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Cytoplasmic delivery of ribozymes leads to efficient reduction in α-lactalbumin mRNA levels in C127I mouse cells

Phillip J.L'Huillier[1], Stephen R.Davis[1] and A.Richard Bellamy

Centre for Gene Technology, School of Biological Sciences, University of Auckland, Private Bag 92019, Auckland and [1]Dairy Research Corporation, Ruakura Agricultural Centre, Private Bag, Hamilton, New Zealand

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Ribozymes targeted to five sites along the α-lactalbumin (α-lac) mRNA were delivered to the cytoplasm of mouse C127I mammary cells using the T7-vaccinia virus delivery system and the amount of α-lac mRNA was monitored 24-48 h post-transfection. Three target sites were selected in the α-lac coding region (nucleotides 15, 145 and 361) and two were located in the 3′ non-coding region (nucleotides 442 and 694). Acting *in trans* and at a target:ribozyme ratio of 1:1000, ribozymes targeting sites 361 and 694 reduced α-lac mRNA by >80%; another two ribozymes (targeting nucleotides 442 and 145) reduced mRNA levels by 80 and 60% respectively; the fifth ribozyme (targeting nucleotide 15, near the AUG) was largely ineffective. The kinetic activity (k_{cat}) of each ribozyme *in vitro* was somewhat predictive of the activity of the two ribozymes that targeted nucleotides 361 and 694, but was not predictive of the *in vivo* activity of the other three ribozymes. Down-regulation of the intracellular levels of α-lac paralleled the ribozyme-dependent reduction achieved for mRNA. For site 442, the reduction in both mRNA and protein was attributed to the catalytic activity of the ribozyme rather than to the antisense effects of the flanking arms, because delivery of an engineered (catalytically-inactive) variant had no effect on mRNA levels and a minimal effect on the level of α-lac present in the cell.

Key words: α-lactalbumin/hairpin ribozyme/hammerhead ribozyme/T7 RNA polymerase/vaccinia virus

Introduction

Ribozymes are small RNA molecules capable of highly specific catalytic cleavage of RNA. Ribozyme-mediated cleavage *in trans* was first demonstrated *in vitro* by Ulhenbeck (1987) and subsequently by Haseloff and Gerlach (1988). In viroids and virusoids, the reaction is intramolecular (Symons, 1989). However, ribozymes that possess a catalytic domain and flanking sequences complementary to the target mRNA can cleave *in trans* provided that a three base sequence (GUX) occurs within the target molecule (Haseloff and Gerlach, 1988).

Targeted cleavage of mRNA by ribozymes acting *in trans* has proved difficult to demonstrate *in vivo*. A high excess (100- to 1000-fold) of ribozyme over target transcripts has been required to achieve a detectable decrease in the level of target RNA transcripts (Cameron and Jennings, 1989; Cotten and Birnstiel, 1989; Sioud and Drlica, 1991). To achieve high endogenous expression of stable ribozymes, Cotten and Birnstiel (1989) inserted a ribozyme into the *Xenopus* $tRNA^{Met}$ gene, whilst Cameron and Jennings (1989) inserted ribozyme sequences into the 3′ non-coding region of the luciferase gene. Both studies showed a reduction of the targeted RNA, but since these authors were unable to detect the products of ribozyme-mediated cleavage, they were unable to eliminate the possibility that an 'antisense action' rather than ribozyme cleavage was responsible for the effects observed.

Recently, Sarver *et al.* (1990) and Scanlon *et al.* (1991) demonstrated a reduction in the level of HIV-1 *gag* RNA and c-*fos* mRNA in cell lines expressing hammerhead ribozymes. Furthermore, Saxena and Ackerman (1990) obtained cleavage of the α-sarcin domain of 28S RNA when both the target and ribozyme RNA was injected into *Xenopus* oocytes. However, a catalytically active ribozyme did not reduce endogenous 28S rRNA synthesis any more than did a catalytically inactive (control) ribozyme. In contrast, Steinecke *et al.* (1992) have shown that transient expression of a catalytically active ribozyme in plant protoplasts completely abolished neomycin phosphotransferase gene expression, but 46% of this effect was attributable to the antisense activity of the flanking sequences.

Here we report the effects of ribozymes delivered to mammalian cells using the T7-vaccinia delivery system (Fuerst *et al.*, 1986). This system utilizes the vaccinia virus to deliver T7 RNA polymerase to the cytoplasm of the cell, and mRNA transcribed under the control of the T7 promoter can constitute up to 30% of total cytoplasmic RNA (Elroy-Stein *et al.*, 1989). We have exploited this transient high level of synthesis to demonstrate ribozyme-dependent reduction of target mRNA within the cell. Our results demonstrate that at inferred target:ribozyme ratios of 1:1000, intracellular α-lactalbumin (α-lac) mRNA levels can be reduced in some cases by >80% and for α-lac mRNA, targeting sites in the 3′ non-coding region or those on exposed stem-loop regions is more effective than targeting the region near the initiation codon.

