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Preparation of HIV TAR RNA with RNA Scissors[1]

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Two hammerhead ribozymes derived from plant pathogenic RNAs were used to cut off the HIV TAR RNA from the T7 RNA transcript through a *cis* cleavage reaction. Stem I of the (+) vLTSV ribozyme comprises 8 nucleotides of the 5′ terminus of TAR RNA, but stem III of the (+)sTRSV ribozyme consists of 8 nucleotides of the 3′ end of TAR RNA. The construct containing two GUC hammerhead ribozyme target sequences identified the cleavage sites to cut off a required RNA molecule. This method was applied for preparation of 35 nt long TAR RNA. Its activity was proved by the complex formation with the Tat protein. It seems that this approach based on RNA scissors can also be used for the generation of required RNA molecules, RNA decoys or RNA aptamers *in vivo*.

Key words: *cis* acting ribozymes, *cis* RNA cleavage, hammerhead ribozymes, HIV TAR RNA, RNA scissors.

The human immunodeficiency virus type 1 (HIV-1) Tat protein is a potent *trans* activator of long terminal repeat gene expression and is essential for viral replication (*1*). Transactivation by the Tat protein is mediated through binding to the transactivation response element (TAR) RNA found at the 5′ end of the mRNA transcript. The NMR-confirmed structure of a 59 nucleotide containing TAR RNA molecule consists of two stem regions separated by three unpaired nucleotides (bulge) and a loop of six nucleotides (*2-4*). Through many studies it has been shown that the carboxyl-terminal fragment of the Tat protein spanning a basic (arginine rich) region binds specifically to TAR RNA containing a trinucleotide bulge (*3*). Because the cysteine-rich part makes the Tat protein difficult to handle, several groups adopted a reductionist approach, trimming the Tat protein down to minimal RNA binding peptides in order to delineate specific residues involved in RNA recognition and to facilitate structural studies (*1, 3, 5*). A TAR RNA-argininamide complex characterised by nuclear magnetic resonance spectroscopy (NMR) exhibited a RNA conformational change upon argininamide addition, indicating that TAR RNA contains a specific arginine binding pocket (*3-5*). A similar conformational change of TAR RNA has been observed upon binding to arginine-rich peptides (*2*). From the results of binding studies on the Tat protein, Tat peptide and arginine one can conclude that a single amino acid of the Tat protein is primarily responsible for the specific recognition of TAR RNA and that a great deal of the specificity interaction is due to the RNA structure formed by TAR (*3-5*). On the other hand, there have been reports that argininamide alone cannot mimic all of the interactions in the full TAR RNA-Tat complex, and careful biochemical analyses of various TAR RNA-Tat interactions have revealed a loss of binding energy and sequence specificity as Tat is truncated (*2*). To resolve this question we decided to study the interactions between TAR RNA and the full length Tat peptide. To do this, large quantities of both RNA and protein are required. RNA up to *ca.* the 50 mer could be rather easily prepared either chemically (*6*) or enzymatically with T7 RNA polymerase (*7-9*). Although the latter method is very efficient, T7 RNA polymerase sometimes incorporates additional nucleotides at the end of the transcript making the product heterogeneous (*7, 10-12*). It is known that naturally occurring enzymatic RNA molecules (ribozymes) catalyse sequence-specific RNA processing (*13*). Their activity and specificity is determined by the ability of ribozymes to form base pairs with nucleotides near the cleavage site of target RNA. This can be achieved by altering of the substrate recognition sequences. Also, intramolecular *cis*-cleaving ribozymes can be engineered to hydrolyse in *trans*. Because of their flexibility in design and their extraordinary sequence specificity, *trans*-cleaving catalytic RNAs such as hammerhead or hairpin ribozymes have been proved to be useful in the regulation of gene expression (*13, 14*). Divalent metal ions are essential for their activity (*15-18*). The hammerhead ribozymes are the smallest catalytic RNAs found to date, consisting of three stems, I, II, and III, connected by a conserved core region (*13, 14, 19*). They recognise substrates containing an XUN base triplet (X can be any base and N can be unpaired A, C, or U) and hydrolyse, through an activated water molecule, the phos-

