Facilitation of hammerhead ribozyme catalysis by the nucleocapsid protein of HIV-1 and the heterogeneous nuclear ribonucleoprotein A1

Edouard L. Bertrand¹ and John J. Rossi²

Beckman Research Institute of the City of Hope, Department of Molecular Genetics, 1500 Duarte Road, Duarte, CA 91010, USA

¹Present address: Institut J. Monod, Tour 43, 2 place Jussieu, 75251 Paris, France

²Corresponding author

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In order to improve the activity of hammerhead ribozymes *in vivo*, we have analyzed the effect of several prototypical RNA binding proteins on the ribozyme cleavage reaction: bacteriophage T4 gene 32 protein (gp32), hnRNP A1 (A1) and the nucleocapsid protein of HIV-1 (NCp7). We show that, while gp32 has no effect on the cleavage reaction, A1 and NCp7 affect different steps of the reaction. Moreover, some of these effects depend upon the ribozyme–substrate hybrid length. A1 and NCp7 inhibit the reaction of the least stable ribozyme–substrate complexes, which have 12 bp of duplex. NCp7, but not A1, inhibits the cleavage of substrates that have long ribozyme–substrate duplexes (17 or 20 bp), while cleavage of complexes having shorter duplexes (13 or 14 bp) is not affected. NCp7 and A1 enhance the turnover of ribozymes by increasing the rate of product dissociation, but only when both cleavage products are bound with ≤ 7 bp. A1 and NCp7 enhance ribozyme binding to long substrates, such as mRNAs, the structure of which otherwise limits ribozyme binding. Therefore, the effects of A1 or NCp7 on the different steps of the cleavage reaction define a length of the ribozyme–substrate duplex which allows enhancement of the rate of binding and product release without inhibiting the cleavage step. Interestingly, this duplex length (14 bases, or 7 on each side of the cleavage site) is identical for A1 and NCp7. Since A1 is thought to interact with most, if not all mRNAs *in vivo*, it may enhance the intracellular activity of ribozymes targeted against any mRNA. On the other hand, since retroviral nucleocapsid (NC) proteins interact only with the viral genomic RNA *in vivo*, these results raise the exciting possibility that NCp7 and other NC proteins may specifically enhance the activity of ribozymes targeted against their cognate retroviruses. Overexpression of A1 or NCp7, and targeting the ribozyme to a cellular compartment rich in A1 or NCp7 may also enhance ribozyme activity *in vivo*.

Key words: hammerhead ribozyme/hnRNP/nucleocapsid protein

Introduction

The hammerhead ribozyme motif (Forster and Symons, 1987) can be engineered to function as a sequence-specific

endoribonuclease (Uhlenbeck, 1987; Haseloff and Gerlach, 1988; Jeffries and Symons, 1989), enabling its use as a specific inhibitor of gene expression (Cameron and Jennings, 1989; Cotten and Birnstiel, 1989; Sarver *et al.*, 1990; Saxena and Ackerman, 1990). However, efficient inhibition often requires high ribozyme concentrations and a large excess of ribozyme over substrate (Cameron and Jennings, 1989; Cotten and Birnstiel, 1989; Saxena and Ackerman, 1990; Sioud and Drlica, 1991; L'Huillier *et al.*, 1992; Sioud *et al.*, 1992; Steinecke *et al.*, 1992). Moreover, the extent of inhibition seen with an inactive ribozyme mutant is often of the same order of magnitude as for the active version, due to antisense effects (Cotten *et al.*, 1989; Saxena and Ackerman, 1990; Scanlon *et al.*, 1991; Weerasinghe *et al.*, 1991; Dropulic *et al.*, 1992; Sioud *et al.*, 1992; Steinecke *et al.*, 1992; Zhao and Pick, 1993). Thus far, it appears that *in vivo* applications of ribozymes have not taken full advantage of their demonstrated *in vitro* potential.

A major difference between the *in vitro* and *in vivo* ribozyme reactions is that most or all RNAs are not freely diffusing in the cell, but normally exist as ribonucleoprotein (RNP) complexes [see Dreyfuss (1986) for a review]. It has recently been shown that many of the major proteins associated with RNAs *in vivo*, such as hnRNPs, splicing factors and translation factors, affect RNA folding by promoting annealing (Kumar and Wilson, 1990; Pontius and Berg, 1990; Lee *et al.*, 1993; Portman and Dreyfuss, 1994), unwinding (Rozen *et al.*, 1990; Wassarman and Steitz, 1991; Pontius and Berg, 1992; Casas-Finet *et al.*, 1993) and strand-exchange (Pontius and Berg, 1992; Casas-Finet *et al.*, 1993). Therefore, interactions between these proteins and either the ribozyme or its substrate may profoundly affect ribozyme activity. Ribozyme binding and cleavage could be inhibited by steric hindrance or via protein mediated unwinding. Proteins may also enhance ribozyme binding via annealing activities, and ribozyme turnover via strand-exchange activities. These considerations have prompted us to analyze the effect of several prototypical RNA binding proteins on the hammerhead cleavage reaction: T4 gene 32 (gp32), HIV nucleocapsid protein (NCp7) and hnRNP A1 (A1). These proteins bind RNA non-specifically and exhibit both annealing and unwinding activities (Karpel *et al.*, 1982; Kumar and Wilson, 1990; Pontius and Berg, 1990, 1992; Khan and Giedroc, 1992; Munroe and Dong, 1992).