Results

Sites in α-lac mRNA targeted with ribozymes

Five sites within the α-lac transcript were targeted with hammerhead ribozymes that possessed two 12 bp flanking sequences complementary to the α-lac transcript (Figure 1A). At one of these sites (nucleotide 442) we also directed a catalytically inactive (control) hammerhead ribozyme (RZ4c) that was identical to ribozyme 4a, but contained one base substitution in the catalytic domain (Figure 1D). This yielded a transcript that enabled us to distinguish antisense effects on mRNA or protein

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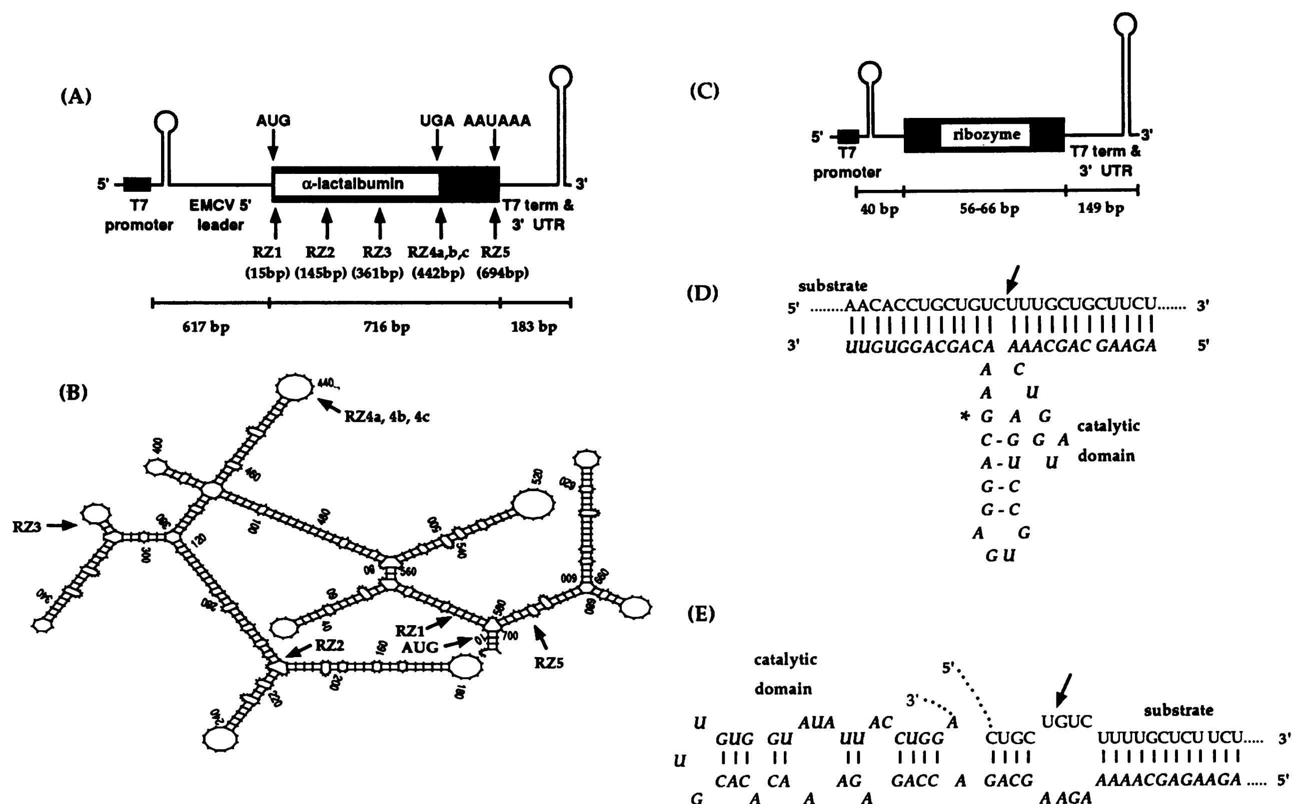

