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IN VITRO NCp7 ENHANCEMENT OF RIBOZYME-MEDIATED CLEAVAGE OF FULL-LENGTH HUMAN IL-6 mRNA

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SUMMARY : We have previously shown that a ribozyme directed against human interleukin-6 (IL-6) mRNA is efficient *in vivo*, despite its poor activity *in vitro* on full-length IL-6 mRNA. We compared the effect of the nucleocapsid protein of HIV-1 (NCp7) on the ribozyme cleavage reaction of a long (1041 nt) and a short (19 nt) substrate IL-6 RNA *in vitro*. At a one to five molar ratio of the long substrate to ribozyme, almost no cleavage is observed after 30 min at 37°C. The NCp7 protein significantly increases the catalytic activity of the ribozyme on this substrate (from 0 to 53% after 7 min at 37°C), but not on the short one. A kinetic analysis of single turnover reactions performed with ribozyme in at least fivefold molar excess over substrate also lead to a stimulation (70-fold) of the reaction rate with long substrate, but not with the shorter one. Preferential increases of the catalytic activity on the long substrate suggests that the NCp7 protein prevents misfolding of RNAs.

Ribozymes are catalytic RNAs that have the ability to cleave the phosphodiester bond of target RNA(s) (1). The simple hammerhead ribozyme found in plant viroids and virusoids can be adapted to cleave specifically an RNA sequence of choice (2-4). Several reports indicate that the hammerhead type of ribozyme functions in living cells: Cotten and Birnstiel (5) and Cameron and Jennings (6), reported ribozyme-mediated inhibition and lowering of specific gene expression in *Xenopus laevis* oocytes and monkey (COS-1) cells, respectively. Sarver et al. (7) showed that a ribozyme directed against HIV-1 gag RNA reduced p24 antigen expression in $CD4^+$ HeLa cells. A hammerhead ribozyme is also active in bacterial cells (8).

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ABBREVIATIONS: Rz: ribozyme; LS: long substrate; SS: short substrate; hIL-6: human interleukin-6; NCp7: nucleocapsid p7 protein; UT: untranslated; aa: amino acid; nt: nucleotide.

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We have previously designed a ribozyme that efficiently cleaves human IL-6 mRNA in U amniotic cells, despite a poor activity on the full-length RNA *in vitro* (9). Others have confirmed that current *in vitro* assays of ribozyme activity are not necessarily helpful in predicting their activity in intact cells: inhibition of HIV-1 replication by ribozymes that show poor activity *in vitro* has been observed *in vivo* (10). In contrast to the *in vitro* ribozyme reactions most RNAs normally exist as ribonucleoprotein (RNP) complexes *in vivo* (11). Thus, as suggested previously (12, 13), interactions between these proteins and either the ribozyme or its substrate may profoundly affect ribozyme activity. Proteins may enhance ribozyme binding via annealing activities (14-16), and ribozyme turnover, via strand-exchange activities (17, 18). Conversely, ribozyme binding and cleavage could be inhibited by steric hindrance or via protein mediated unwinding (17-19). Tsuchihashi et al. (12) have shown that non specific RNA binding proteins (gp32, A1 and NCp7) enhance ribozyme binding and turnover. NCp7 is a nucleocapsid protein of HIV-1 which binds RNA with broad specificity (20-22).

To evaluate the effect that RNAs associated proteins may have on the *in vivo* ribozyme-mediated cleavage of substrates with local secondary structure, a comparison of the cleavage efficiency of human IL-6 mRNA derived 19 nt synthetic substrate and 1041 nt long transcript was undertaken *in vitro*, in the presence of NCp7. This study shows a strong facilitating effect of this NCp7 protein on the ribozyme mediated cleavage with a long substrate, that more closely mimics the *in vivo* transcript and not with the shorter one. Thus even when ribozymes exhibit low reaction kinetics and do not truly act as enzymes *in vitro*, their *in vivo* activity may be enhanced by protein facilitation.

MATERIALS AND METHODS

Ribozyme and substrate RNAs

The 74 nt IL-6 trans-acting hammerhead ribozyme was prepared by *in vitro* transcription from *Eco* R I digested pBS IL-6 Rz plasmid as described previously (9). The substrates RNAs used in this study were prepared either by *in vitro* transcription (for the 1041 full-length non polyadenylated human IL-6 mRNA) (23) or by direct synthesis, using an Applied Biosystems 392 DNA Synthesizer for the shorter 19 nt substrate. This latter oligoribonucleotide was prepared using phosphoramidites from Milligen Biosearch, as described previously (24), and ^{32}P-labeled at the 5'-end with T4 polynucleotide kinase (Gibco BRL) and γ^{32}P(ATP) (Amersham).

