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Ribozyme mediated destruction of RNA *in vivo*

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Previous studies have demonstrated that high ribozyme to substrate ratios are required for ribozyme inhibitory function in nuclear extracts. To obtain high intracellular levels of ribozymes, tRNA genes, known to be highly expressed in most tissues, have been modified for use as ribozyme expression cassettes. Ribozyme coding sequences were placed between the A and the B box, internal promoter sequences of a *Xenopus* tRNA^{Met} gene. When injected into the nucleus of frog oocytes, the ribozyme tRNA gene (ribtDNA) produces 'hammerhead' ribozymes which cleave the 5′ sequences of U7snRNA, its target substrate, with high efficiency *in vitro*. Oocytes were coinjected with ribtDNA, U7snRNA and control substrate RNA devoid of a cleavage sequence. It was found that the ribtRNA remained localized mainly in the nucleus, whereas the substrate and the control RNA exited rapidly into the cytoplasm. However, sufficient ribtRNA migrated into the cytoplasm to cleave, and destroy, the U7snRNA. Thus, the action of targeted 'hammerhead' ribozymes *in vivo* is demonstrated.

Key words: gene expression/ribozyme/RNA degradation

Introduction

Ribozymes are RNAs which are capable of catalyzing RNA cleavage reactions (Cech, 1987). From early studies of the self-cleaving plant viroids and satellite RNAs (Buzayan *et al.*, 1986) Haseloff and Gerlach (1988) have established simple rules for the design of short RNA molecules with ribozyme activity which are capable of cleaving other RNA molecules in *trans* in a highly sequence specific way. Since such 'hammerhead' ribozymes can be targeted to many different kinds of sequences, in fact to virtually all kinds of RNA (Koizumi *et al.*, 1988a,b), these custom-designed ribozymes provide highly flexible tools to inhibit the expression of specific genes. Ribozymes may therefore supply an attractive alternative to antisense constructs (reviewed by Weintraub *et al.*, 1985) whose capacity to inhibit translation is of potential therapeutic value (reviewed by Zon, 1988; Marcus-Sekura, 1988).

The targeted cleavage of RNA by ribozymes in *trans* has not as yet been observed *in vivo*. It has been shown, however, that the cleavage of U7snRNA by U7snRNA-targeted ribozymes requires a relatively high concentration of ribozymes to inhibit 3′ processing of histone pre-mRNA in *in vitro* extracts, as compared for instance to complementary DNA or RNA oligomers, despite the catalytic properties of ribozymes (Cotten *et al.*, 1989). The build-up of a concentration of ribozymes sufficient to elicit biological effects may in most instances become a limiting factor *in vivo*. If it were possible to introduce ribozyme synthesizing genes into the cell rather than the ribozyme itself, a considerable amplification would be achieved because such genes would produce a great many ribozymes and thus replenish the pools of ribozymes destroyed by nuclease activity.

We have chosen to test ribozyme tRNA genes in a *Xenopus* oocyte system. The large size of the oocyte nucleus allows one to introduce test genes and substrates by microinjection bypassing the variability of standard transfections into tissue culture systems, and eliminating the high background of untransfected cells obtained with most transient transfection methods. The large size of the cell facilitates the fractionation of nucleus and cytoplasmic material so that we can determine the cellular compartmentalization of gene products. Furthermore, the microinjection technique allows us to precisely control the amount of DNA and RNA that we are introducing into each cell. Frog oocytes have previously been used to great advantage for the study of antisense oligonucleotide dependent mRNA cleavage (Jessus *et al.*, 1988; Shuttleworth and Coleman, 1988; Shuttleworth *et al.*, 1988).

Here we show that the transfer RNA genes, transcribed by pol III, are suitable 'cassette' genes to express such ribozymes. Their small size (less than two hundred base pairs including the ribozyme coding sequence), their high rate of transcription and ubiquitous expression in different kinds of tissues make them good candidates for expressing ribozyme sequences. Earlier studies have demonstrated the utility of the pol III VAI gene for the expression of antisense RNA (Jennings and Molloy, 1987).

We find that a very compact ribozyme producing gene unit can be constructed by simply placing the ribozyme coding sequences between the A and B block (Galli *et al.*, 1981; Hofstetter *et al.*, 1981) of a tDNA^{Met}-gene. This gene, when injected into frog oocyte nuclei produces ribozymes *in vivo*. The ribozymes thus generated remain localized, in the main, inside the cell nucleus and are shown to be capable of cleaving the target sequence both *in vivo* and *in vitro*.