T4 gp32 and HIV NCp7 bind cooperatively and non-specifically to nucleic acids, through stretches of basic residues (Khan and Giedroc, 1992; Rocquigny *et al.*, 1992; Casas-Finet and Karpel, 1993; Surovoy *et al.*, 1993). They bind preferentially and stably to single-stranded RNA, and this is thought to be responsible for their helix-destabilizing activity (Karpel *et al.*, 1982; Khan and Giedroc, 1992). Their unwinding activity can directly lead to RNA–RNA annealing, since denaturation of intramolecular structures promotes intermolecular renaturation (Karpel *et al.*, 1982).

Therefore, unlike A1 (see below), the annealing properties of gp32 and NCp7 are dependent upon the RNA structure, and require a stoichiometric amount of protein to completely unwind and coat the RNA prior to renaturation.

A1 contains three RNA binding domains: two ribonucleo-protein consensus sequences (RNP-CS: Kenan *et al.*, 1991) that are part of an N-terminal domain, and a C-terminal domain that contains repeats of the RGG sequence (RGG box: Cobianchi *et al.*, 1986; Kiledjian and Dreyfuss, 1992). A1 binds preferentially and non-specifically to single-stranded RNAs, and exhibits moderate cooperativity (Cobianchi *et al.*, 1988). The N-terminal domain of A1 accounts for the preferential binding to single-stranded RNAs and for the unwinding activity, while the RGG domain is responsible for the cooperative binding and for the annealing activity (Kumar and Wilson, 1990; Casas-Finet *et al.*, 1993). This annealing activity is very efficient (1000-fold enhancement: Kumar and Wilson, 1990; Casas-Finet *et al.*, 1993), and is thought to occur by a different mechanism than for gp32 and NCp7 (Pontius and Berg, 1990; Pontius, 1993), and involves protein-protein interactions which stabilize the transient state of the annealing reaction, allowing more RNA-RNA encounters to produce double-stranded RNA.

A1 shares a number of structural features with several other proteins. The RNP-CS domains and the RGG box are present in numerous RNA binding proteins (Kenan *et al.*, 1991; Kiledjian and Dreyfuss, 1992). In some cases, this structural similarity reflects similar biochemical activities (Portman and Dreyfuss, 1994). On the other hand, NCp7 shares structural and functional characteristics with NC proteins from other retroviruses (Covey, 1986; Rocquigny *et al.*, 1993). A detailed analysis of the effect of A1 and NCp7 on each step of the ribozyme reaction could therefore serve as a conceptual framework to evaluate the effect of other cellular and retroviral proteins on ribozyme activity.

We have used a previously characterized set of hammerhead ribozymes and substrates (Bertrand *et al.*, 1994) to analyze the effect of purified gp32, A1 and NCp7 on the cleavage reaction. All the substrates have the same target sequence, but differ in the length of the ribozyme-substrate duplex (from 11 to 20 bp). In agreement with the study of Tsuchihashi *et al.* (1993), we show that NCp7 enhances ribozyme binding and turnover. Moreover, we show that A1 is also able to enhance ribozyme binding and turnover, and that the effects of A1 and NCp7 are dependent upon the length of the ribozyme-substrate hybrid. These results suggest several principles for optimizing ribozyme activity in the presence of these proteins, and perhaps in an intracellular environment. They also provide a basis for the development of new strategies to suppress gene expression with *trans*-acting ribozymes.

Results

To evaluate the effect of gp32, NCp7 and A1 on the hammerhead reaction, we have performed cleavage reactions in the presence of the purified proteins. To mimic physiological conditions, the reactions were performed at 37°C in the presence of 140 mM NaCl (a parameter which critically influences the binding of these proteins to RNA: Nadler *et al.*, 1991).

Fig. 1. Binding of S14 by BSA, gp32, A1 and NCp7. S14 RNA (20 nM final concentration) was incubated with the indicated concentration of protein. Free RNA (F) is separated from the bound RNA (B) by native gel electrophoresis. Lane C is a control lane with S14 RNA alone.

RNA binding activity of gp32, A1 and NCp7

In all of the experiments described below, the purified proteins were present at a concentration of 50 ng/μl. Except for the experiment involving the *cis*-acting ribozyme, the concentration of RNA was adjusted to maintain an excess of protein over available binding sites on RNA between 12- and 28-fold for gp32 and A1, and between 28- and 115-fold for NCp7. A gel-shift assay, performed under conditions identical to the cleavage reactions (identical protein and RNA concentration, identical buffer and incubation temperature), demonstrated that binding of gp32, NCp7 and A1 completely shifts the substrate RNA present in the cleavage reaction

Substrates:		Length of hybrid formed with:	
		R3	R3b
S20	5' GGG--30 bases--UGUAGAGGA <u>AAGUAGUA</u> <u>AGAGUGUGGG</u> CUGCA 3'	20	14
S17	5' GGG--30 bases--UGUAGAUG <u>AAGUAGUA</u> <u>AGAGUGUGG</u> UCUGCA 3'	17	14
Pit1	5' GGG--770 bases--AUAAAA <u>AAGUAGUA</u> <u>AGAGUGUGGU</u> -150 bases- 3'	17	14
S14	5' GGG--30 bases--UGUAGAUUA <u>AAGUAGUA</u> <u>AGAGUGUC</u> CUGCA 3'	14	20
S13	5' GGG---30 bases----UGUA <u>GAGGUAGUA</u> <u>AGAGU</u> UCUGCA 3'	13	12
S12	5' GGG---30 bases --UGUAGACC <u>AGUAGUA</u> <u>AGAGUG</u> CCUGCA 3'	12	12
S11	5' GGG---30 bases---UGUAGACC <u>AGUAGUA</u> <u>AGAGU</u> CCUGCA 3'	11	11

Ribozymes:

Fig. 2. Schematic representation of the ribozymes and substrates used in this study. The ribozyme cleavage site in the RNAs is indicated by an arrow. The base-pairing between the substrates and R3 is underlined in the substrate sequence, while the base-pairing between the substrates and R3b is overlined. The 30 base leader sequence is identical for all the RNAs except CisR3–15, and derives from the polylinker region of Bluescript KS+.