Fig. 1. Target sites, transcript structures and ribozymes. **(A)** Structure of α-lac transcript and position of cleavage sites. RZ1, 2, 3, 4a and 5 are hammerhead ribozymes (Haseloff and Gerlach, 1988) that possess two 12 bp antisense flanking sequences. RZ4b is a hairpin ribozyme (Hampel *et al.*, 1990) with 4 and 12 bp antisense flanking sequences. RZ4c is a hammerhead ribozyme with a one base substitution (see panel D) rendering it catalytically inactive. Numbers in brackets indicate the location of the U in each GUX target site, with numbering taken from the start of the α-lac sequence (nucleotides 9–11 represent AUG initiation codon). **(B)** Predicted secondary structure for α-lac mRNA showing position of ribozyme cleavage sites. Folding was inferred from the algorithm of Zuker and Stiegler (1981). Arrows indicate cleavage sites targeted. **(C)** Structure of pTF29 ribozyme transcripts. Ribozyme sequences were cloned into vector pTF29 cut with *Kpn*I and *Sal*I (or *Kpn*I and *Pst*I for RZ2). Transcripts possess 40 bp of (5') and 149 bp of (3') flanking stem–loop prokaryotic sequences. **(D)** Structure of hammerhead ribozyme, RZ4a (Haseloff and Gerlach, 1988) containing two 12 bp flanking sequences (asterisk indicates single base substitution (G to U) used to inactivate catalytic activity in the variant form RZ4c, see text). **(E)** Hairpin ribozyme, RZ4b (Hampel *et al.*, 1990) with four (5') and 12 (3') flanking bases. Arrows in (D) and (E) indicate cleavage sites; bold italics type indicates ribozyme sequences, normal type indicates α-lac target sequences. EMCV indicates sequences derived from encephalomyocarditis virus (Elroy-Stein *et al.*, 1989). For details of ribozyme target sequences see Table II.

accumulation from those which resulted from true ribozyme catalysis. A hairpin ribozyme (Hampel *et al.*, 1990), which possessed 4 and 12 bp antisense flanking sequences directing it to site 442 (RZ4b), was also synthesized (Figure 1E).

Accessibility of target sites to ribozymes may be an important factor influencing ribozyme activity (Heidenreich and Eckstein, 1992; Xing and Whitton, 1992). Furthermore, different mRNAs appear to differ in their susceptibility to antisense RNA or oligonucleotides, as do different regions within a particular mRNA (Izant and Weintraub, 1984; Melton, 1985; Strickland *et al.*, 1988). For this reason we targeted ribozymes # 1 and # 5 (sites at nucleotides 15 and 694 respectively) to regions near the AUG start codon and the 3' polyadenylation signal respectively (Figure 1A). The target sites for the remaining three ribozymes were identified from examination of a putative secondary structure of the α-lac transcript generated by a computer algorithm (Zuker and Stiegler, 1981). Two of these ribozymes (RZs 3 and 4) targeted sites that were located in or near apparent exposed stem–loop regions within the transcript (nucleotides 361 and 442), whilst the remaining ribozyme target site (RZ2, nucleotide 145) was located in a region of predicted base pairing near the 5' end of the transcript (Figure 1B).

Ribozymes cleave α-lac transcripts in vitro

To confirm that all ribozymes were capable of cleaving the α-lac target mRNA *in vitro*, the catalytic activity of each ribozyme was investigated using 'runoff' transcripts synthesized *in vitro*. The target α-lac transcript expressed from plasmid pTM1α-lac possessed the same upstream 5' (leader) sequences present on the intracellular transcripts, but only 40 bp of the (3') 183 bp intracellular T7 terminator untranslated sequence. To compare the cleavage efficiency of each ribozyme on an equimolar basis, transcripts were synthesized in the presence of [α-^{32}P]UTP, purified by electrophoresis on an acrylamide gel (when necessary) and equivalent amounts of each ribozyme were used to determine catalytic activity.

All ribozymes cleaved the substrate *in vitro* and generated fragments of the expected sizes (Figure 2). For example RZ1 (nucleotide 15) and RZ2 (nucleotide 145) generated the anticipated fragments (762 bp and 605 bp or 632 bp and 735 bp respectively, Figure 2). As RZ5 cleaves at the 3' end of the α-lac sequence, it was difficult to separate the 5' cleavage product from the full length transcripts (Figure 2). However, the 5' cleavage product could be detected on gels run for a longer length of time, confirming the catalytic