Results

Rationale for the use of tDNA genes as cassettes for the expression of ribozymes

tDNA genes with their gene internal regulatory sequences have a high density of genetic information. This was observed initially, when it was found that a tDNA unit with as little as 22 base pairs 5′ to the structural gene supports a high level of transcription in frog oocytes (Telford *et al.*, 1979). Linker scan mutants further suggest that in most, but not all (reviewed by Sharp *et al.*, 1985), tDNA genes, essentially all that is required for faithful production of tRNA

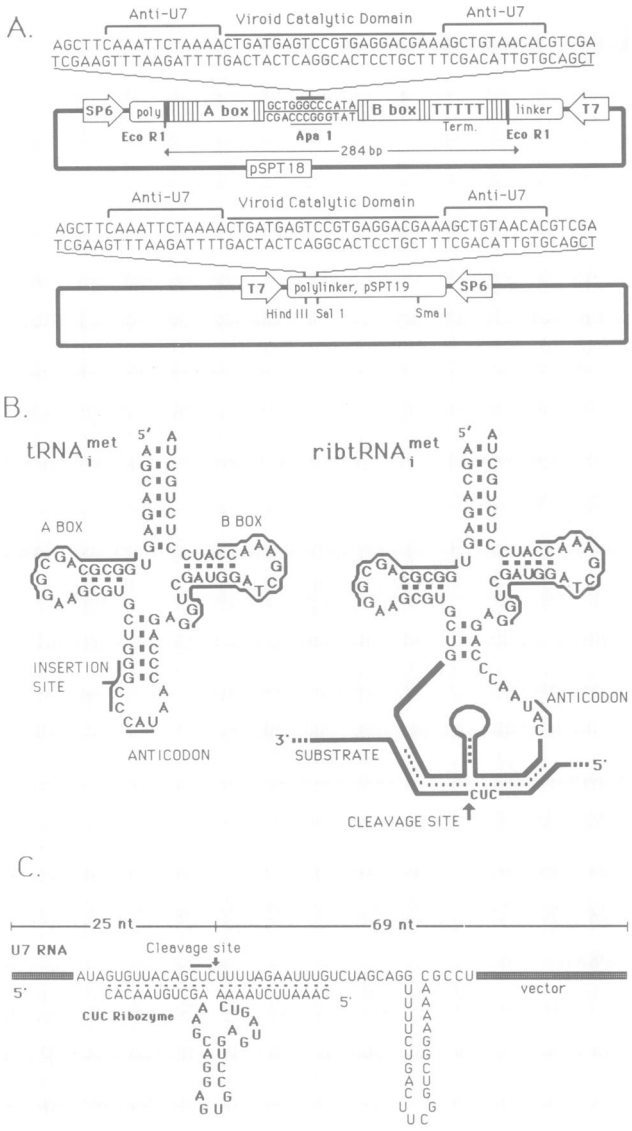

Fig. 1. Sequences used in this study. **(A) Upper portion**. The sequence of ribozyme encoding DNA oligonucleotides inserted into the *Apa*I site of *Xenopus* $tRNA^{Met}_i$ gene to generate $pribtRNA^{Met}_i$. The catalytic domain and U7 complementary portions of the ribozyme gene are indicated. **Lower portion**. The same ribozyme encoding oligonucleotides were inserted into the *Hin*dIII/*Sal*I sites of pSPT19 (Boehringer Mannheim) for use in generating a linear (non-tRNA) ribozyme. T7 polymerase transcription was performed with a *Sma*I-linearized plasmid. **(B) Left portion**. The cloverleaf structure of $tRNA^{Met}$ indicating the folding pattern, the A and B box, the anticodon and the insertion site of the ribozyme. **Right portion**. A possible secondary structure for the $ribtRNA^{Met}$. Note that in the cloning procedure used here the internal GGCC sequence of the *Apa*I site was removed before addition of the ribozyme sequence. Not shown is the 3′ terminal CCA sequence found in the mature tRNA. **(C)** The sequence of the target U7snRNA indicating the base-pairing interactions between the ribozyme and substrate RNA. 10 nucleotides of 5′ vector sequence and 21 nucleotides of 3′ vector sequence are indicated by the filled box.

is the tRNA coding sequence followed by a run of T residues, the latter functioning as a terminator signal of transcription (reviewed by Geiduschek and Tocchini-Valentini, 1988). The promoter of the tRNA genes is split (Kressmann *et al.*, 1979) and is, in the main, made up from the A and the B box sequences (Galli *et al.*, 1981; Hofstetter *et al.*, 1981). The DNA intervening between the A and the B box appears not be be crucial for promotion of transcription. However, when the A and the B box are placed too close to one another in the tDNA unit, transcription is reduced (Hofstetter *et al.*, 1981). When the distance is increased by insertion of a small amount of DNA between them there is no major effect on transcription (Hofstetter *et al.*, 1981), but when more than 50 intervening base pairs are added, the accumulation of mutant tRNA is much reduced (Ciliberto *et al.*, 1982).

tRNA genes are small, are transcribed at a high rate in almost all tissues and would not be expected, upon integration into the chromosome, to enhance transcription of adjacent genes nor permit read-through transcription into adjacent genes (any run of 5 or more T residues acting as a stop signal; see Geiduschek and Tocchini-Valentini, 1988). Due to these properties tDNA genes make ideal, very compact, cassettes for the expression of ribozyme sequences. We have tested this concept by constructing a ribozyme-containing $tRNA^{Met}$ gene ($ribtRNA^{Met}$) targeted to the 5′ sequences of the U7snRNA which is known to be essential for the 3′ processing reaction during histone pre-mRNA maturation (reviewed by Birnstiel *et al.*, 1985). We have also synthesized U7 RNA *in vitro* and coinjected both the ribtDNA gene and U7snRNA molecules into the nucleus of the oocyte to measure the ribozyme mediated cleavage and destruction of the U7snRNA *in vivo*.