In vitro transcription

The transcription reactions were carried out using 1 µg linearized DNA template, in 0.4 mM each ATP, GTP, UTP, 0.2 mM cold CTP, 8 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol (DDT), 40 mM Tris-HCl (pH 8), 25 mM NaCl, in presence of 80 units of RNasin and 2 U/µl T7 RNA polymerase. If ^{32}P-labeled transcripts were needed, 15 µCi of [α^{32}P]-CTP (400 Ci/mmol) were included. The reactions were carried out for 1 h at 37°C, treated 15 min with RNase-free DNase to degrade the template DNA and the products were isolated by phenol chloroform extraction and ethanol precipitation or by purification on a nick column for the short or long substrate respectively.

NCp7 protein

The NCp7 protein was purified by Z. Tsuchihashi, as described previously (25). Briefly, the gene encoding the 71 amino acid form of HIV-1 NCp7 was amplified by

polymerase chain reaction from pNL4-3 plasmid DNA. For the purpose of one-step purification using a nickel-nitrilotriacetic acid column (Quiagen, USA), the sequence Met-$(His)_6$ was added to the N-terminal end of the native NCp7 gene. This hybrid gene was cloned into T7-7 vector (26) and expressed in *E. coli* strain BL21(DE3) which has an IPTG-inducible T7 RNA polymerase gene. It was > 95% pure, as judged from Coomassie brilliant blue staining of SDS gels, and was free of detectable RNase activity. As there was a loss of activity with storage, the reported concentrations may overestimate the amount of fully active NCp7 required for stimulation.

***In vitro* ribozyme reactions**

Reactions were carried out in 50 mM Tris HCl pH 8, 20 mM $MgCl_2$. Reactants (ribozyme and target RNA) were mixed together at the desired molar ratios and the reactions were initiated by adding $MgCl_2$. For the short substrate, the reactions were stopped by the addition of an equal volume of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. For the long substrate, the reactions were stopped with 25 mM EDTA, 0.5% SDS and ethanol precipitated in presence of 2.5 µg of linear polyacrylamide (27) after extraction by phenol chloroform, to circumvent problems of migration observed in presence of nucleoprotein. The pellet was redissolved in water and mixed with an equal volume of 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF. Reaction products were then separated on a 20% (short substrate) or 4% (long substrate) polyacrylamide/8M urea gel. The substrates and cleavage products (P1 and P2) were quantitated with an "Instant Imager" (Packard-Canberra Company). The extent of cleavage was calculated as P1+P2/P1+P2+S. All values are averages of two to four independant determinations. The observed first-order rate constants were obtained under single turnover reactions. With the short substrate (SS), the reaction is performed using 6.5 nM (SS) and 65 nM (Rz) over a 10 min time course following initiation of the reaction. With the long substrate (LS), constants were determined at a concentration of 3.4 nM (LS) and 17 nM (Rz) over a 15 min time course.

RESULTS AND DISCUSSION

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 (13, 28). In the case of our IL-6 Rz, the difference between the short (19 bases long) and the long (1041 bases long) substrate in cleavage efficiency is about 175-fold. The effect of NCp7 on the cleavage of these two substrates is described below.

Ribozyme and substrates are depicted in Figure 1. The IL-6 ribozyme has been previously shown to efficiently cleave IL-6 messenger *in vivo* (9). The long transcript, which is not completely represented in this figure, corresponds to the entire coding region (including 10 nt of the 3' UT region) of the human IL-6 mRNA. It is slightly different in the length of the complementary region with the ribozyme (Figure 1).