(Figure 1). This shows that every RNA molecule is bound by at least one molecule of protein. The negative control protein, bovine serum albumin (BSA), did not interact with the RNA (Figure 1). Moreover, different complexes increasing in molecular weight can be observed with increasing concentrations of A1 and NCp7, suggesting that one RNA molecule can be bound by several protein molecules. Interestingly, the four different complexes observed with A1 correlate with the four potential A1 binding sites present on the RNA molecule (the RNA is 60 bases long and one molecule of A1 covers ~15 bases of RNA; Cobiainchi *et al.*, 1988; Nadler *et al.*, 1991), suggesting that the four different complexes correspond to one, two, three and four molecules of A1 bound per RNA. Under our cleavage reaction conditions, the occupancy of the binding site is ~40% for A1, and between 50 and 100% for NCp7.

gp32, A1 and NCp7 have no effect on the cleavage rate of CisR3–15

To eliminate the variables of binding and product release in the ribozyme cleavage reaction, we used a *cis*-cleaving

ribozyme (CisR3–15; Figure 2). *Cis*-acting ribozymes self-cleave during transcription, and the ratio of full-length ribozyme transcript (L) over full-length ribozyme transcript plus cleaved ribozyme products (L + S) is solely dependent on the rate of the cleavage step (k), provided that the transcription rate remains constant [D.Long and O.Uhlenbeck, personal communication; $L/(L + S) = (1 - e^{-kt})/kt$]. Therefore, a simple time course of transcription allows measurement of the rate of the cleavage step. The rate of the chemical cleavage step as measured by this method, or determined from Michaelis–Menten kinetics, has been shown to be very similar over a wide range of values (from 1 to 0.1 min^{-1}), demonstrating the reliability of this method (D.Long and O.Uhlenbeck, personal communication). Another advantage of this procedure is that the protein:RNA ratio is very high at the beginning of the reaction, and then decreases continuously. Thus, if these proteins require a very high protein:RNA ratio to inhibit the cleavage step, or if a narrow range of protein concentration is required to enhance it, we should be able to detect such effects with this simple assay. Varying with

Fig. 3. Cleavage rate of the *cis*-acting ribozyme CisR3-15 in the presence of BSA, gp32, A1 or NCp7. (A) Time course of transcription of pCisR3-15 in the presence of BSA. The uncleaved CisR3-15 (U) and cleaved product (C) are indicated. (B) The ratio of uncleaved CisR3-15 (L) over cleaved plus uncleaved CisR3-15 (L + S) is plotted against transcription time (*t*). The theoretical curve of a *cis*-acting ribozyme with a cleavage rate *k* of 0.13 min⁻¹ is shown. Filled squares: BSA; open squares: gp32; filled circles: A1; open circles: NCp7.

the amount of RNA synthesized during transcription, the excess of protein over available binding sites on RNA molecules is between 20- and 0.5-fold for A1 and gp32, and between 80- and 2-fold for NCp7. The results obtained with CisR3-15 in the presence of BSA fit well with a theoretical curve of a cleavage rate constant of 0.13 min⁻¹. More importantly, no significant differences between BSA, gp32, NCp7 and A1 were observed (Figure 3). We conclude that 50 ng/μl of BSA, gp32, A1 and NCp7 do not influence the rate of the chemical cleavage of the CisR3-15 ribozyme.

The effect of A1 and NCp7 on single turnover reactions depends on the enzyme-substrate hybrid length

In order to analyze the effect of gp32, A1 and NCp7 on the binding and on the cleavage step as a function of the enzyme-substrate hybrid length, single turnover reactions with a *trans*-acting version of CisR3-15, R3, and a set of substrates, S11, S12, S13, S14 and S17, were conducted. These substrates have identical sequences, except for a few nucleotides flanking the pairing region, and are depicted in Figure 2. Substrates S11, S12, S13, S14 and S17 hybridize to R3 with 11, 12, 13, 14 and 17 bases, respectively. The R3-substrate hybrids have the following distribution of

Table I. Single turnover k_{cat}/K_m values^a of different substrates of R3

	BSA	gp32	A1	NCp7
S11	0.11 ± 0.02	0.15 ± 0.01	n.d. ^b	n.d. ^b
S12	1.5 ± 0.3	1.4 ± 0.25	0.8 ± 0.24	0.8 ± 0.32
S13	1.9 ± 0.28	1.9 ± 0.25	1.5 ± 0.29	2.1 ± 0.40
S14	1.5 ± 0.13	1.6 ± 0.40	1.6 ± 0.17	2.0 ± 0.20
S17	1.1 ± 0.10	1.2 ± 0.30	1.1 ± 0.20	(-) ^c

^aExpressed in 10⁻³ nM⁻¹min⁻¹.

^bNot determined.

^cThe reaction was inhibited by NCp7.

complementary pairings: 5+6 for S11, 6+6 for S12, 8+5 for S13, 7+7 for S14 and 8+9 for S17. To detect any effects of gp32, A1 or NCp7 on the binding or the cleavage step, we determined the k_{cat}/K_m for all the R3-substrate combinations under single turnover conditions. These results are presented in Table I. For the substrates S12, S13 and S14, the k_{cat}/K_m values are similar for each of the tested proteins, although a slight inhibition (1.9-fold) is observed with S12 in the presence of A1 and NCp7. Such similar k_{cat}/K_m values could reflect identity in both the k_{cat} and K_m values, or could be fortuitous, for example if a decrease in k_{cat} is compensated for by an increase in K_m . Since CisR3-15 and R3-S14 form identical base-pairings, the absence of any effect of gp32, A1 or NCp7 on the cleavage rate of CisR3-15 under identical concentrations of protein and RNA (see above) strongly suggests that the k_{cat} of the R3-S14 combination is not changed by the presence of these proteins. Therefore, nearly identical k_{cat}/K_m values reflect identity in both the k_{cat} and K_m values. Thus, gp32, A1 and NCp7 have no effect on single turnover reactions involving S13 and S14.