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phodiester bond on the 3′ side of N. However, the GUC sequence is cleaved the most efficiently. The small size of the hammerhead molecule, which is able to form an active self-cleaving structure, promoted an explosion of efforts at its characterisation and manipulation both *in vitro* and *in vivo*. The self-cleaving activity has been discovered and characterised in the virusoid of the lucerne transient streak virus (+vLTSV) and in the satellite RNA of the tobacco ringspot virus (+sTRSV) (*13*). Ribozymes are very active *in vitro* but exhibit much lower activity *in vivo* (*14*). They are considered to be potential therapeutic agents and have been used in studies on gene expression, mostly in eukaryotic cells. Only a few cases have been reported of ribozyme activity in a natural bacterial system (*13, 14*). The core sequence of the hammerhead structure of (+)sTRSV RNA has been used in many cases *in vitro* for cleavage in *trans* (*13, 14*). There have been some studies in which a similar approach involving *cis*-acting hammerhead ribozymes was newly designed and used (*20-33*). These works demonstrated successful trimming of short RNAs by one or two *cis*-acting ribozymes. A different strategy based on the use of RNase H for the same purpose has also been described (*34*).

In this paper we describe the use of two hammerhead ribozymes acting in *cis* as scissors to produce TAR RNA for structural studies. This approach could be used *in vitro* for the production of RNA aptamers, *e.g.* TAR decoys and other active RNA molecules of interest, and probably *in vivo* for the generation of RNA targets to bind strongly and inactivate various proteins. In contrast to many reports we show that application of the ribozymes strategy on a large scale may be limited due to the formation of a stable intermediate non-ribozymic active structure.

MATERIALS AND METHODS

Preparation of a DNA Construct Containing the Sequence of HIV TAR RNA-Flanked by Hammerhead Ribozymes Derived from (+)vLTSV and (+)sTRSV RNA on the 5′ and 3′-Sides Respectively—The double-stranded oligodeoxynucleotide, 183 mer (Figs. 1 and 2), was obtained by ligation of the chemically synthesised short overlapping oligodeoxynucleotides, of U1-U3, for one strand and L1-L3 for the complementary one (Fig. 2A). The synthesised DNA fragment was inserted into the pT7/T3α-18 plasmid at the *Eco*RI and *Hin*dIII restriction sites (Fig. 2B). The NovaBlue (Novagen) bacterial strain was used as the host for transformation. To confirm the correctness of ligation of the DNA fragments, the plasmid was isolated from transformants and sequenced using a dideoxy chain termination kit (Pharmacia).

RNA Transcription—RNA was synthesised by T7 RNA polymerase transcription. After linearisation of 200 μg of plasmid (40 μg/ml) with *Hin*dIII, transcription was carried out in 80 mM Hepes buffer, pH 7.5, containing 25 mM $MgCl_2$, 1 mM spermidine, 5 mM dithiothreitol, 0.12 mg/ml BSA, 4 mM NTP_s (C, G, U, A), and 40 U/μl T7 RNA polymerase for 5 h at 37°C. When the reaction was completed, the DNA template was digested with RNase-free DNase. The reaction mixture was treated with phenol and