For the construction of the ribozyme containing tRNA genes a unique *Apa*1 site in the *Xenopus* $tRNA^{Met}$ gene, lying between the A and the B box provided a suitable site of insertion for ribozyme coding sequences (see Figure 1 and Materials and methods). Note that this same insertion site was used for the initial studies outlining the tRNA gene promoter (Kressmann *et al.*, 1979). Although for the experiments described in this paper we have used the complete 284 bp *Eco*RI fragment, we have subsequently removed 5′ and 3′ nonessential sequences thus generating a 95 bp minimum tRNA gene, and a 145 bp ribtRNA gene (unpublished results).

Time course of $ribtRNA^{Met}$ and $tRNA^{Met}$ synthesis and intracellular location of gene products

We wanted to compare the relative transcriptional activity of the wt RNA^{Met} and $ribtRNA^{Met}$ genes and assess the stability of the respective transcripts in frog oocytes. For this we injected wt and ribtDNA in a molar ratio of 1:10 together with ^{32}P[GTP] and measured the pools of radioactive RNA over several days.

For the interpretation of the results it should be taken into consideration that injected ^{32}P[GTP] is diluted out by the endogenous pool of 150 pmol GTP (Woodland and Pestell, 1972; La Marca *et al.*, 1973) and that the initial specific radioactivity of the GTP is diminished during the course of incubation due to the synthesis of new GTP by the oocyte. Thus, after approximately 2 days incubation 'chase' conditions are established as witnessed previously by the disappearance of labeled, short-lived pre-$tRNA^{Met}$ (Kressmann *et al.*, 1979).

The apparent initial rate of GTP incorporation into $ribtRNA^{Met}$, on a per gene basis, is about 14 times lower compared to $tRNA^{Met}$. This may be an underestimate of the real rate for two reasons: first, $ribtRNA^{Met}$ has a short half life and is considerably less stable than $tRNA^{Met}$ (see below). At 6 h a considerable portion of the newly synthesized $ribtRNA^{Met}$ must therefore have already turned over. Second, a situation may prevail in these experiments

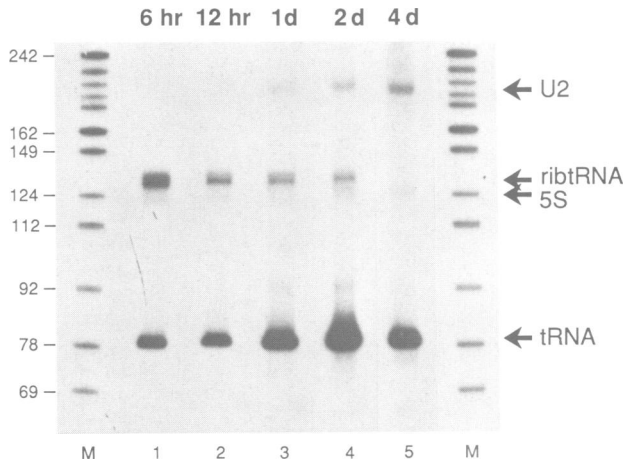

Fig. 2. Time course of tRNA and ribtRNA accumulation. (**A**) Oocytes were injected, in the nucleus, with a solution containing 2 μCi/μl ^{32}P[GTP], 0.1 μg/μl pSTP18tDNA^{Met} and 1 μg/μl pSTP18ribtDNA^{Met}. At the indicated times, pools of 6–14 oocytes were harvested and the nucleic acids were purified. The nucleic acid equivalent of two oocytes were loaded in each lane. **Lane 1**, material from oocytes at 6 h post-injection. **Lane 2**, 12 h post-injection. **Lane 3**, 1 day post-injection. **Lane 4**, 2 days post-injection. **Lane 5**, 4 days post-injection. **Lanes M**, *Hpa*II-cut pBR322 labeled with α-^{32}P[dGTP] and Klenow, the molecular weights (in nucleotides) of some of the fragments are indicated at the left of the gel. The migration of the tRNA^{Met}, the ribtRNA endogenous U2 (**U2**) and endogenous 5S RNA (**5S**) are indicated to the right of the figure.