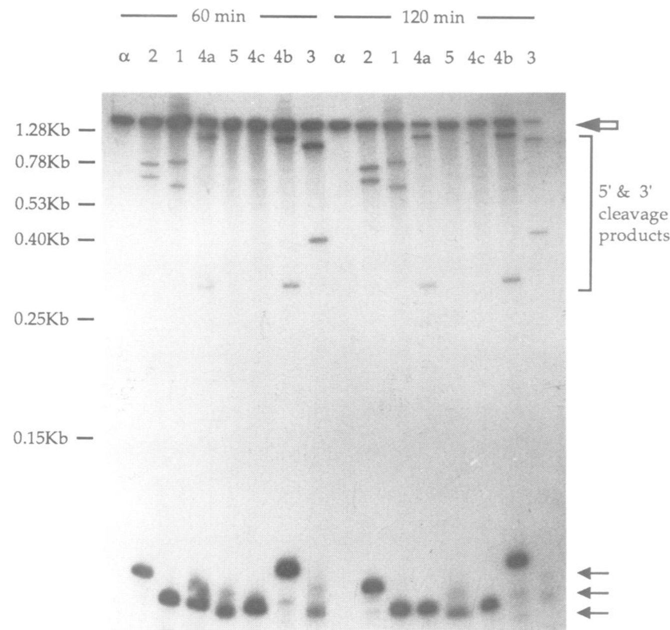

Fig. 2. Cleavage of α-lac *in vitro* by six different catalytically active ribozyme. ^{32}P-labelled α-lac transcripts (5.0 pmol) were incubated at 37°C in 50 mM Tris–HCl pH 8.0, 20 mM $MgCl_2$ buffer with ^{32}P-labelled ribozymes (15.0 pmol). After incubation for 60 or 120 min, EDTA was added to 25 mM, the samples fractionated on a 5% polyacrylamide–7M urea gel, autoradiographed, the bands excized and the radioactivity measured. Numbers refer to the six catalytically active ribozymes (1, 2, 3, 4a, 4b and 5) and the catalytically-inactive variant 4c. α-lac transcripts (α) are in lanes 1 and 9 (no ribozyme present). For details of ribozyme design and target sites, see Figure 1, Table II and text. Solid arrows, ribozyme transcripts; open arrow, full length α-lac transcript. Position of molecular weight markers are shown on the left (Life Technologies).

activity of this ribozyme (data not shown). Thus the five hammerhead ribozymes targeting different sites within the α-lac transcript were all found to be catalytically active, but they appeared to show different cleavage efficiencies *in vitro*.

A priori, the *in vitro* catalytic activity of a particular ribozyme should be influenced by factors such as the accessibility of the target site and the ability of the flanking arms of the ribozyme to dissociate from the target sequences (Fedor and Uhlenbeck, 1990). The marked variation in activity observed *in vivo* (see below) therefore might reflect differences in the intrinsic K_m and k_{cat} of each ribozyme. Alternatively, the different activities observed may result from the secondary structure of the mRNA. We therefore derived K_m and k_{cat} values (Table I) for each ribozyme *in vitro* using labelled transcripts and methods similar to those summarized in Figure 2. The highest k_{cat} values were obtained for RZ5 and RZ3 which targeted the 3′ non-coding region (nucleotide 694) and a putative stem–loop region (nucleotide 361) respectively. As will become apparent below, the k_{cat} values determined *in vitro* were not entirely predictive of the ribozyme-mediated reduction in mRNA achieved *in vivo*.

Table I. *In vitro* ribozyme kinetics and *in vivo* reduction in α-lac mRNA

Ribozyme (#)	K_m (μM)[a]	k_{cat} (mol/min)[a]	k_{cat}/k_m (μM^{-1} min^{-1})[a]	*In vivo* mRNA (% of control)[b]
1	0.17	0.3	1.8	85
2	0.27	0.4	1.5	39
3	0.62	1.1	1.8	18
4a	0.28	0.3	1.1	29
4b	0.28	0.5	1.8	19
4c	–	–	–	90
5	0.41	1.5	3.7	15

[a]Determined at 37°C in 50 mM Tris pH 8.0 and 20 mM $MgCl_2$ buffer (see Materials and methods).
[b]Normalized against control (no ribozyme) data from Figure 3C.
– represents not determined.

Ribozymes reduce mRNA levels in vivo

Ribozyme and α-lac plasmid DNA were transfected into vvTF7-3-infected C127I cells to examine the activity of ribozymes *in vivo*. Total RNA was extracted after 24–48 h incubation and examined by Northern analysis. Preliminary experiments showed that target mRNA levels were greatest at 24–48 h after transfection (data not shown).

The degree of reduction of target transcripts achieved by the different ribozymes varied from site to site. RZs 3, 4a and 5 exerted the greatest effect, estimated to represent an ~70–90% reduction compared with the control (Figure 3a and 3C; Table II). In contrast, RZ2 reduced α-lac mRNA by ~60%, whilst RZ1, which targeted the 5′ end of the transcript, yielded only a small reduction (~15%) in the level of α-lac mRNA. At site 442 (near the termination codon, in the 3′ non-coding region) both the hammerhead and hairpin ribozymes (RZ4a and 4b respectively) reduced the amount of mRNA present in the cell, with the hairpin ribozyme exerting a somewhat greater effect than did the hammerhead ribozyme (Figure 3C).