For the S17 RNA, the k_{cat}/K_m value is unchanged in the presence of BSA, gp32 or A1, but the reaction is strongly inhibited by the presence of NCp7 (~5-fold). This inhibition could be due to a particular feature of the S17 substrate, or could be related to the length of the ribozyme-substrate duplexed region. To discriminate between these possibilities, we constructed another substrate, S20, that hybridizes to R3 with 20 bases, as well as another ribozyme, R3b, that hybridizes to S20 with 14 bases, and to S14 with 20 bases (Figure 2). Thus, if the inhibition is due to the longer hybrid length and not to a particular substrate or ribozyme, NCp7 should inhibit the R3-S20 and R3b-S14 combinations, but not R3-S14 and R3b-S20. Indeed, combinations that provide 14 bases of hybrid length (S14-R3 and S20-R3b) are not inhibited by the presence of BSA, gp32, A1 or NCp7 (Table II). The S14-R3 combination has a cleavage rate of 0.05 min⁻¹ in the presence of BSA, gp32, A1 or NCp7, while the combination S20-R3b gives a rate of 0.019 min⁻¹ in the presence of BSA, gp32 or NCp7, but appears to be slightly stimulated by A1 (1.5-fold). In contrast, the combinations that provide 17 or 20 bases of hybrid length are cleaved less efficiently in the presence of NCp7 than in the presence of BSA, gp32 or A1 (Table II). The S14-R3b combination gives a cleavage rate of 0.025 min⁻¹ with BSA, gp32 and A1 but only 0.005 min⁻¹ with NCp7, while the S20-R3 combination gives a rate of 0.012 min⁻¹ with BSA, gp32 and A1 but only 0.002 min⁻¹ with NCp7. Moreover, the combination S17-R3 (17 bases of hybrid) is also cleaved ~5-fold more slowly in the presence of NCp7

Table II. Effect of the length of the ribozyme–substrate duplex on single turnover cleavage rate^a

Enzyme–substrate combination	Duplex length (bp)	k_{obs}			
		BSA	gp32	A1	NCp7
R3-S14	14	50 ± 6	51 ± 6	57 ± 7	55 ± 5
R3-S17	17	34 ± 4	28 ± 3	28 ± 3	6.0 ± 2
R3-S20	20	11 ± 2	15 ± 3	11 ± 2	2.4 ± 1
R3b-S14	20	30 ± 4	25 ± 3	23 ± 3	5.2 ± 2
R3b-S20	14	21 ± 3	18 ± 2	30 ± 4	16 ± 3

^aMeasured at 20 nM ribozyme and 2 nM substrate in the presence of 50 ng/μl of the indicated protein. The observed cleavage rate (k_{obs}) is given in 10^{-3} min^{-1} .

than in the presence of BSA, gp32 or A1. Thus, it appears that NCp7 inhibits association and/or cleavage of the ribozyme–substrate combinations which have a hybrid length greater than or equal to 17 base pairs.

A1 and NCp7 can enhance the ribozyme–substrate binding step

To examine the effect of gp32, A1, and NCp7 on the ribozyme–substrate annealing step, we analyzed the cleavage of Pit1 mRNA (Bodner *et al.*, 1988; Ingraham *et al.*, 1988) by R3b, which hybridizes to Pit1 mRNA with 14 bases (Figure 2). We have previously shown that Pit1 mRNA (950 bases long) is cleaved 10-fold less efficiently than S17 (60 bases long), despite an identical pairing with the ribozyme R3 (Bertrand *et al.*, 1994). We attributed this lower efficiency to an inhibition of ribozyme binding by intramolecular folding of the mRNA, which is due to its greater length. Single turnover reactions, performed under non-saturating concentrations of ribozyme, show that A1 and Ncp7 enhance the rate of cleavage of Pit1 mRNA ~4-fold (Figure 4). Since Pit1-R3b and R3-S14 form exactly the same complexes, and since A1 and NCp7 do not change the rate of the cleavage step of the complex S14-R3 (see above), we conclude that A1 and NCp7, but not gp32, are able to enhance binding of R3b to the long Pit1 RNA substrate.

The effect of A1 and NCp7 on multiple turnover reactions depends on the length of enzyme–cleavage product hybrids

To analyze the effects of gp32, A1 and NCp7 on the product dissociation step, we performed multiple turnover reactions with the R3-S12, R3-S13, R3-S14 and R3-S17 combinations. The steady-state velocities (determined using a 10-fold excess of substrate) were compared with the initial velocities of single turnover reactions containing a 10-fold excess of ribozyme. Since the total concentration of ribozyme plus substrate was identical in both cases, any differences between the single and multiple turnover velocities can be attributed to the product dissociation step. As shown in Table III, the product dissociation step is rate-limiting for S12, S13, S14 and S17 in the presence of either BSA or gp32. The multiple turnover rate is 3–4 times lower than the single turnover rate. Addition of A1 or NCp7 enhanced the turnover rate of S12 and S14 by 3- to 4-fold (Table III), making the velocities of multiple and single turnover conditions virtually identical. In this case, the product dissociation step is no longer rate-limiting. NCp7 also enhanced the turnover rate of S13 by 2-fold, but the velocity was still 2-fold lower than under single turnover conditions, showing that the product

Fig. 4. Effect of BSA, gp32, A1 and NCp7 on the single turnover cleavage rate of a long RNA (Pit1 mRNA). Cleavage reactions containing 50 ng/μl of the indicated protein, 20 nM R3b and 0.2 nM Pit1 were incubated for 45 min at 37°C. The 950 base long Pit1 mRNA (S) and the 850 base long cleavage product (P) are indicated. Lane C is a control lane with Pit1 mRNA alone.

dissociation step is rate-limiting with S13. A1 did not enhance the cleavage rate of S13 or S17 under multiple turnover conditions.