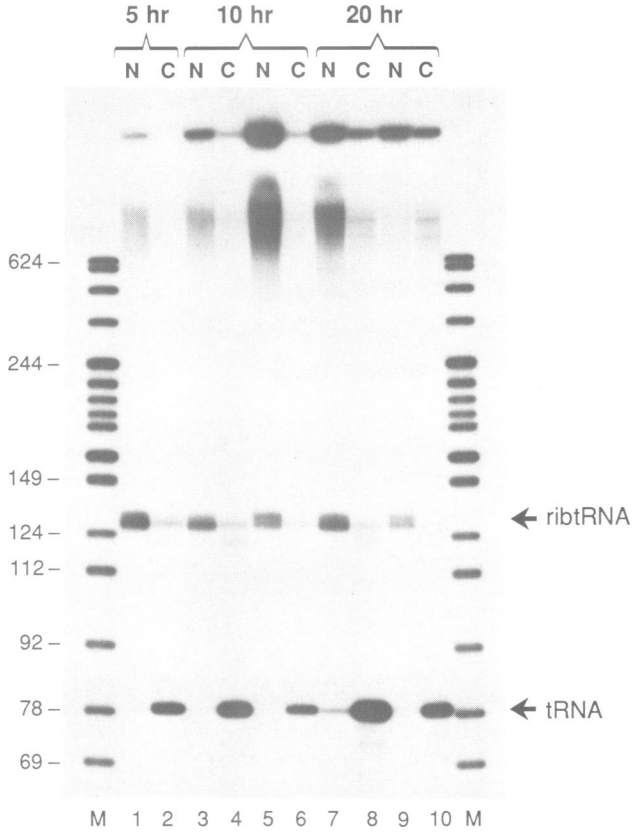

Fig. 3. Time course for the nuclear exit of tRNA ribozyme. Oocytes were injected, in the nucleus, with a solution containing 2 μCi/μl ^{32}P[GTP], 0.1 μg/μl pSPT18tDNA^{Met} and 1 μg/μl pSPT18ribtDNA^{Met}. At the indicated times individual oocytes were harvested and submerged in a boiling bath for 3 min. The nucleus of each oocyte was removed by microdissection and the nucleic acids in the nucleus and in the cytoplasm were purified and resolved by electrophoresis (Georgiev *et al.*, 1984). **Lanes 1** and **2**, the nuclear and cytoplasmic material from an oocyte 5 hours post-injection. **Lanes 3–6**, two pairs of nuclear and cytoplasmic material from oocytes 10 h post-injection. **Lanes 7–10**, two pairs of nuclear and cytoplasmic material from oocytes 20 h post-injection. **Lanes M**, molecular weight markers as in Figure 2. The migration of the wild-type tRNA^{Met} and the ribtRNA^{Met} are indicated at the right of the figure.

in which competition between mutant and wt tRNA genes for the same transcription factors put the mutant gene at a disadvantage for transcription. It has been demonstrated using an *in vitro* transcription system, that mutant tRNA genes containing insertions between the A and the B box have a decreased affinity for transcription factors (Dingermann *et al.*, 1983).

Note that in Figure 2, the labeled pools of tRNA^{Met} which has a half life of several days (Kressmann *et al.*, 1979) increase in amount till day 2 and then decrease. Even at early time points synthesis and turnover of the ribtRNA^{Met} largely counterbalance each other, and in this way the labeled RNA pools remain more or less constant until such time as chase conditions are established. At day 4 no ribtRNA^{Met} remains, but note the presence of tRNA^{Met} and of the stable endogenous 5S and U2 RNA.

Next we determined the intracellular location of the labeled RNAs. For this, injected oocytes incubated for the periods indicated in Figure 3 were boiled for 3 min and nucleus and cytoplasm prepared manually (Georgiev *et al.*, 1984). As can be seen from the distribution of the heterogenous nuclear RNA, the procedure yields a cytoplasm free from nuclear RNA (compare lanes 1 and 2 of Figure 3). After 5 h ribtRNA^{Met} is mainly nuclear, while tRNA^{Met} nearly exclusively cytoplasmic. Only a minor portion of ribtRNA^{Met} migrates into the cytoplasm and its cytoplasmic pool remains small, presumably as a consequence of high turnover and/or because of a diminishing migration into the cytoplasm.

ribtRNA^{Met} cleaves the substrate in vitro

We then wanted to establish that the ribozyme placed within the tRNA moiety and generated *in vivo* within frog oocytes was still capable of cleaving an appropriate substrate.

In order to compare the cleavage efficiency on a molar basis, ^{32}P[ribtRNA] was generated in the oocyte, isolated after 5 h incubation and purified by electrophoresis. The specific activity of the GTP in the oocyte was calculated from the amount of ^{32}P[GTP] injected and the GTP pool of the oocyte (see Kressmann *et al.*, 1978 and refs therein). Ribozyme in linear, non-tRNA form, was produced by *in vitro*, T7 polymerase transcription of a linear plasmid containing the ribozyme encoding DNA (Figure 1a.) and was isolated in OD quantities by gel electrophoresis. Both types of ribozymes, in approximately equal concentration, were reacted *in vitro* with U7snRNA generated by *in vitro* transcription. As shown in Figure 4, on a molar basis, both ribozyme and ribtRNA^{Met}, were similarly efficient in cleaving the U7snRNA. The 69 and 25 nucleotide cleavage products are clearly visible in this *in vitro* reaction. Hence the oocyte produced a ribtRNA^{Met} which on a molar basis was equally active in substrate cleavage as the linear ribozyme produced *in vitro* (see Materials and methods section).