Reduction in mRNA in vivo is dependent on the ratio of ribozyme to target

The ratio of ribozyme to target has previously been shown to influence significantly the efficiency of both antisense RNA and ribozyme-mediated reduction of target mRNA

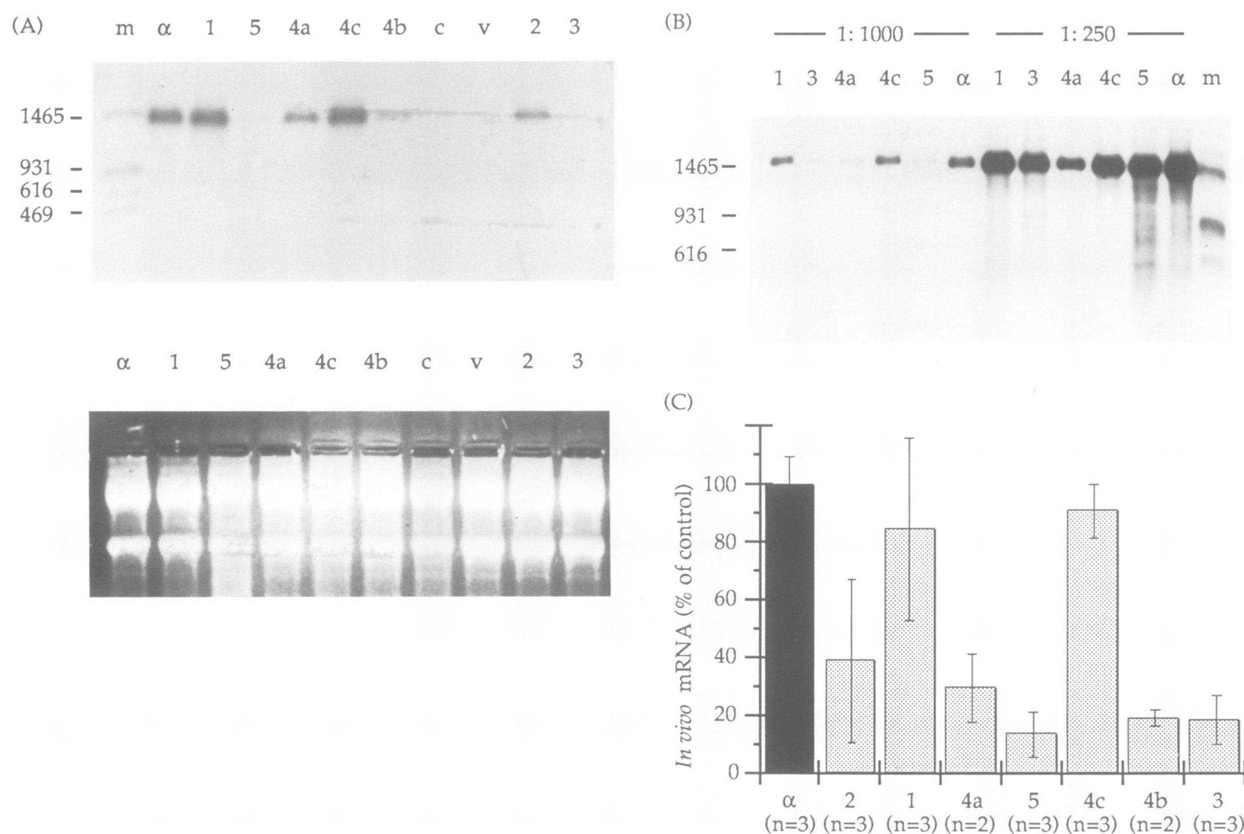

Fig 3. Ribozyme-mediated reduction of α-lac mRNA transcripts *in vivo*. C127I cells were infected with vvTF7-3 followed by transfection with a mixture of pTM1 (α-lac) and pTF29 (ribozyme) plasmids. After 24–48 h incubation, RNA was extracted and Northern analysis carried out using a ^{32}P-labelled α-lac cDNA probe. **(A)** Upper panel: Northern analysis of α-lac mRNA in cells synthesizing both target and ribozyme transcripts. Transfections used a target:ribozyme plasmid DNA ratio of 1:1000. Lower panel: formaldehyde gel stained with ethidium bromide prior to blotting showing the 28S and 18S ribosomal RNA, and indicating approximately equal loading for all samples on the gel. **(B)** A separate experiment but using the two different target:ribozyme ratios indicated. The ratios were achieved by holding the amount of 'ribozyme' plasmid constant and increasing the amount of 'target' plasmid. **(C)** Results from three separate experiments [data from (A) and (B) have been included] using target:ribozyme (plasmid) ratios of 1:1000. Transcripts remaining intact 24–48 h after transfection of the ribozyme plasmid have been normalized against α-lac transcripts in cells that did not receive ribozyme. Error bars indicate range in densitometric values between experiments. C, cells only; v, cells plus vvTF7–3. Other lanes received mRNA from cells receiving α, α-lac or α-lac plus hammerhead ribozymes (1, 2, 3, 4a and 5), hairpin ribozyme (4b) or catalytically inactive hammerhead ribozyme (4c). M, markers of DNA of known molecular weight.