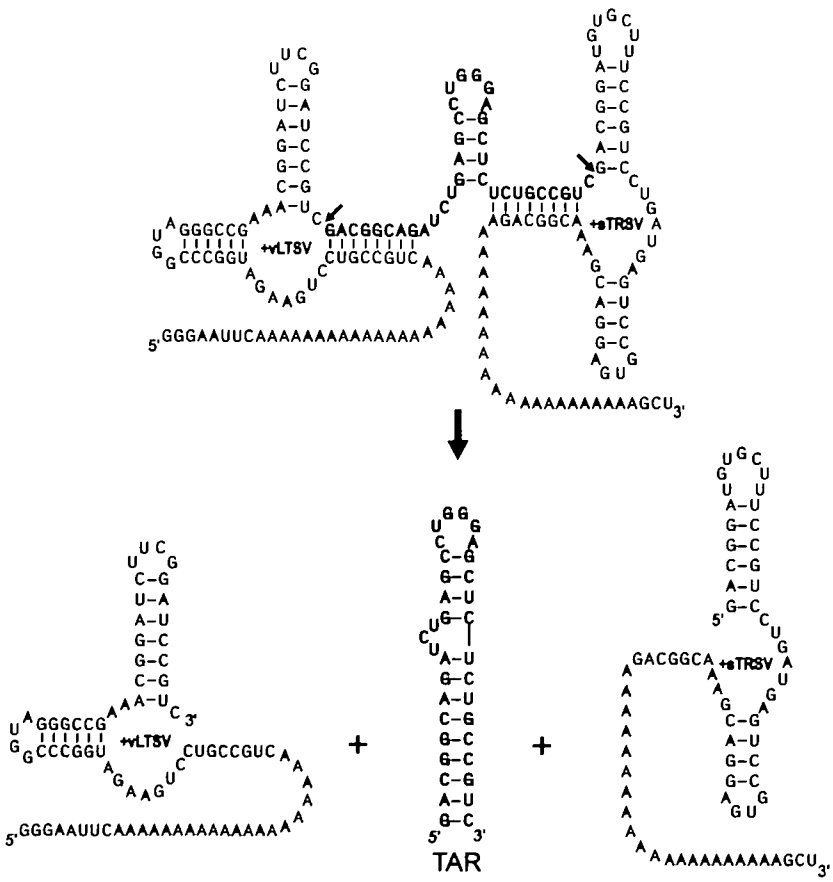

Fig. 1. **The secondary structure of the T7 RNA transcript containing two hammerhead ribozymes derived from lucerne transient streak virusoids (+vLTSV) and tobacco ringspot virus satellite RNA (+sTRSV RNA) located at the 5′ and 3′ends of the HIV TAR RNA (35 nt) nucleotide sequence, respectively.** Eight nucleotides of the 5′ and 3′ ends of the TAR RNA element form stem I of vLTSV and stem III of sTRSV ribozymes, respectively. At both termini of the hammerhead ribozymes, oligo (A) tails were added. Additionally, the 5′ oligo (A) tail is capped with a T7 RNA polymerase promoter sequence. The cleavage sites are indicated by arrows.

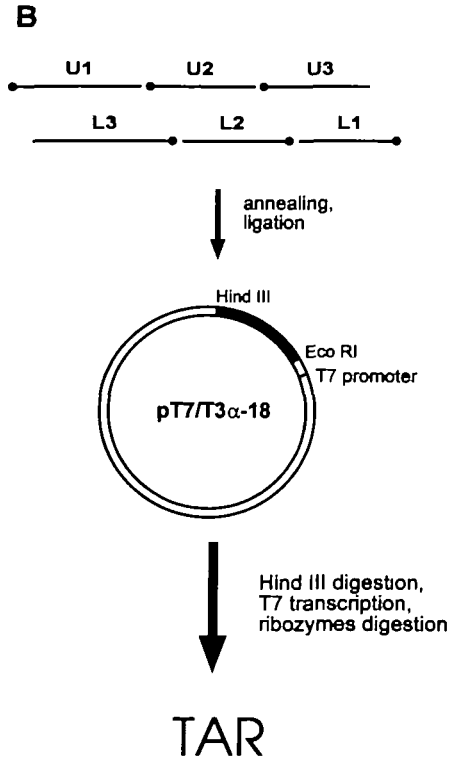

Fig. 2. **A: The nucleotide sequence of a synthetic DNA construct of 183 nt which contains sequences corresponding to two hammerhead ribozymes, the TAR-RNA element and oligo(A) tails.** The composite oligodeoxynucleotides numbered U1-U3 and L1-L3 were synthesised chemically. **B: Scheme for preparation of the plasmid.** pT7/T3α-18 was digested with *Eco*RI and *Hind*III, and then chemically synthesised DNA fragments were inserted and ligated to the vector. Filled circles indicate 5'-phosphate. The hatched bar denotes the T7 construct, which was inserted at the *Eco*RI and *Hind*III sites of pT7/T3α-18.

then chloroform, and the RNA was precipitated with ethanol (*7, 11*).

Cleavage Reaction of the T7 Transcript—The T7 transcript is a RNA resulting from T7 transcription of the 183 nt DNA template. Ribozyme intramolecular cleavage reaction of the T7 RNA transcript (160 μg) was carried out in 50 mM Hepes buffer, pH 7.5, containing 25 mM $MgCl_2$, with incubation at 37°C for 4 h, and RNA was renatured by heating to 70°C for 3 min every 1 h. The RNA sample was precipitated with ethanol. The pellet was dried and resuspended in 10 μl of deionised formamide. RNA was separated by 12% polyacrylamide gel electrophoresis in the presence of 7 M urea. The band corresponding to RNA of 35 nt was cut out, eluted from gel with the buffer (*7*), and recovered by ethanol precipitation. RNA length markers were prepared by chemical synthesis (*6*).