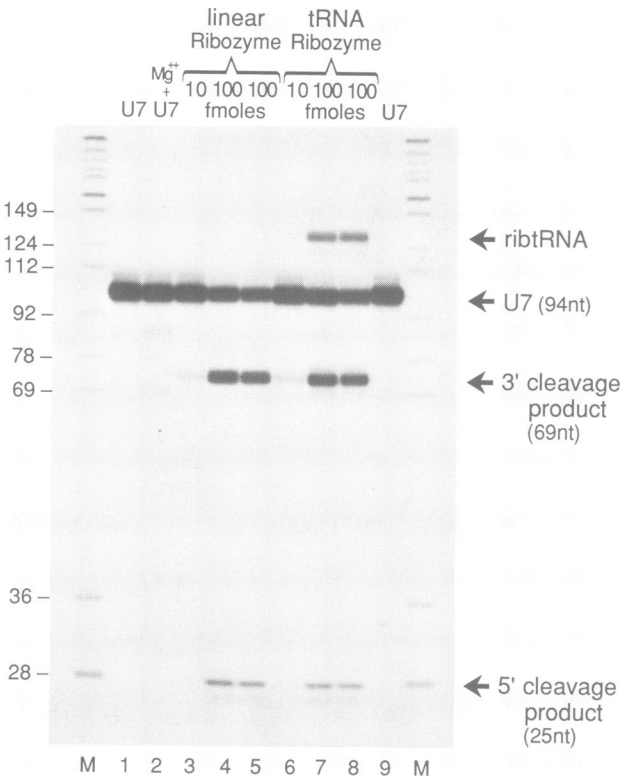

Fig. 4. *In vitro* activity of *in vivo* synthesized ribozyme. The ribtRNA was purified from oocytes injected with ribtDNA and quantified as described in the Materials and methods section. A ^{32}P-labeled RNA molecule (100 fmol) containing the U7 sequence was incubated at 37°C in 150 mM NaCl, 20 mM Tris, pH 7.5, 10 mM $MgCl_2$ in the presence of various amounts of an *in vitro* synthesized (T7 polymerase) linear ribozyme or an *in vivo* synthesized tRNA ribozyme. After incubation for 90 min, EDTA was added to 20 mM, the samples were dried and resolved by electrophoresis. **Lanes 1** and **9** U7 RNA, unincubated. **Lane 2**, U7 RNA incubated at 37°C with magnesium. **Lane 3**, U7 plus 10 fmol of linear ribozyme. **Lanes 4** and **5**, U7 incubated with 100 fmol of linear ribozyme. **Lane 6**, U7 with 10 fmol of ribtRNA. **Lanes 7** and **8**, U7 with 100 fmol of ribtRNA. **Lanes M**, molecular weight markers as in Figure 2. The migration of the ribtRNA, the intact U7 RNA and the 3′ and 5′ cleavage products are indicated at the right of the figure.

ribtRNA^{Met} cleaves the substrate in vivo

In order to test the activity of the ribtRNA *in vivo* a mixture of plasmids containing ribtDNA^{Met} and tDNA^{Met} (in a molar ratio of 10:1) was coinjected together with ^{32}P[GTP] labeled U7snRNA (substrate for the ribtRNA) and a labeled control RNA (a sequence from the human EGF receptor message) not capable of being cleaved by the ribozyme. After 5, 10 and 20 h nuclei and cytoplasm were prepared and analyzed separately (Figure 5).

We observe that the control RNA as well as the *in vitro* synthesized injected U7snRNA exit from the nucleus within the first 5 h and from then on these RNA species are found exclusively in the cytoplasm of the oocyte. The behaviour of ribtRNA and tRNA^{Met} is as described in the experiment of Figure 2. Labeled 5S and U2 RNA are seen in most oocytes mainly in the cytoplasm in which they are assembled into or stored as RNP particles (Mattaj, 1988).

