleic Acids Res.*, **20**, 5017–5025.

Calnan,B.J., Tidor,B., Biancalana,S., Hudson,D. and Frankel,A.D. (1991) *Science*, **252**, 1167–1171.

Casas-Finet,J.R., Smith,J.D.,Jr, Kumar,A., Kim,J.G., Wilson,S.H. and Karpel,R.L. (1993) *J. Mol. Biol.*, **229**, 873–889.

Cech,T.R. (1988) *J. Am. Med. Assoc.*, **260**, 3030–3034.

Cobianchi,F., Karpel,R.L., Williams,K.R., Notario,V. and Wilson,S.H. (1988) *J. Biol. Chem.*, **263**, 1063–1071.

Cobianchi,F., Calvio,C., Stoppini,M., Buvoli,M. and Riva,S. (1993) *Nucleic Acids Res.*, **21**, 949–955.

Coetzee,T., Herschlag,D. and Belfort,M. (1994) *Genes Dev.*, in press.

Cole,P.E., Yang,S.K. and Crothers,D.M. (1972) *Biochemistry*, **11**, 4358–4368.

Connell,G.J., Illangesekare,M. and Yarus,M. (1993) *Biochemistry*, **32**, 5497–5502.

Davis,J., Scherer,M., Tsai,W.P. and Long,C. (1976) *J. Virol.*, **18**, 709–718.

DeRocquigny,H., Gabus,C., Vincent,A., Fournie-Zaluski,M.-C., Roques,B. and Darlix,J.L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 6472–6476.

Dib-Hajj,F., Khan,R. and Giedroc,D.P. (1993) *Protein Sci.*, **2**, 231–243.

Dreyfuss,G. (1986) *Annu. Rev. Cell. Biol.*, **2**, 459–498.

Dreyfuss,G., Matunis,M.J., Pinol-Roma,S. and Burd,C.G. (1993) *Annu. Rev. Biochem.*, **62**, 289–321.

Ellis,R.J. (1991) *Curr. Biol.*, **1**, 177–178.

Fang,G. and Cech,T.R. (1993) *Cell*, **74**, 875–885.

Fedor,M.J. and Uhlenbeck,O.C. (1992) *Biochemistry*, **31**, 12042–12054.

Fischer,G. and Schmid,F.X. (1990) *Biochemistry*, **29**, 2205–2212.

Frankel,A.D. (1994) In Nagai,K. and Mattaj,I.W. (eds), *RNA–Protein Interactions: Frontiers in Molecular Biology*. Oxford University Press, in press.

Gartland,W.J. and Sueoka,N. (1966) *Proc. Natl Acad. Sci. USA*, **55**, 948–956.

Goodchild,J. (1992) *Nucleic Acids Res.*, **20**, 4607–4612.

Guo,Q. and Lambowitz,A.M. (1992) *Genes Dev.*, **6**, 1357–1372.

Hardy,S.J.S. and Randall,L.L. (1991) *Science*, **251**, 439–443.

Haseloff,J. and Gerlach,W.L. (1988) *Nature*, **334**, 585–591.

Herschlag,D. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 6921–6925.

Hertel,K.J., Herschlag,D. and Uhlenbeck,O.C. (1994) *Biochemistry*, **33**, 3374–3385.

Ishida,T. and Sueoka,N. (1968) *J. Biol. Chem.*, **243**, 5329–5336.

Jencks,W.P. (1981) *Proc. Natl Acad. Sci. USA*, **78**, 4046–4050.

Karpel,R.L., Swistel,D.G., Miller,N.S., Geroch,M.E., Lu,C. and Fresco,J.R. (1974) *Brookhaven Symp. Biol.*, **26**, 165–174.

Karpel,R.L., Miller,N.S. and Fresco,J.R. (1982) *Biochemistry*, **21**, 2102–2108.

Karpel,R.L., Henderson,L.E. and Oroszlan,S. (1987) *J. Biol. Chem.*, **262**, 4961–4967.

Khan,R. and Giedroc,D.P. (1992) *J. Biol. Chem.*, **267**, 6689–6695.

Kumar,A. and Wilson,S.H. (1990) *Biochemistry*, **29**, 10717–10722.

Kumar,A., Casas-Finet,J.R., Lunneau,C.J., Karpel,R.L., Merrill,B.M., Williams,K.R. and Wilson,S.H. (1990) *J. Biol. Chem.*, **265**, 17094–17100.

Lambowitz,A.M. and Perlman,P.S. (1990) *Trends Biochem. Sci.*, **15**, 440–444.