The product dissociation rate is limited by the dissociation rate of the most stably bound cleavage product. These are duplexed with the ribozyme with 6, 7, 8 and 9 bases respectively, for S12, S14, S13 and S17. Since A1 enhanced turnover of S12 and S14, but not of S13 and S17, we conclude that A1 promotes dissociation of stems shorter than or equal to 7 bp under our reaction conditions. Similarly, we conclude that NCp7 is able to efficiently dissociate stems shorter than or equal to 7 bases, and to improve slightly the dissociation of the 8 bp long stem.

Discussion

We have analyzed the effects of several prototypical RNA binding proteins (gp32, A1 and NCp7) on the hammerhead cleavage reaction. We found that A1 and NCp7 are able to increase binding, specificity and turnover of certain ribozymes, without inhibiting cleavage. Moreover, we showed that the effect of these proteins on each step of the cleavage reaction depends upon the length of the ribozyme–substrate duplexed region, and that the combination of these effects defines an optimal length for the base-pairing arms. These results have implications both for gene suppression studies involving hammerhead ribozymes and for A1 function *in vivo*.

Effects of A1 and NCp7 on the cleavage step

A self-cleaving ribozyme was used to analyze the effects of gp32, A1 and NCp7 on the chemical cleavage step. An advantage of the self-cleaving ribozyme is that it allowed us to test, in a straightforward manner, a continuous range

Table III. Ratio of single versus multiple turnover velocity^a

	Longest hybrid R3/product	BSA	gp32	A1	NCp7
S12	6 bases	2.9 ± 0.3 (0.22)	3.4 ± 0.1 (0.24)	1.2 ± 0.1 (0.15)	1.2 ± 0.1 (0.15)
S14	7 bases	2.8 ± 0.2 (0.20)	4.3 ± 0.6 (0.24)	1.0 ± 0.1 (0.27)	0.9 ± 0.1 (0.26)
S13	8 bases	3.2 ± 0.3 (0.20)	4.1 ± 0.2 (0.24)	3.7 ± 0.4 (0.23)	1.8 ± 0.2 (0.25)
S17	9 bases	3.0 ± 0.3 (0.12)	4.5 ± 0.6 (0.9)	3.5 ± 0.2 (0.9)	n.d. ^b

^aFor single turnover reactions, the initial velocity was measured at 20 nM R3 and 2 nM substrate; for multiple turnover reactions the steady-state velocity was measured at 2 nM R3 and 20 nM substrate. The number given in parenthesis is the initial velocity of single turnover reactions in $\text{nM}^{-1} \text{min}^{-1}$.

^bNot determined because the reaction was inhibited by NCp7.

of protein:RNA ratios. Although the protein concentration used was high enough to bind all the RNA present in the reaction (Figure 1), gp32, A1 and NCp7 did not inhibit or enhance the rate of the cleavage step of this ribozyme (Figure 3). We next analyzed the effect of the same proteins on *trans*-cleavage reactions. We used one ribozyme (R3) and a set of substrates (S11, S12, S13, S14 and S17) that hybridize to the ribozyme with a total length of 11, 12, 13, 14 and 17 bases, respectively (Figure 2). It is important to note that the R3-S14 combination forms a hammerhead complex that has exactly the same ribozyme-substrate pairing as the *cis*-acting ribozyme. The $k_{\text{cat}}/K_{\text{m}}$ values determined under single turnover conditions confirmed the results obtained with the *cis*-acting ribozyme: ribozyme-substrate combinations having a hybrid length of 13 or 14 bases have similar values in the presence of BSA, gp32, A1 or NCp7. Moreover, increasing A1 concentrations up to 300 ng/ μl (10 μM), which is high enough to completely saturate the RNA (Figure 1), did not inhibit the reactions with S13 and S14 (data not shown). Therefore, although gp32, A1 and NCp7 interact strongly with the ribozyme or the substrate under these reaction conditions, they do not alter the structure of the catalytic center. This could be due to the failure of these proteins to bind to the catalytic center formed by the ribozyme-substrate hybrid. The structure of the catalytic center is unknown, but many of the bases not involved in secondary base-pairing interactions might be involved in tertiary interactions (for reviews, see Castanotto *et al.*, 1992; Symons, 1992). Since each of these proteins has a higher affinity for single-stranded than for double-stranded RNA (Karpel, 1990; Casas-Finet *et al.*, 1993; Surovoy *et al.*, 1993), the ordered structure of the catalytic center may prevent binding of these proteins. An alternative possibility is that these proteins do interact with the catalytic domain, but their binding does not inhibit formation of a catalytically active structure.

Cleavage activity of the ribozyme-substrate combination with a hybrid length of 12 bp is slightly inhibited by A1 and NCp7. Although we have not determined whether this inhibition is due to an impairment in the binding or in the cleavage step, it is probably related to the fact that this is the least stable combination which supports optimal cleavage activity, since more stable combinations are not inhibited. This effect may be related to the cleavage inhibition elicited by NCp7 on a mismatched ribozyme-substrate complex,

which has a sub-optimal stability for ribozyme activity (Tsuchihashi *et al.*, 1993).