Table II. Target sequences for ribozymes

Ribozyme (#)	Type	Target site (nucleotide)	Location	Ribozyme flanking sequences[a] (5'/3')	Target sequences
1	Hammerhead	15	near AUG	12/12	5'-CCAAAATGATGTC*CTTTGTCTCTCT-3'
2	Hammerhead	145	coding	12/12	5'-GCCTGAATGGGTC*TGTACCACGTTT-3'
3	Hammerhead	361	coding/stem–loop	12/12	5'-TCTGGATAAAGTA*GGAATTAACTAC-3'
4a	Hammerhead	442	near UGA/stem–loop	12/12	5'-AACACCTGCTGTC*TTTGCTGCTTCT-3'
4b	Hairpin	442	near UGA/stem–loop	4/12	5'-CTGCT*GTCTTTGCTGCTTCT-3'
4c	Hammerhead	442	near UGA/stem–loop	12/12	5'-AACACCTGCTGTC*TTTGCTGCTTCT-3'
5	Hammerhead	694	3' non-coding	12/12	5'-TTCCACTTTTGTC*CCGAATAAAAGC-3'

[a]Number of nucleotides in 5' and 3' flanking arms that are complementary to the α-lac target sequence.
*Represents the 3'-phosphate on the 3'-terminal nucleotide following the cleavage reaction.

(Cotten *et al.*, 1989). Although the ratio of input plasmid DNAs can be controlled precisely in transfection experiments, the ratio of the ribozyme RNA and target transcripts can only be inferred. Within this constraint, we varied the ratio of ribozyme to target by altering the ratio of plasmid DNA transfected into the cells. The ratios were achieved by holding the amount of 'ribozyme' plasmid constant and increasing the amount of target plasmid. Only when the ratio 250:1 (ribozyme:target) was reached was a small reduction in the amount of α-lac mRNA observed (Figure 3b). However, increasing the ratio to yield an inferred 1000-fold excess of ribozyme over target resulted in a significant effect on target RNA (Figure 3b). A 500-fold excess of ribozyme over target transcripts also yielded a detectable effect on the α-lac transcripts, particularly for RZs 4A, 4B and 5 (data not shown).

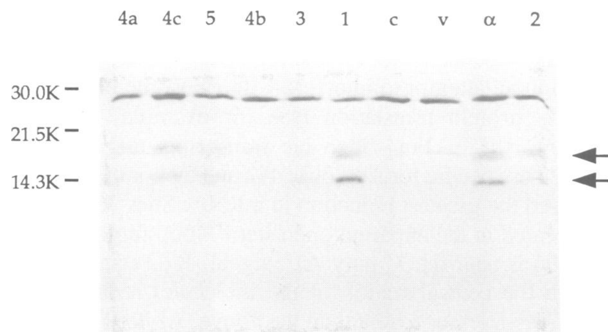

Fig. 4. Reduction in α-lac in cells transfected with ribozyme plasmids. C127I cells were infected with vvTF7-3 and transfected with plasmid DNA as described for Figure 3. After 48 h incubation, cells were harvested and proteins resolved on a 15% SDS–polyacrylamide gel followed by Western analysis to detect α-lac. C, cells only; v, cells plus vvTF7-3; α, α-lac. Other lanes received extracts from cells receiving α-lac plus hammerhead ribozymes (1, 2, 3, 4a and 5), hairpin ribozyme (4b) or catalytically inactive hammerhead ribozyme (4c). Arrows indicate glycosylated and non-glycosylated forms of α-lac (Barman, 1970). Molecular weights estimated from the positions of proteins of known molecular weights (Rainbow markers, Amersham), shown at left. A cellular protein at 30 kDa cross-reacts with the anti-α-lac antibody and is present in all lanes (see lane c).

Ribozyme-dependent reduction in α-lac accumulation in vivo

To investigate whether the reduction achieved in α-lac mRNA was paralleled by a reduction in the α-lac accumulating in the cell, extracts were prepared from cells harvested 48 h after transfection and examined by Western analysis. For most samples examined, the ribozyme-mediated reduction in α-lac paralleled that found for mRNA (Figure 4). The 5′ cleavage fragment produced by cleavage at nucleotide 694 contained an intact α-lac coding sequence and this may account for the slightly higher amount of α-lac present in cells that received ribozyme #5 than might be anticipated on the basis of the full length mRNA transcript detected in the cells (compare results for RZ5 in Figures 3A and 4 respectively).