We have determined the NC concentrations required to stimulate cleavage of the SS and LS (Figure 2). As the ratios of protein to RNA binding sites are different for these two substrates, a large range of concentrations (up to 20 µM NCp7) was tested. No stimulatory effect is observed for the SS (Figure 2A) and a slight decrease of cleavage is obtained with high concentrations of protein. With concentrations varying between 0 and 400 nM, the amplitude of cleavage is unchanged (~50%). These results are in opposition to those of Tsuchihashi et al. (12), where an enhancement of 10- to 20-fold was obtained in presence of the protein. As a window for stimulation exists with NCp7 concentrations high enough

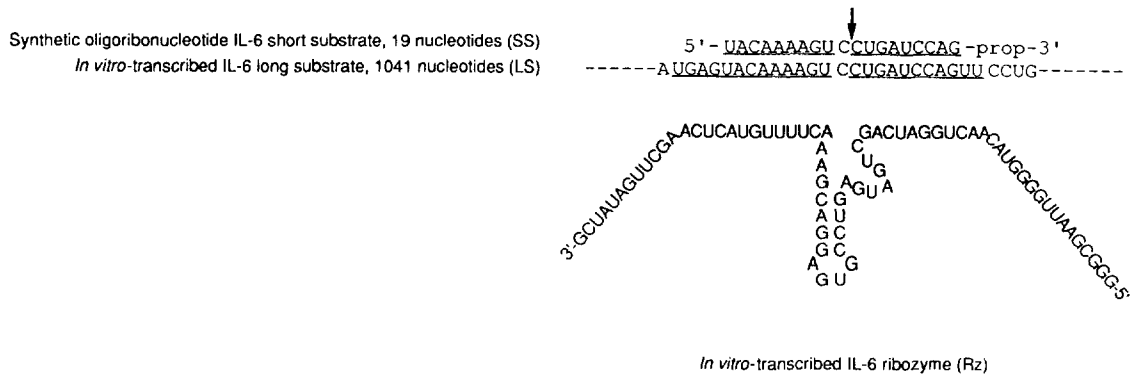

FIGURE 1. Schematic representation of the IL-6 ribozyme and IL-6 RNA substrates used in this study.
The ribozyme cleavage site in the RNAs is indicated by an arrow. The base-pairing between substrates and Rz is underlined in the substrate sequence. "Prop" attached at the 3'-end of the SS corresponds to a propanediol moiety, rendering stability against 3'-exonucleolytic attack. It has no effect on the cleavage efficiency. The *in vitro*-transcribed Rz contains vector-derived flanking sequences, indicated in oblique. The IL-6 mRNA target region contains a GUC cleavage site at 510 nucleotides from the cap site on IL-6 mRNA, corresponding to aa 121 of the mature IL-6.

to allow stimulation of substrate binding and product dissociation without also shutting down the cleavage step because of disruption of the catalytic core of the ribozyme, we have compared the NCp7 concentrations used in both studies. The concentrations used in this study were ~30-fold lower than that utilized by Tsuchihashi et al., suggesting that thermodynamic equilibrium without shutting down the RNA's ability to function varies from ribozymes to ribozymes. The autoradiogram in Figure 2B shows the high stimulatory effect of NCp7 on the LS. No cleavage of the LS is observed in absence of protein. However, 32 or 53% of cleavage is obtained when 1 μM or 2 μM of NCp7, respectively, is added to the reaction. In the following experiments, concentrations used in ribozyme reactions with the SS and the LS are 400 and 2000 nM NCp7, respectively.

To evaluate the effect of NCp7 protein, we have analysed the first-order rate constants under single turnover conditions in presence of the SS and LS. As cleavage efficiency with long substrates is very low, multiple turnover reactions do not reach saturation kinetics within reasonably short incubation periods and the k_{cat} cannot be determined. Moreover, unspecific degradations of substrate and products cleavage have been observed after long time incubation. Thus, for these two reasons, k_{cat} and K_m cannot be determined under multiple turnover conditions where substrate is in excess over ribozyme. Even the presence of the NCp7 protein is not sufficient to detect a cleavage under these conditions. It is only visible when the ribozyme is in at least 2-fold molar excess over the substrate (data not shown). The Figure 3 shows the reaction time course (and related autoradiogram) for LS RNA cleavage under single turnover conditions in the presence and absence of NCp7, in non-saturating concentrations of Rz. The results show the average of three independent experiments (variation: 10%). There was a large increase (~70-fold) in the rate of products