The ribozyme-substrate combinations that have a duplex length of 17 or 20 bases are inhibited 5-fold by NCp7. We have shown that this is not related to a particular substrate or ribozyme, but is dependent solely upon the hybrid length. Since NCp7 does not inhibit binding of oligonucleotides hybridizing with a similar length (Prats *et al.*, 1988), this inhibition likely involves the cleavage step, rather than the binding step.

Effect of A1 and NCp7 on the binding step

It has been previously shown that ribozymes cleave longer substrate RNAs less efficiently than shorter ones, because of an impairment in the ribozyme binding step caused by the structure of the long RNAs (Heidenreich and Eckstein, 1992; Bertrand *et al.*, 1994). In the case of Pit1 mRNA (950 bases long) and S17 (60 bases long), the difference in cleavage efficiency ($k_{\text{cat}}/K_{\text{m}}$) is 10-fold. A1 and NCp7, but not gp32, are able to relieve much of this inhibition, and increase by 5-fold the cleavage rate of Pit1 mRNA. This shows that A1 and NCp7 are able to facilitate the annealing of ribozymes to a longer substrate. Two different mechanisms have been formulated to explain the interstrand annealing activity of NCp7 and A1. An unfolding of the long substrate by NCp7 would increase the accessibility of the target sequence (Khan and Giedroc, 1992), or A1 could stabilize the transient state of the annealing reaction via A1-A1 interactions (close encounter mechanism: Pontius, 1993). We believe that the effect we observe on the ribozyme binding step is due to an unfolding of the longer substrate, with both A1 and NCp7. Indeed, the amplitude of the facilitation (5-fold) corresponds more closely to an unfolding effect than a close encounter effect, which typically is in the range of 100- to 1000-fold (Pontius, 1993). Moreover, this effect is seen with the long substrate and not with the shorter one, a result best explained by the unfolding of the substrate. The fact that A1 can facilitate annealing by an unfolding mechanism is not surprising, since it has been shown that A1 has helix destabilizing properties (Pontius and Berg, 1992). The absence of effect of gp32 might be explained by a weaker unwinding activity. Indeed, it has been shown that, in contrast to A1 and NCp7, gp32 does not lower the melting temperature of natural double-stranded DNA (Karpel, 1990), and that gp32 is unable to unwind tRNA

under conditions where NCp7 does (Khan and Giedroc, 1992).

Effect of A1 and NCp7 on the product dissociation step

We have performed multiple and single turnover reactions where the concentrations of ribozyme and substrate were inverted. The steady-state velocity, measured under multiple turnover conditions, was compared with the initial velocity as measured under single turnover conditions. A multiple turnover velocity lower than a single turnover velocity indicates that the product dissociation step is rate-limiting. Under our reaction conditions, the product dissociation step is rate-limiting in the presence of BSA or gp32, even when the ribozyme–substrate duplex length is only 12 bp long (the shortest length that supports ribozyme activity under our conditions: Table I). However, A1 and NCp7 are able to enhance the turnover rate, so that the product dissociation step is no longer rate-limiting. Since a step other than the product release step becomes rate-limiting in the presence of the A1 or NCp7, the enhancement in the rate of product release is probably higher than the observed enhancement in turnover rate (4-fold).

More importantly, we have also shown that this enhancement is dependent upon the length of the longest ribozyme–cleavage product hybrid. A1 enhances the turnover rate so that the product release step is no longer rate-limiting when the ribozyme–cleavage product hybrid length is 6 or 7 bases. If one of the cleavage products bound to the ribozyme has ≥ 8 bases, A1 does not enhance turnover of the ribozyme. Similarly, in the presence of NCp7, the product release step is accelerated and is not rate-limiting when the ribozyme–cleavage product duplex length is 6 or 7 bases, but is rate-limiting when it is 8 bases (although an enhancement in the turnover rate can still be observed). These results suggest that at 37°C and under physiological salt conditions, NCp7 and A1 are able to melt helices rapidly, but only if they are ≤ 7 bp. However, this limitation in helix length is likely to be dependent upon the helix stability rather than on its length *per se*, since it has been shown that A1 is able to melt longer duplexes at higher temperatures (Pontius and Berg, 1992). Thus, according to the sequence of the ribozyme–substrate complexes we have used, A1 and NCp7 should be able to rapidly melt helices that have a free energy of binding (ΔG°) of ≥ -12 kcal/mol, but should be unable to melt those that have a ΔG° of ≤ -13.5 kcal/mol.

This limit of -12 kcal/mol in the stability of helices unwound by A1 appears to be the lowest possible, since increasing A1 concentration does not allow A1 to unwind more stable stems. Indeed, the highest concentration of A1 we have tested (300 ng/ μ l, or 10 μ M) does not promote dissociation of the product bound with a ΔG° of ≤ -13.5 kcal/mol, although this concentration is 6-fold higher than the one required to produce the maximal effect on the release of the product bound with a ΔG° of ≥ -12 kcal/mol (data not shown). In contrast, the rate of product dissociation appears to be dependent upon the protein concentration for helices that have a $\Delta G^\circ -12$ kcal/mol. Indeed, a significant effect of A1 on ribozyme turnover requires a concentration of A1 of 20 ng/ μ l (0.6 μ M). Above this concentration, the ribozyme turnover rate increases linearly with A1

concentration and becomes saturated at a concentration of A1 of 50 ng/ μ l, or 1.7 μ M (data not shown).