As far as the ribtRNA and the U7snRNA substrate are concerned an interesting correlation exists. At early times of incubation both may coexist in the cytoplasm (Figure 5, lane 3) but where there is an appreciable amount of ribtRNA, no U7snRNA can be detected even at early times (Figure

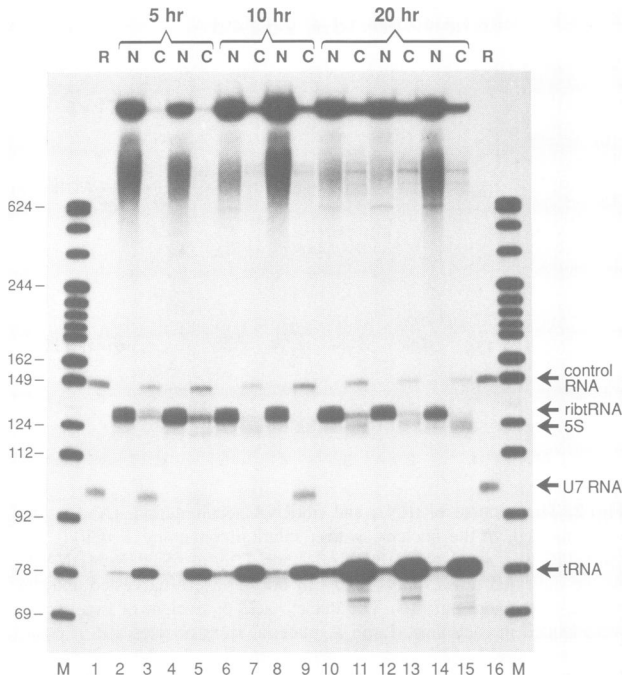

Fig. 5. *In vivo* cleavage activity of ribtRNA. Oocytes were injected, in the nucleus, with a solution containing 2 μCi/μl ^{32}P[GTP], 0.1 μg/μl pSPT18tDNA^{Met}, 1 μg/μl pSPT18ribtDNA^{Met}, 30 000 c.p.m./μl *in vitro* synthesized U7 RNA and 30 000 c.p.m./μl *in vitro* synthesized control RNA. At the indicated times, individual oocytes were harvested and immersed in a boiling water bath for 3 min. The nucleus was removed by microdissection and the nucleic acids from the nucleus and cytoplasm were purified and resolved by electrophoresis. The target substrate was synthesized by *in vitro* T7 polymerase transcription using GTP at 400 Ci/mMol. Hence the specific activity of the target transcript is approximately 1000-fold higher than that of RNA synthesized in the oocyte. The control RNA is a similarly prepared transcript corresponding to 91 nucleotides spanning the human EGF receptor message translation initiation codon (nucleotides 181 to 265 in Ullrich *et al.*, 1984). **Lanes 1** and **16**, an aliquot of the *in vitro* synthesized U7 RNA and control RNA. **Lanes 2–5**, nuclear and cytoplasmic material from two oocytes harvested at 5 h post-injection. **Lanes 6–9**, nuclear and cytoplasmic material from 2 oocytes, 10 h post-injection. **Lanes 10–15**, nuclear and cytoplasmic material from 3 oocytes, 20 h post-injection. **Lanes M**, molecular weight markers as in Figure 2. The migration of control RNA, U7 RNA, the ribtRNA ribozyme and the tRNA^{Met} are indicated at the right of the figure.

5, lane 5). The U7snRNA cleavage products cannot be detected but note the specificity of the reaction in that neither the coinjected control RNA without a cleavage sequence nor the 5S nor the U2 RNA are cleaved. At 10 h and 20 h, in general, no U7snRNA can be detected on the gels with the exception of lane 9 where, for reasons unknown, there is no detectable ribtRNA in the cytoplasm of the oocyte. Furthermore, in oocytes in which the injection was into the cytoplasm and therefore no transcription of the ribozyme tRNA gene occurred, both the target and the control RNA are equally present at 20 h post-injection (results not shown). Thus, there is an excellent correlation between the appearance of ribtRNA in the cytoplasm and the destruction of the cytoplasmically located U7snRNA. The reaction of the ribozyme with the substrate after homogenization and during RNA extraction is excluded by the presence of high levels of EDTA throughout the sample preparation (see Cotten *et al.*, 1989). The *in vivo* action of ribozyme generated within the oocyte is thus demonstrated.

Discussion

In this paper we investigate the question of whether ribozymes can be used to cleave an RNA target *in vivo*. *In vitro* nuclear extract studies (Cotten *et al.*, 1989) have previously suggested that relatively high ratios of ribozyme to substrate concentration may be necessary to obtain cleavage in a proteinacious milieu and that the stability of the ribozyme could be a major issue. We argued that on theoretical grounds tRNA genes would provide appropriate cassettes for expressing ribozymal RNA sequences at high levels within the living cell, in a tissue non-specific manner, and also considered it possible that a ribozyme embedded in a tRNA might prove more stable than its linear form.

From the incorporation of GTP it can be calculated (Kressmann *et al.*, 1978 and refs therein) that an oocyte injected with ribtDNA genes accumulates ribtRNA molecules of the order of 10^{10} after a 5 h incubation. In other experiments (M.Cotten and M.Zenke, unpublished results) we observed pools of approximately 10^5 ribtRNA molecules in tissue culture cells transfected with ribtDNA constructs. Thus, as predicted, high intracellular levels of ribtRNA are easily attained. However, we have as yet little evidence that placing the ribozyme within a tRNA structure enhances its stability since ribtRNA^{Met} appears to decay more rapidly than tRNA^{Met}. However, the ribtRNA^{Met} has a greater stability than linear ribozyme when assayed in a nuclear extract (results not shown).