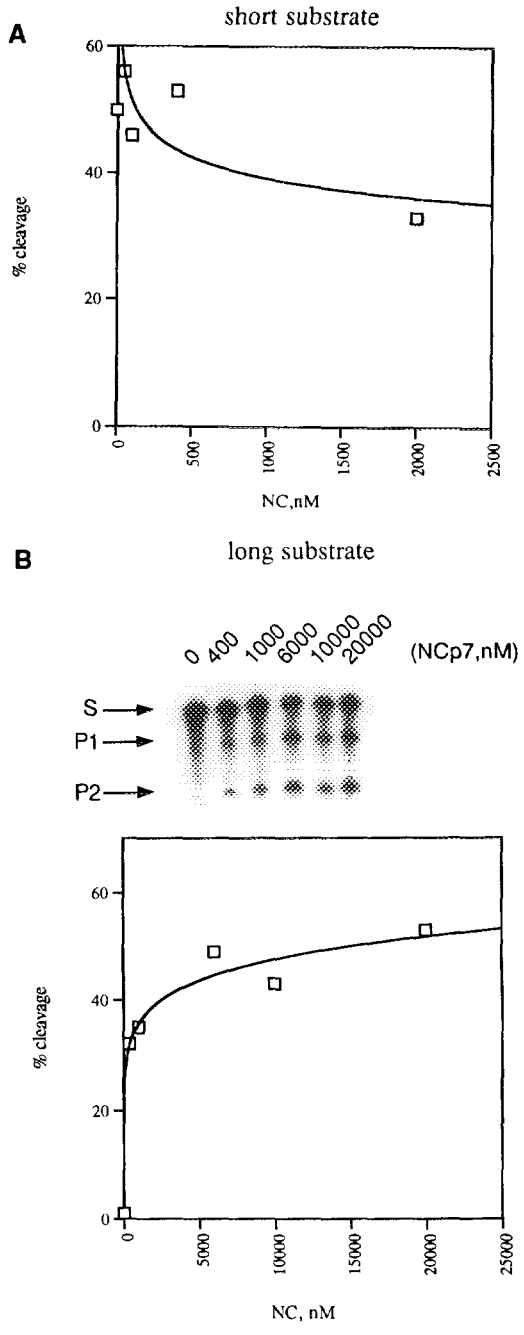

FIGURE 2. Effect of NCp7 on ribozyme-mediated cleavage.
Single turnover cleavage reactions of the short (A) and the long IL-6 RNA substrate (B) were performed with increasing concentrations of NCp7 protein. A- With the short substrate, excess of ribozyme over substrate (10X) and incubation time of 10 min at 37°C are used. B- With the long substrate, the excess of ribozyme was 5-fold and the incubation time, 7 min at 37°C. The autoradiogram shows the *in vitro* cleavage of the long IL-6 RNA by the Rz. The sizes of the fragments were 662 (P1) and 379 (P2) nucleotides as predicted by the location of the single site of cleavage (Figure 1). The graph is a representation of these data.

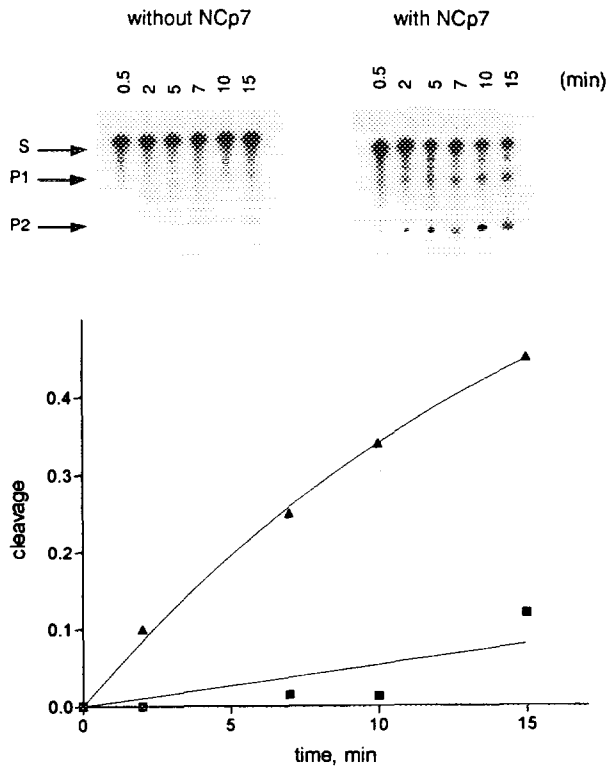

FIGURE 3. Effect of NCp7 on single turnover reactions (LS).
The autoradiogram corresponds to the cleavage reactions and substrate and products were quantitated by scann as described in Methods. Time-course using a fivefold molar excess of ribozyme (17 nM) over substrate (3.4 nM) in the absence (■) and presence (▲) of 2000 nM NCp7. The lines are non-linear least squares fits that give $k = 0.8 \times 10^{-3}$ min^{-1} and 59.4×10^{-3} min^{-1} in the absence and presence of NCp7, respectively. For the curve corresponding to experiments without NCp7 protein, the coefficient of determination R^2 (that reflects goodness of fit) was only 0.63 (compared to 0.99 in presence of NCp7), which could be due to the relative inaccuracy of measurement in this low range of activity.