Several lines of evidence have implicated A1 in pre-mRNA splicing: A1 influences both 5' splice site selection and exon skipping *in vitro* (Mayeda and Krainer, 1992; Mayeda *et al.*, 1993), and the interaction of A1 with pre-mRNA is influenced by both U1 and U2 snRNPs (Mayrand and Pederson, 1990; Buvoli *et al.*, 1992). Therefore, it has been suggested that the RNA annealing activity of A1 may promote base pairing of U1 and/or U2 snRNP to the 5' splice site and to the branch site, respectively (Buvoli *et al.*, 1992). Our finding that A1 unwinds helices that have a stability below a threshold value, adds another potential role to this function of A1. Indeed, the base pairing between U1 snRNP and pre-mRNAs is within the stability range for the unwinding activity of A1 (usually between -10 and -14 kcal/mol for U1 snRNP). Therefore, if U1 snRNP binds to a poorly matched sequence in the pre-mRNA, A1 would promote its rapid dissociation, preventing spliceosome assembly. On the other hand, if U1 snRNP binds to a site that provides a high binding stability, either because of good sequence complementarity or because the interaction with other splicing factors also bound to the pre-mRNA is providing additional stabilization, A1 would not be able to promote its dissociation. In this case, spliceosome assembly would proceed further. Such a mechanism might increase the discrimination of U1 snRNP between authentic and cryptic splice sites, but it may also account for the observation that A1 can regulate 5' splice site selection *in vitro* in a concentration-dependent manner (Mayeda and Krainer, 1992). Since the rate of unwinding increases with A1 concentration, high A1 concentrations would prohibit stable binding of U1 snRNP to the weak splice donor site, but not to the strong one, while low A1 concentrations would allow stable binding of U1 snRNP to both splice sites. In support of this possibility, it has been shown that high concentrations of A1 can inhibit U1 snRNP binding to 5' splice sites (Buvoli *et al.*, 1992), and that SC35 and SF2, splicing factors that counteract the effect of A1 on 5' splice site selection (Fu *et al.*, 1992), stabilize U1 snRNP binding to the pre-mRNA by promoting interactions between U1 snRNP and the pyrimidine tract (Fu and Maniatis, 1992; Wu and Maniatis, 1993).

Implications for the use of trans-acting ribozymes *in vivo*

A recent report (Tsuchihashi *et al.*, 1993) demonstrated that NCp7 enhanced a hammerhead ribozyme reaction in a fashion similar to what we have observed for both A1 and NCp7. We both demonstrated specific enhancement in the binding step. However, we observed this effect only when binding is hindered by the length of the RNA substrate, suggesting that this effect is most likely due to the unfolding of the substrate. In concurrence with the previous report, we demonstrate that NCp7 inhibits the reaction of sub-stable ribozyme–substrate complexes [12 bp of hybrid in this study and 13 bp, including one mismatch, in Tsuchihashi *et al.* (1993)], therefore enhancing ribozyme specificity. In both studies, the cleavage step was either unaffected or inhibited by NCp7, depending upon the reaction conditions. However, the inhibition did occur under different conditions: a long ribozyme–substrate hybrid length of 17 and 20 bp (this study) versus a high NCp7 concentration (Tsuchihashi *et al.*,

1993). These results are not contradictory, but suggest that long ribozyme–substrate hybrids are more sensitive to the inhibition of cleavage produced by high NCp7 concentration than short ones. Finally, we observed an enhancement of the product dissociation step, in agreement with the previous study, such that this step is no longer rate-limiting under multiple turnover conditions. However, we showed that this effect is dependent on the length of the ribozyme–substrate arms, longer arms (≥ 8 bases) being poorly dissociated by NCp7. Therefore, the combination of the effects of NCp7 on each step of the cleavage reaction allows us to define an optimal ribozyme arm length of 7 bases on each side of the catalytic domain. We also showed that A1 has similar effects on the hammerhead reaction and that these effects are also dependent upon the length of hybrid arms. In fact, despite different primary structure and RNA binding domains, the effects of A1 and NCp7 are similar except for: (i) A1, in contrast to NCp7, does not inhibit cleavage of ribozyme–substrate complexes that have long hybrid arms; (ii) A1 does not promote turnover of the ribozyme that binds to its cleavage product with 8 bp, while NCp7 can promote some turnover with this combination.

These results raise the possibility that other proteins with similar biochemical activities (i.e. non-sequence-specific annealing and unwinding activities) may exert similar effects on the hammerhead reaction, including enhancement of ribozyme binding, specificity and turnover. Interestingly, A1 and NCp7 require the same length of ribozyme–substrate duplex for optimal facilitation.

A1 and NCp7 could potentially enhance ribozyme activity *in vivo*. Indeed, A1 is one of the most abundant proteins involved in RNA processing, and is thought to bind to most, if not all, mRNAs *in vivo*. A1 is involved in both splicing (Mayeda and Krainer, 1992; Mayeda *et al.*, 1993) and nucleo-cytoplasmic transport of mRNAs (Pinol-Roma and Dreyfuss, 1992). Moreover, the concentration of A1 in the cell nucleus (10 μM in a nuclear extract; Mayeda *et al.*, 1993) is higher than the concentration that produced an effect on ribozyme cleavage reactions *in vitro* (0.6 μM). Therefore, A1 is likely to interact with both the ribozyme and its mRNA substrate *in vivo*, and could enhance both ribozyme binding and turnover. In contrast, the NC proteins are thought to interact only with the genomic viral RNA *in vivo*. NC zinc fingers specifically recognize high affinity binding sites within the *psi* region of the viral RNA (Darlix *et al.*, 1990; Berkowitz *et al.*, 1993; Dorfman *et al.*, 1993; Sakaguchi *et al.*, 1993), coat the viral RNA and promote annealing of the tRNA primer to the primer binding site (Prats *et al.*, 1988; Darlix *et al.*, 1990). Therefore, NC proteins might specifically enhance the activity of ribozymes targeted against the genomic retroviral RNA. In other words, retroviruses might promote their own destruction via a *trans*-acting ribozyme. However, the ribozyme–substrate hybrid should have a free energy of binding of ≥ -12 kcal/mol on each side of the cleavage site.