TAR/Tat Binding Assay—The Tat 2 protein expressed and purified from *E. coli* (EW, unpublished work) was used for the binding reaction to TAR RNA. For the assay (total volume, 10 μl) we used 3×10^{-11} M (10,000 cpm) of 3'-^{32}P-labeled TAR-RNA and $2.5\text{-}10\times10^{-9}$ M the Tat 2 protein. The reaction was carried out in 50 mM Tris-HCl buffer, pH 7.5, containing 70 mM NaCl, 1 mM EDTA, and 0.1% Nonidet for 30 min at room temperature (23°C). Analysis of complex formation was performed on a 0.7% agarose gel.

Agarose Gel Electrophoresis—Analysis of the TAR RNA/Tat protein complex formation was carried out on 0.7% agarose gels. The samples were loaded on the gels in 4 mM Tris HCl buffer, pH 7.4, containing 4% glycerol and 0.02% bromophenol blue (BB). The gel was run at 30 mA for 45 min (BB at the bottom of the gel) in 1×TEB (89 mM Tris, 89 mM borate, 2.5 mM EDTA). The gel was dried under vacuum at 60°C and then exposed to X-ray film.

Labeling of RNA—TAR RNA purified as described above was labeled at the 3' end with [^{32}P]pCp (3,000 Ci/mmol) (Amersham) and T4 ligase (Pharmacia), and separated on a 10% polyacrylamide gel containing 7 M urea. The radioactive band was cut out, and RNA was eluted with the buffer and then precipitated with ethanol (*7*).

RESULTS AND DISCUSSION

In this paper we describe an approach for the preparation of any RNA molecules with homogenous ends, taking advantage of the RNA self-cleavage reaction in *cis* with two hammerhead ribozymes. This method was used here for preparation of the HIV TAR RNA element. The DNA construct for T7 RNA polymerase transcription was synthesized. It contains one ribozyme derived from the positive strand of a lucerne transient streak virusoid (+vLTSV) located upstream to the TAR RNA segment having a 5' cleavage site. The second hammerhead ribozyme is a part of the (+) strand of tobacco ring spot virus satellite RNA (+sTRSV) placed at the 3' end of the TAR RNA sequence having a 3' cleavage site. In this constuct 8 nucleotides at the 5' end of TAR RNA form stem I of the vLTSV ribozyme, and 8 nucleotides at the 3' end of TAR RNA form stem III of sTRSV (Fig. 1). The poly(A) tail at the 3' end of the transcript made additional A-U base pair formation between TAR RNA and the ribozyme moiety possible (Fig. 1). The numbers of base pairs between TAR RNA and the ribozymes were programmed by taking into account data on the association and dissociation of both

molecules *in vitro*, as well as the number of paired nucleotides on either side of the catalytic domain which should be 6-8 (*35-37*). An oligodeoxynucleotide duplex containing the TAR RNA sequence and the ribozymes was prepared by ligation of the six shorter chemically synthesised fragments (U1-U3 and L1-L3), and cloned into the pT7T3α-18 plasmid. Sequencing analysis confirmed the correctness of the nucleotide sequence of the template (Fig. 2). The recombinant plasmid, containing a construct of 183 base pairs of DNA, inserted downstream of the T7 RNA polymerase strong promoter, was used as a template for *in vitro* synthesis of the transcript: GGGAAUUC-poly(A)-(vLTSV)-TAR RNA-(sTRSV)-poly(A) (Fig. 1). The template was cut at a unique *Hin*dIII site downstream of the insert (Fig. 2). This procedure is a reliable easy and rapid way of obtaining a relatively long template for *in vitro* transcription using T7 RNA polymerase (*7, 11*). We engineered a construct for preparation of the TAR RNA element consisting of 35 nucleotides derived from HIV-1 mRNA. Two cleavage sites are included in a single RNA chain. In order to restrict random double strand formation and misfolding as well as to differentiate the lengths (*e.g.* migration on the gel) of the final products, oligo(A) tails were included at both ends of the RNA transcript (Fig. 1). There have been some suggestions that the activity of a ribozyme can be impaired by additional sequences at the 5′ and 3′ termini of the catalytic sequence (*24*). However, experiments involving a multifunctional ribozyme expression plasmid have not confirmed these observations (*7, 8, 19*). To confirm the self-cleavage activity of the ribozymes and splitting off of the TAR RNA element, the full length RNA transcript was incubated with magnesium ions under various conditions (*35-37*). We showed that during the transcription reaction at 37°C for 5 h, TAR RNA molecule was not observed (Fig. 3A, lane 5, and Fig. 3B, lane 6). However, on incubation of the RNA primary transcript for 1 or 2 h with 25 or 50 mM $MgCl_2$, a TAR RNA band of 35 nt was observed on the gel (Fig. 3A, lanes 1-4). It was observed that there is no significant difference in the effect of a high $MgCl_2$ concentration on the intramolecular reaction, but its presence is absolutely required. The cleavage reaction depends on time as well temperature. The effects of incubation overnight and for 4 h with heating at 70°C for 3 min every hour were similar (Fig. 3B, lanes 1 and 4). Therefore, 25 mM $MgCl_2$ and a 4 h incubation time with heating at 70°C for 3 min every hour are the best for spliting off TAR RNA, which was purified on a large scale a 12% PAGE (Fig. 4). The overall yield of TAR RNA was 15 μg from 100 μg T7 transcript. From the results of analysis under different reaction conditions it seems that folding of the hammerhead ribozyme active site is thermodynamically controlled. The appearance of the band corresponding to an RNA chain of 35 nt confirmed that both ends of TAR RNA were trimmed. However, on the gel (Fig. 3) there were still some bands corresponding to lengths of *ca.* 100 nt. It is reasonable to think that they are TAR RNA intermediate products having a ribozyme moiety at the 5′ or 3′ end of the transcript (Figs. 3 and 4), which comprise 45% of the total T7 transcript. The TAR RNA element