formation in the presence of 2 μM NCp7. The observed rate constant k increases from 0.8×10^{-3} min^{-1} to 59.4×10^{-3} min^{-1} with addition of the protein. Comparable analysis were performed for the short substrate, and the deduced k values are presented in Table I. These rate constants are unchanged by NCp7 (110×10^{-3} min^{-1} and 140×10^{-3} min^{-1} with and without NCp7 protein, respectively). If results obtained by Bertrand et al. (13) could be extrapolated to hammerhead ribozymes in general, the increase of the k could be attributed to an enhancement of the binding of Rz to the substrate. This conclusion is supported by an earlier observation showing that the enhancement of NCp7 disappeared when the Rz and S have been pre-annealed (12). Under these conditions the chemical cleavage step limits the rate of reaction. Thus the higher effect observed in this study on the LS was expected, as an unfolding of this substrate by NCp7 would increase the accessibility of the target sequence. However, even after enhancement with NCp7, the rate constants of the LS cannot compare to those of short substrate, which are still 2-fold higher (Table I).

Table I : Influence of NCp7 on the cleavage rate of long (LS) and short (SS) hIL-6mRNA substrates

	Short Substrate		Long Substrate	
NCp7 (μM)	0	0.4	0	2
k ($x10^{-3}$ min^{-1})	140	110	0.8	59.4

Reaction conditions were as described under Methods; k were calculated as in Fig. 3.

Surprisingly, RNA and DNA facilitators that presumably also disrupt inhibitory intramolecular structures by base-pairing to substrate oligonucleotides outside of the region recognized by a Rz (29), do not facilitate the LS cleavage (not shown).

Moreover, in contrast to other reports (13), the long ribozyme-substrates duplexes (18 and 24 for the SS and LS, respectively) do not represent an obstacle to the beneficent effect of the protein. Previous results demonstrated that NCp7 and hnRNP A1 are able to melt helices rapidly but only if they are <7bp. However, as suggested, this limitation in helix length is likely to be dependent upon the helix stability rather than on its length per se. In addition, the low efficiency and therefore the inability to work under multiple turnover conditions could also be due to the design of the ribozyme itself (with long flanking sequences) (Figure 1) rather than to the size of the substrate. Additional experiments with the SS under similar conditions (single turnover) show a slight enhancement of the cleavage rate when the ribozyme contains deoxyribonucleotides arms (not shown). This result is in agreement with a slight NCp7 effect on the binding under single turnover. Moreover, it supports the importance of the global helix stability rather than the helix length, as discussed above, to obtain an effect of the NCp7, since interactions are more stable between RNA-RNA than between RNA-DNA.

CONCLUSION

In agreement with others (12) (13), we suggested specific NCp7 enhancement in the binding step. However, we only observed this effect when binding is hindered by the length of the RNA substrate, suggesting that it is most likely due to the unfolding of the substrate. Indeed, the amplitude of facilitation corresponds more closely to an unfolding effect than a close encounter effect, which typically is in the range of 100 to 1000-fold (30).

The NCp7 stimulation of single turnover, may not represent the maximum possible rate stimulation. Indeed, in multiple turnover reaction, NCp7 should overcome the rate-limiting product dissociation (but likely not completely as we have not be able to work under multiple turnover conditions). As long arms are too poorly dissociated by NCp7, an optimal ribozyme arm length of 7 bases on each side of the catalytic domain would give rise to more efficient cleavage of the LS in presence of the protein (13).

Moreover, as the NCp7 protein used in this study shows a higher effect than facilitators which presumably also disrupt inhibitory intramolecular structures, we think that NCp7 or related proteins could preferentially be helpfull for ribozyme activity *in vivo* and could be used in gene therapy. The hnRNP A1 is probably a better candidate, as it is one of the most abundant proteins involved in RNA processing, and it is thought to bind to most, if not all, mRNAs *in vivo*, in contrast to nucleocapsid protein which interacts mainly with the genomic retroviral RNA *in vivo* (13).

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