Does the reduction in α-lac result from ribozyme cleavage or from antisense action?

The substantial flanking sequences present on the ribozymes (12/12 bp and 12/4 bp for the hammerhead and hairpin ribozymes respectively) raises the possibility that the effects on protein accumulation may result from antisense effects rather than true ribozyme catalysis. We therefore investigated this aspect by constructing a mutated form of the (otherwise) active ribozyme #4 (targeting nucleotide 442). This mutated ribozyme (RZ4c) is identical to RZ4a but contains a catalytic domain that has been altered by a single base substitution at nucleotide 5 of the catalytic domain (see Figure 1D). This base substitution yielded no detectable effect on the inferred secondary structure of the ribozyme when the modified molecule was examined using the computer algorithm of Zuker and Stiegler (1981; data not shown) and the mutation completely abolished catalytic activity *in vitro* (Figure 2). When the catalytically inactive ribozyme (RZ4c) was delivered to cells, it produced only a small reduction in the level of α-lac mRNA (Figure 3C) compared with that achieved by the catalytically active molecules targeted to the same site (RZs 4a and 4b, Figure 3C). The catalytically-inactive ribozyme did, however, show some reduction in

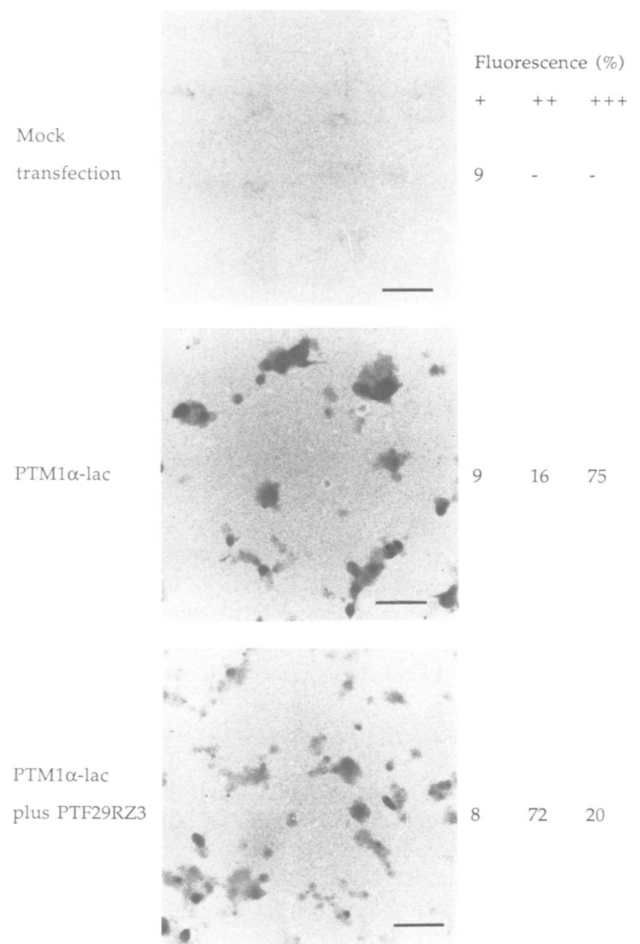

Fig. 5. Immunofluorescent staining of α-lac in individual cells transfected with ribozyme plasmids. C127I cells were infected with vvTF7–3 to deliver T7 RNA polymerase followed by transfection with plasmid DNA. After 48 h, cells were fixed, permeabilized and stained with an anti-human α-lac antiserum (see text). 250 randomly-selected, but 'intact' cells were scored for the intensity of fluorescence using an arbitrary three point scale (values are recorded at right). Extensive cell cytopathic effect is evident at 48 h and is caused by the vaccinia infection. Low levels of fluorescence in control cells (mock transfection, upper panel) presumably reflects endogenous α-lac present in C127I mammary cells (see Figure 4, lanes 7 and 8). The scale bar is 50 μm.

α-lac protein (Figure 4), an effect we attribute to antisense effects created by the extensive complementary flanking sequences. However, the reduction observed for protein synthesis represented only ~25% of the total effect observed for the corresponding catalytically active ribozymes targeted at the same site.

Individual cells show evidence for ribozyme activity

The ribozyme-dependent reduction in α-lac RNA and protein observed above could be interpreted in two possible ways. On the one hand, mRNA in all cells could be reduced, for example, by 75%. On the other hand, 75% of cells could have transcripts completely cleaved, with 25% of the cells remaining totally refractory to the presence of the ribozyme. We therefore examined the effect of ribozymes on individual cells by staining cells to detect α-lac using a fluorescein-conjugated antibody (Figure 5, panel 2).