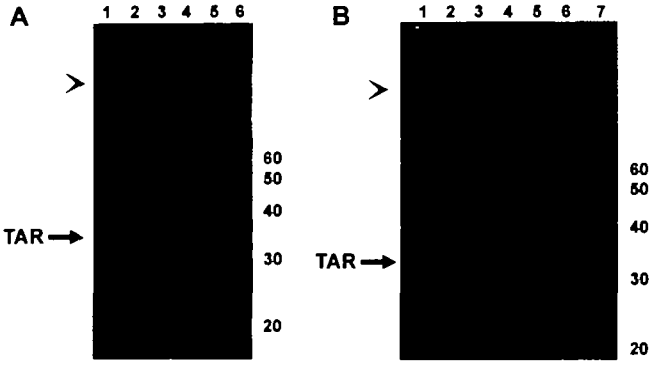

Fig. 3. **Polyacrylamide (12%) gel electrophoresis of HIV TAR RNA after cleavage of the T7 RNA transcript with two ribozymes at the GUC target sequence.** The reactions were performed at 37°C with different magnesium ion concentrations (A): lane 1, 25 mM $MgCl_2$, 1 h; lane 2, 50 mM $MgCl_2$, 1 h; lane 3, 25 mM $MgCl_2$, 2 h; lane 4, 50 mM $MgCl_2$, 2 h; lane 5, T7 RNA transcript (control); lane 6, RNA length markers. The numbers indicate the lengths of relevant RNA fragments. (B) Effect of incubation time on cleavage off of the TAR RNA fragment: lane 1, 25 mM $MgCl_2$, overnight (12 h), 37°C; lane 2, 25 mM $MgCl_2$, 4 h, 37°C; lane 3, 25 mM $MgCl_2$, 4 h, 37°C with heating at 70°C for 3 min every 2 h; lane 4, 25 mM $MgCl_2$, 4 h, 37°C with heating at 70°C for 3 min every 1 h; lane 5, 25 mM $MgCl_2$, 2 h, 37°C, lane 6, T7 transcript (control); lane, 7 RNA length markers. The numbers indicate the lengths of relevant RNA fragments. The bands corresponding to the 35 nt TAR RNA element and total T7 transcript are indicated by arrows.

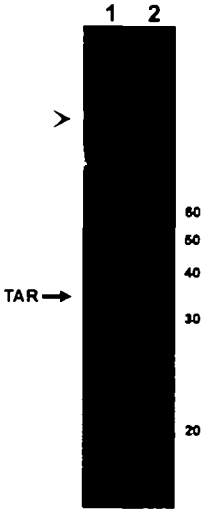

Fig. 4. **Purification of the TAR RNA element by 12% PAGE in 7 M urea after the hammerhead ribozyme-mediated cleavage reaction.** Arrows as in Fig. 3.