The ribtRNA, on a molar basis, proved to have a similar cleavage activity to the linear ribozyme synthesized *in vitro*. This is surprising because posttranscriptional nucleotide modifications are common in tRNAs (reviewed by Geiduschek and Tocchini-Valentini, 1988). If there are any modifications in the ribtRNA, these must have occurred in regions where such changes are tolerated without effect on cleavage activity. If the tRNA molecule had preserved its folded structure, the ribozyme sequences would have been under topological constraints preventing intertwining with the substrate RNA. Since the ribtRNA appears to be fully active we must assume that probably it is in a relatively unfolded form. This open structure may explain several additional features of the ribtRNA. First, we observe that the ribtRNA remains predominantly in its pre-tRNA form which is poorly processsed. Thus, the precursor ribtRNA may be hindered in its interaction with nuclear factors required for both 5′ and 3′ processing and for 3′ terminal CCA addition (Melton *et al.*, 1980). Second, we note a lower stability of the RNA in the living cell as compared to the genuine tRNA^{Met}; third, the ribtRNA is sufficiently altered in its structure to remain inside the nucelus with only a small proportion transported into the cytoplasm. Such a faulty compartmentalization has been previously observed for certain mutant tRNAs (Zasloff *et al.*, 1982). Nevertheless, the accumulation of ribtRNA in the nucleus will be beneficial in those experiments in which the ribozyme is targeted to splice sites, introns or polyadenylation signals, but will be less desirable where the RNA to be cleaved is located in the cytoplasm.

The coinjected U7snRNA, bearing a mono-methyl G cap and terminating in a palindrome structure is relatively stable in the oocyte presumably being protected against the action of exonuclease through the structural features. This is apparently not true once the U7snRNA is cleaved, but the cleavage products are easily detected after cleavage of the RNA in a nuclear extract (Cotten *et al.*, 1989).

In contrast to the ribtRNA, the U7snRNA exits rapidly into the cytoplasm and in this it conforms to the behaviour of other U RNAs injected into the frog oocyte nucleus, as reviewed by Mattaj (1988). It is possible that similarly the U7snRNP returns into the nucleus after cytoplasmic assembly, where it would be efficiently destroyed by the large nuclear pools of ribtRNA.

Fortuitously, injected oocytes differ from one another in their capacity to release ribtRNA into the cytoplasm. This allows us to gauge the efficiency of ribozymes cleaving the substrate *in vivo*. In some oocytes, and especially during shorter incubation times, ribozymic molecules and substrate may coexist in the cytoplasm as shown for instance in lane 3 of Figure 5. In lane 3 a similar level of radioactivity is detected autoradiographically for both ribtRNA and its cleavable RNA. The cleavable substrate was synthesized *in vitro* with GTP at 400 Ci/mMol undiluted by the internal GTP pools of the oocyte while, for ribtRNA synthesis *in vivo*, we have injected 60 nCi into an oocyte containing approximately 150 pmol GTP producing a specific activity of GTP of approx. 0.4 Ci/mMol. Therefore, the specific activities of these RNAs differ by a factor of approximately 1000. It can be calculated from the radioactivity that $\sim 10^9$ cytoplasmic ribozymes are found in the same cellular compartment as $\sim 5 \times 10^6$ (of 2×10^7 injected) molecules of its substrate, i.e. that the cleavage reaction must be relatively inefficient. This finding is consistent with our previous *in vitro* results (Cotten *et al.*, 1989) which showed, unexpectedly, that a 500 to 1000-fold excess of ribozymes over U7snRNA substrate was required to cleave U7snRNA and to eradicate 3′ processing *in vitro*. The persistence of the substrate at long incubation times is exceptional, as for instance in lane 9, and probably due to the unusually low level of ribtRNA in the cytoplasm of that particular oocyte.

tRNA genes can also be used to express antisense RNA at high level (X.Fu and M.L.Birnstiel, unpublished results). The ribtRNA contains complementary sequences to the substrate and can be viewed as such antisense molecules. Since we have not been able to demonstrate the presence of specific cleavage products in the oocyte, it remains a possibility that the ribtRNA acts here through an antisense mechanism. We think this to be less likely because at least in nuclear extracts antisense inhibition of U7 RNA is a reversible reaction not leading to the destruction of the substrate (Cotten *et al.*, 1989).

The requirement for relatively high levels of ribozymes *in vitro* (Cotten *et al.*, 1989) and *in vivo* (these experiments) means that the success of the approach of inhibiting specific gene activities with ribozymes, will depend critically on a high level of import or of expression of ribozymal sequences in the cell. Such a high level of expression is provided by the tRNA cassette genes. Since these genes are compact, all information lying within 200 nucleotides (or less), it should be possible to use concatemers of ribtRNA genes targeted to multiple sites within pre-mRNAs. Thus, it may be possible to elicit an intracellular immunity (Baltimore, 1988) against viruses in cells and organisms by means of introducing appropriate clusters of ribtRNA genes into somatic cells or into the germ line of animals and plants.