Lapadat-Tapolsky,M., DeRocquigny,H., Roques,B., Plasterk,R. and Darlix,J.-L. (1993) *Nucleic Acids Res.*, **21**, 831–839.

Leis,J., McGinnis,J. and Green,R.W. (1978) *Virology*, **84**, 87–98.

Lindahl,T., Adams,A. and Fresco,J.R. (1966) *Proc. Natl Acad. Sci. USA*, **55**, 941–948.

Lohman,T.M. and Mascotti,D.P. (1992) *Methods Enzymol.*, **212**, 424–458.

Mayeda,A. and Krainer,A.R. (1992) *Cell*, **68**, 365–375.

McPheeters,D.S., Stormo,G.D. and Gold,L. (1988) *J. Mol. Biol.*, **201**, 517–535.

Meric,C., Darlix,J.-L. and Spahr,P.-F. (1984) *J. Mol. Biol.*, **173**, 531–538.

Milligan,J.F. and Uhlenbeck,O.C. (1989) *Methods Enzymol.*, **180**, 51–62.

Munroe,S.H. and Dong,X. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 895–899.

Nissen-Meyer,J. and Abraham,A.K. (1980) *J. Mol. Biol.*, **142**, 19–28.

Page,M.I. (1977) *Angew. Chem. (Engl. Ed.)*, **16**, 449–459.

Page,M.I. and Jencks,W.P. (1971) *Proc. Natl Acad. Sci. USA*, **68**, 1678–1683.

Pontius,B.W. (1993) *Trends Biochem. Sci.*, **18**, 181–186.

Pontius,B.W. and Berg,P. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8403–8407.

Pontius,B.W. and Berg,P. (1991) *Proc. Natl Acad. Sci. USA*, **88**, 8237–8241.

Pontius,B.W. and Berg,P. (1992) *J. Biol. Chem.*, **267**, 13815–13818.

Portman,D.S. and Dreyfuss,G. (1994) *EMBO J.*, **13**, 213–221.

Prats,A.C., Sarih,L., Gabus,C., Litvak,S., Keith,G. and Darlix,J.L. (1988) *EMBO J.*, **7**, 1777–1783.

Prats,A.-C., Housset,V., deBilly,G., Cornille,F., Prats,H., Roques,B. and Darlix,J.-L. (1991) *Nucleic Acids Res.*, **19**, 3533–3541.

Puglisi,J.D., Tan,R., Calnan,B.J., Frankel,A.D. and Williamson,J.R. (1992) *Science*, **257**, 76–80.

Pyle,A.M., McSwiggen,J.A. and Cech,T.R. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 8187–8191.

Reich,C., Olsen,G.J., Pace,B. and Pace,N.R. (1988) *Science*, **239**, 178–181.

Sigal,N., Delius,H., Kornberg,T., Gefter,M.L. and Alberts,B. (1972) *Proc. Natl Acad. Sci. USA*, **69**, 3537–3541.

Smith,B.J. and Bailey,J.M. (1979) *Nucleic Acids Res.*, **7**, 2055–2072.

Sullenger,B.A. and Cech,T.R. (1993) *Science*, **262**, 1566–1569.

Sundquist,W.I. and Heaphy,S. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 3393–3397.

Surovoy,A., Dannull,J., Moelling,K. and Jung,G. (1993) *J. Mol. Biol.*, **229**, 94–104.

Swanson,M.S. and Dreyfuss,G. (1988a) *Mol. Cell. Biol.*, **8**, 2237–2241.

Swanson,M.S. and Dreyfuss,G. (1988b) *EMBO J.*, **11**, 3519–3529.

Sykora,K.W. and Moelling,K. (1981) *J. Gen Virol.*, **55**, 379–381.

Symons,R.H. (1992) *Annu. Rev. Biochem.*, **61**, 641–671.

Tsuchihashi,Z., Khosla,M. and Herschlag,D. (1993) *Science*, **262**, 99–102.

Walstrum,S.A. and Uhlenbeck,O.C. (1990) *Biochemistry*, **29**, 10573–10576.

Weeks,K.M., Ampe,C., Schultz,S.C., Steitz,T.A. and Crothers,D.M. (1990) *Science*, **249**, 1281–1285.

Yarus,M. (1988) *Science*, **240**, 1751–1758.

Yarus,M. (1989) *Biochemistry*, **28**, 980–988.

You,J.C. and McHenry,C.S. (1993) *J. Biol. Chem.*, **268**, 16519–16527.

Zaug,A.J., Grosshans,C.A. and Cech,T.R. (1988) *Biochemistry*, **27**, 8924–8931.

Received on February 1, 1994