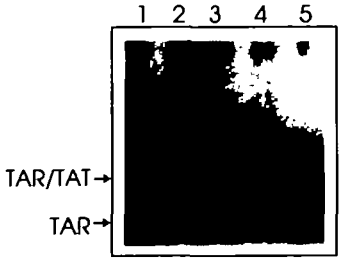

Fig. 5. **Autoradiogram of TAR RNA/Tat2 complex formation on a 0.7% agarose gel.** Lanes 1-5, TAR (3×10^{-11} M) in the binding buffer. Lanes 2-5 also contained 2.5, 5, 7.5, and 10×10^{-9} M Tat2 protein, respectively.

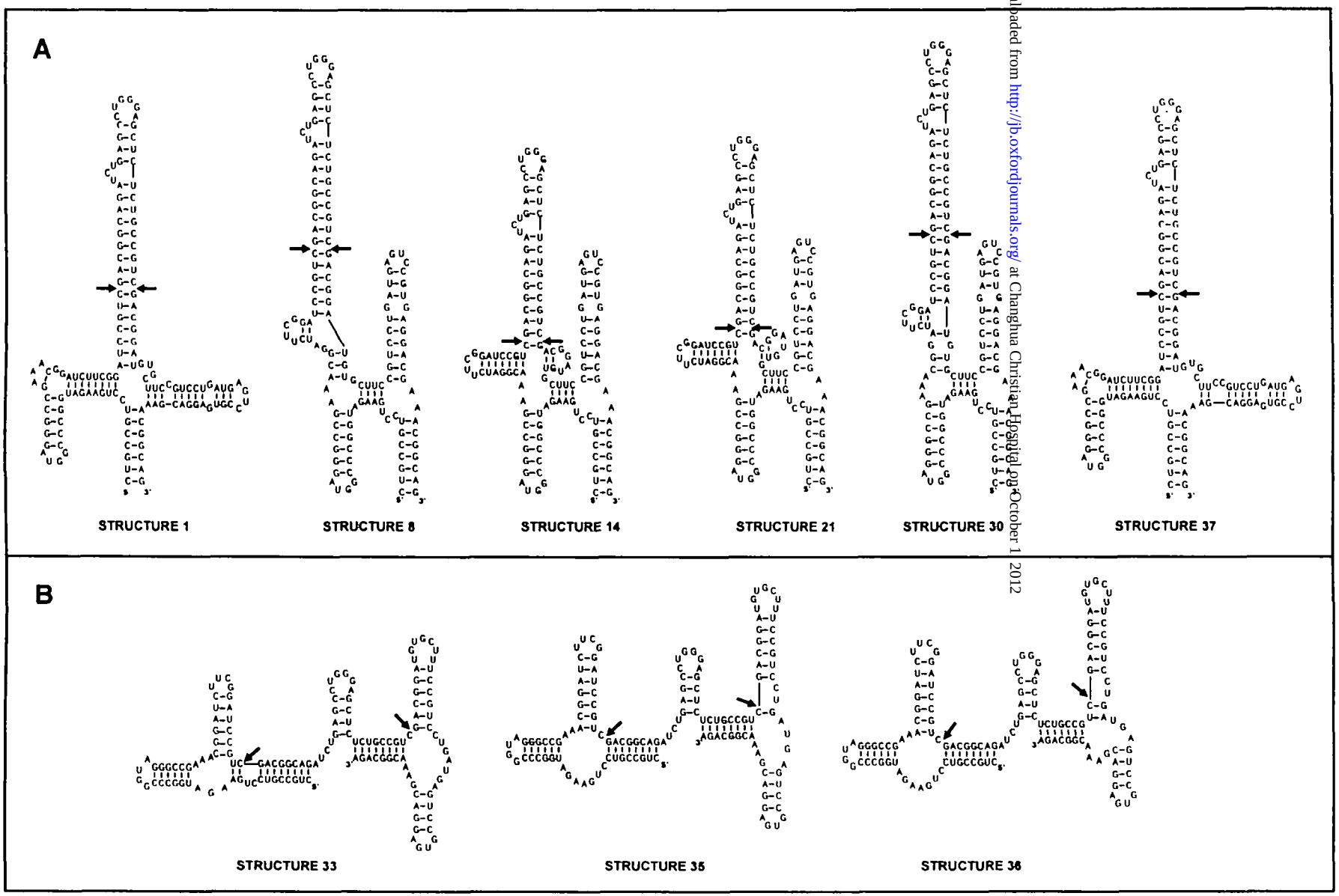

Fig. 6. **The calculated (*32*) putative secondary structures of the T7 RNA transcript containing (+)vLTSV and (+)sTRSV RNA derived ribozymes flanked by the HIV TAR RNA element.** At both ends of the transcript oligo (A) tails are ligated to prevent random base pairing. A: Structures in which the GUC target sequence (C-cleavage site) occurs in a double stranded region. B: Structures in which C is not paired.