New strategies might be devised which take advantage of the activity of these proteins. For example, it is possible to target the ribozyme to compartments containing high protein concentrations, such as the nucleus for A1, and the viral capsid for NCp7. It might also be advantageous to link the ribozyme and the target to a high-affinity binding site for A1 or NCp7, so that the interaction between the protein and ribozyme or the target will be greatly facilitated *in vivo*.

Interestingly, it has recently been shown that linkage of the ribozyme and the target to the packaging signal of the Moloney murine leukemia virus, which both have a high affinity binding site for the NC protein (Dupraz and Sphar, 1992; Berkowitz *et al.*, 1993) and localize the RNA to the viral capsid, significantly increased ribozyme activity *in vivo* (Sullenger and Cech, 1993). Alternatively, these proteins could be overexpressed *in vivo*. We are currently investigating such an approach for NCp7.

Materials and methods

Plasmid constructions and RNA synthesis

pS11, pS12, pS13, pS14, pS17, pPit and pR3 have been previously described (Bertrand *et al.*, 1994). pS20, pR3b and pCisR3–15 (Figure 2) were constructed by cloning synthetic oligonucleotides into the polylinkers of Bluescript KS+ (pS20 and pR3b) or pGem 9z– (pCisR3–15).

The sequences of the RNAs transcribed from these plasmids linearized by digestion with *Pst*I, except pPit which was digested with *Kpn*I, are shown in Figure 2. Transcription reactions were performed as previously described (Bertrand *et al.*, 1994), with the following modifications. [^{32}P]UTP was the radioactive nucleotide, and the concentration of cold UTP was 100 μM for the plasmids pR3, pR3b and pPit, and 10 μM for the others. After transcription, the RNAs were treated with RNase-free DNase I (Promega) for 15 min in 5 mM MgCl_2 , 50 mM Tris, pH 8, followed by phenol and chloroform extraction, and ethanol precipitation. The RNA concentration was determined by measuring the incorporated radioactivity, as described previously (Bertrand *et al.*, 1994).

Ribozyme cleavage assays

Ribozyme and substrate were heated independently for 1 min at 90°C in water. After cooling to room temperature, the reaction buffer was added to a final concentration of 20 mM MgCl_2 , 140 mM KCl, 20 mM Tris–HCl, pH 7.5. The RNAs were further incubated for 5 min at room temperature, and each protein was added to a final concentration of 50 ng/ μL . Ribozyme and substrate were then combined and incubated at 37°C. The reactions were stopped by mixing them with an equal volume of stop solution (0.5% SDS, 25 mM EDTA), and then with 100 μL of phenol. The aqueous phase was brought to 100 μL , extracted, and precipitated with ethanol in the presence of 2.5 μg of linear polyacrylamide (Gaillard and Strauss, 1990). The RNAs were then analyzed on denaturing polyacrylamide gels, and subsequently scanned on either an AMBIS Radioanalytic imager or a Molecular Dynamics Phosphorimaging system. All values are averages of two to four independent determinations.

BSA (DNase- and RNase-free) was purchased from Pharmacia, gp32 was purchased from USB, and recombinant A1 and NCp7 were kindly provided by D. Portman and G. Dreyfuss (Portman and Dreyfuss, 1994), and R. Kahn and D.P. Giedroc (Khan and Giedroc, 1992), respectively. The NCp7 concentration used in our assays (50 ng/ μL) was ~ 10 -fold higher than that utilized by Tsuchihashi *et al.* (1993) in their study. Our requirement for a higher concentration is probably due to the 4- to 20-fold higher RNA concentrations present in our assays (when expressed in moles of RNA nucleotide per volume), since the ratios of protein to RNA binding sites are similar in both studies.

Kinetics

The steady-state cleavage rates under multiple turnover conditions were determined using 2 nM ribozyme and 20 nM substrate. The reactions were linear for the first 20 min, during which time aliquots were sampled. The initial cleavage velocities under single turnover conditions were determined at a concentration of 2 nM substrate and 20 nM ribozyme over a 10 min time course following initiation of the reaction.

The single turnover k_{cat}/K_m values were determined as previously described (Heidenreich and Eckstein, 1992). The substrate concentration was 4 nM and the ribozyme concentration varied between 8 and 24 nM. The reaction time was 7 min for S12, S13, S14 and S17, and 60 min for S11.

The cleavage rate constant (k) of CisR3–15 was measured by calculating the ratio of full-length ribozyme (L) over full-length plus cleaved ribozyme (L+S) as a function of the transcription time (t), and by visual fitting of these plots to theoretical curves that follow the equation $L/(L+S) = (1 - e^{-kt})/kt$. The transcription reactions for the self-cleaving ribozyme were performed as above, except that they contained 100 ng of template plasmid linearized with *Eco*RI, 100 μM UTP and 50 ng/ μL of the indicated protein. Aliquots of the reaction were withdrawn at various time intervals (from 1 to 40 min), and processed as for the *trans*-cleavage reactions.

RNA binding assays

The RNA was processed as for the cleavage reaction, until the protein was added. After 15 min incubation at 37°C, glycerol was added to a final concentration of 10%, and the samples were immediately loaded onto a 1% agarose gel buffered with 0.5 × TBE for A1 and NCp7, or onto a 6% polyacrylamide gel buffered with 1 × TBE for gp32. The samples were electrophoresed at room temperature, with a voltage of 6 V/cm for the agarose gels, and of 12 V/cm for the polyacrylamide gels.

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