Materials and methods

Construction of tRNA ribozyme genes

The methionine initiator 1 tRNA gene of *Xenopus*, present on a 284 bp *Eco*RI fragment cloned into pBR322 (the *Hin*fI H and G fragments, Telford *et al.*, 1979; Hofstetter *et al.*, 1981) was isolated by *Eco*RI digestion of the pBR322 vector, gel purified and ligated into the *Eco*RI site of the bacterial plasmid pSPT18 (Boehringer Mannheim) such that a sense tRNA transcript could be obtained with an SP6 polymerase transcription reaction of the plasmid (see Figure 1). Standard cloning procedures, essentially as described by Maniatis *et al.*, (1982), were used to obtain pSPT18tDNA^{Met1}.

The tRNA gene of pSPT18tDNA^{Met1} was cleaved at the unique *Apa*I site in the anticodon stem and loop (see Figure 1), the single stranded DNA at the cleavage site was removed by treatment with T4 DNA polymerase in the presence of deoxynucleotide triphosphates and the 5′ phosphates were removed by treatment with calf intestinal phosphatase. Double-stranded synthetic DNA oligonucleotides encoding the viroid-derived cleavage sequence (Haseloff and Gerlach, 1988) flanked by complementarities to the target RNA sequences (see Figure 1) were obtained as single-stranded oligonucleotides synthesized by standard phosphoramidite chemistry. Complementary oligonucleotides were phosphorylated, annealed, ligated into *Apa*I-cleaved pSPT18tDNA^{Met1} and cloned using standard methods (Maniatis *et al.*, 1982) to yield pSPT18ribtDNA^{Met1}. The presence of active ribozyme sequences on the cloned plasmid DNA was ascertained in two ways: (i) RNA molecules derived from *in vitro* SP6 transcription of cloned DNA plasmids were incubated with a labeled RNA containing the ribozyme target sequence and assayed for specific cleavage of the target RNA. (ii) The presence of correctly inserted DNA sequences was verified by dideoxyDNA sequencing across the insertion site.

Oocyte microinjection

Transcription of wild-type and ribtRNA genes microinjected into *Xenopus* oocytes was performed as previously described (Kressman *et al.*, 1978; Kressmann and Birnstiel, 1980; Hofstetter *et al.*, 1981). A brief description follows: stage VI oocytes were obtained from adult *Xenopus laevis* injected two weeks earlier with 50 to 100 units/frog of Pregnyl. The oocytes were centrifuged to position the nucleus at the top of the oocyte. Each oocyte nucleus was injected with 30 nl of a solution containing 1 mg/ml supercoiled plasmid DNA containing the ribtRNA gene plus 2 μCi/μl ^{32}P[GTP] (400 Ci/mMole) in 80 mM NaCl, 10 mM Tris−HCl, pH 7.5. In these experiments the wild-type methionine tRNA gene was present at 1/10 the ribtRNA gene concentration. All plasmid DNAs used for this study were purified by two CsCl gradient centrifugations followed by an extensive dialysis against 20 mM HEPES, pH 8, to remove CsCl and EDTA after the second gradient. After incubation at 20−23°C, injected oocytes were homogenized in 1% SDS, 1 mg/ml proteinase K, 300 mM NaCl, 20 mM Tris−HCl, pH 8, 20 mM EDTA (110 μl/oocyte) digested at 56°C for 45 min with frequent vortex agitation, extracted once with phenol, once with phenol−chloroform and precipitated with ethanol. The collected ethanol precipitates were dissolved in 80% deionized formamide−1X TBE, heated for 30 s at 95°C to denature and resolved by electrophoresis on a preheated 9.7% acrylamide, 8.3 M urea, TBE gel and exposed to X-ray film. TBE buffer (Tris, Borate, EDTA) was prepared as described in Maniatis *et al.* (1982).

In vitro ribozyme cleavage

RNA molecules were generated by transcription with T7 RNA polymerase of linear DNA templates and purified by gel electrophoresis. A 94 nt RNA molecule containing the U7 sequence (Figure 1a, cf. Cotten *et al.*, 1989) was used as a test substrate for the anti-U7 ribozyme. A sample of 100 fmol of the target RNA (10 000 cpm) was incubated with various quantities of test ribozymes, at 37°C in the presence of 150 mM NaCl, 10 mM $MgCl_2$, 20 mM Tris, pH 7.5 for 90 min. The reactions were stopped by the addition of EDTA to 20 mM, the samples were dried, dissolved in 80% deionized formamide−1X TBE, heated to 90°C for 30 s to denature and resolved by electrophoresis as described above.

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