TABLE I. **The localisation of two target GUC hammerhead ribosome sequences in the double (D) or single (S) stranded regions of some calculated structures with the Zuker algorithm (32) (Fig. 6).** The energy (kcal/mol) for each structure is also shown.

Calculated structure	Energy (kcal/mol)	Localisation of GUC of (+)vLTSV	Localisation of GUC of (+)sTRSV RNA
1	−74.3	D	D
8	−72.7	D	D
14	−71.6	D	D
21	−71.3	D	D
30	−70.9	D	D
33	−70.6	D	S
35	−70.5	S	D
36	−70.5	S	D
37	−70.5	D	S

prepared by this method forms a stem-loop structure (Fig. 1), as already proposed by several groups (*13-15*). We checked its activity in the complex formation with the HIV-2 Tat protein. An RNA-protein complex was observed (Fig. 5). The above results indicate that two RNA hammerhead ribozymes are sufficient for the intramolecular self-cleavage of TAR RNA and that this intron molecule acquires an active structure required for interactions with protein(s).

The method described here involves RNA scissors and is based on manipulation with GUC hammerhead ribozymes. It can be used to prepare any homogeneous RNA with predetermined termini. Such an approach offers a new possibility for the generation *in vivo* any RNA molecule of interest. One would expect them to act as decoys to sequester some proteins *e.g.* Tat binding proteins. On the other hand, there is a big problem with ribozymes targeting to cells due to their sensitivity towards ribonucleases, and therefore it is difficult to predict whether or not a ribozyme will arrive at the target sequence. A longer RNA molecule with a strong folded structure should be generally more resistant to nucleases (*38*) and the probability is much higher of getting it at the right site, where *e.g.* an RNA aptamer should be cleaved off.

As one can conclude from the conditions for the ribozyme catalysed reaction, it required renaturation to acquire the RNA active conformation. This means that the RNA transcript folds preferentially into a non-ribozymic structure without catalytic activity. This is reasonable, because it is well known that TAR RNA forms a stable stem-loop structure. If it is so, we can ask the question of how the T7 RNA transcript is folded. We applied the Zuker algorithm (*39*) to calculate the secondary structures of the T7 RNA transcript of 183 nt long (Fig. 1). Each calculated secondary structure can be verified because from the results of many biochemical and crystallographic studies it is known that an efficient cleavage reaction occurs only when the GUC target sequence of the ribozyme is in the extended conformation and the C residue is unpaired (*13-15*). Surprisingly none of the calculated structures with the lowest energy fulfilled the structural requirements for the GUC hammerhead ribozyme activity (Fig. 6A). We selected 9 structures for further examination (Fig. 6 and Table I). Generally, two families of putative structures appeared. Those with the lowest energy contain the GUC sequence in the double-stranded portion of RNA that forms an extension of the TAR-element stem. These RNA molecules do not show catalytic activity (Fig. 6A). In the second group, each TAR RNA structure contains only one ribozyme at either the 5′ or 3′ end, *i.e.* not at both (Fig. 6B). Inspection of the gel bands (Fig. 3) confirmed that longer precursors of 115 nt (cleavaged at the 3′ site) or 103 nt (cleavaged at the 5′ site) are formed. This suggests that in this case folding of the hammerhead ribozymes is not a very favourable process, probably due to the strong secondary structure of TAR. It is noteworthy that the structures containing the extended TAR-RNA stem have about 4 kcal/mol lower energies than those with hammerhead motifs. The formation of the stable RNA structure within the transcript (non-ribozymic) clearly explains the low efficiency of the cleavage and the required repeated renaturation conditions for TAR-RNA preparation. There is also the possibility of unusual interactions between cleavage products (*40*).

Our calculations also show that some variability in the structures of stems I and III may influence folding of the catalytic core of the ribozymes. There is also the general conclusion that computer calculations do not provide the correct secondary structure of a transcript with two active GUC hammerhead ribozymes. Therefore, one has to be very careful when only using folding programs to predict the secondary structure of a long RNA and on that basis the functional consequences of a molecule.

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