Even though the activity of the T7–vaccinia virus creates considerable cytopathic effect, ~75% of cells clearly had

expressed α-lac at a high level over the preceding 48 h as judged by the intensity of fluorescence (Figure 5). For cells receiving ribozyme, the number of cells fluorescing at the same high level was reduced to 20% of the total. These 'refractory' cells, which retained the ability to express α-lac, fluoresced just as strongly as did control cells, indicating that the accumulation of α-lac in some cells was totally prevented, whereas in others it appeared to be unaffected. The presence of these refractory cells may be due to the fact that the uptake of transfected DNA by cells probably follows a Poisson distribution. Thus, a low proportion of cells would incorporate only one plasmid whilst others probably receive both plasmids. The ratio of target:ribozyme plasmid therefore would vary over a wide range (Wigler *et al.*, 1979). An alternative explanation for the presence of refractory cells could be that some cells are differentially susceptible to ribozyme-mediated reduction of protein synthesis due to differences in the stage of the cell cycle that they have reached. Thus the cell fluorescence results suggest that the ribozyme-mediated reduction in α-lac mRNA transcripts and protein observed by Northern and Western analysis almost certainly represent an underestimate of the overall efficacy of the individual ribozymes studied.

Discussion

Delivery of ribozymes to mammalian cells using the transient T7−vaccinia system has a number of advantages for the study of the intracellular activity of ribozymes in vertebrate cells. First, vaccinia virus replicates in the cytoplasm of the cell, a simpler environment for the study of ribozyme action in view of the complications created by nuclear RNA processing and the consequential transport of spliced mRNA to the cytoplasm. Delivery of transcripts to the cytoplasm may therefore avoid the complications observed by Cotten and Birnstiel (1989) who found that while the target RNA rapidly exited from the nucleus, the ribozyme transcripts remained almost exclusively intra-nuclear. Secondly, the (prokaryotic) 5′ and 3′ flanking sequences associated with both the target and ribozyme transcripts of the T7−vaccinia delivery system are relatively small and well defined. Thus, ribozyme activity can be examined in a situation where the 5′ and 3′ flanking sequences are both able to be defined and unlikely to interfere either with the folding of the catalytic domain or with the antisense flanks which direct the ribozyme to its target site. Thirdly, the 5′ stem−loop sequences associated with the T7−vaccinia transcripts are known to provide enhanced stability to mRNA transcripts (half-life of ~75 min for β-galactosidase cDNA, Fuerst and Moss, 1989). Other workers (Cameron and Jennings, 1989) have found it necessary to incorporate their ribozyme sequences within the 3′ untranslated region of the luciferase gene to provide for transcript stability.

Within the cell, the substrate mRNA presumably is in a highly folded structure that is extensively base paired. The mRNA may also be protected by protein bound to a substantial length of the molecule. Given this context, the question of accessibility of the substrate to a ribozyme is of particular relevance. Individual target mRNA transcripts are differentially susceptible to interaction with antisense oligonucleotides or RNA (Melton, 1985; Izant and Weintraub, 1984) and regions within a particular transcript may be differentially accessible (Goodchild *et al.*, 1988; Strickland *et al.*, 1988). Many studies have targeted antisense RNA or oligonucleotides to the region around the AUG start codon in an attempt to interfere with ribosome binding and thereby protein translation (see for example, Stout and Caskey, 1987). For the α-lac transcripts targeted here, ribozymes specific for sites 361, 442 and 694 of the transcript achieved the greatest reduction in mRNA. Sites 361 and 442 are located in the apparent open stem−loop regions, whilst site 694 is near the 3′ poly(A)$^+$ signal. RZ2 (targeting site 145 in the central region of the molecule) reduced α-lac mRNA to a lesser extent than the above, while RZ1 (which targeted the 5′ AUG region) showed no measurable effect on α-lac mRNA levels. The targeting of similar regions of other mRNAs with ribozymes should provide information concerning the extent to which the preliminary accessibility rules developed here for α-lac can be applied to other mRNA transcripts. For example, Xing and Whitton (1992) studied the cleavage of arenavirus RNA and found that three out of four apparent stem−loop regions were cleaved efficiently *in trans*. However these authors studied cleavage *in vitro* and it is not clear to what extent sites accessible *in vitro* will also be accessible *in vivo*.

Although RZ1 did not reduce the level of target mRNA *in vivo* it nevertheless was able to cleave the target RNA *in vitro* (Figure 2) and thus it appears that this transcript is able to fold into a catalytically active structure. Therefore, the 5′ AUG region of the α-lac transcript appears to be inaccessible to ribozymes within the cell. This could reflect the presence of ribosomal or protein binding sites around the AUG; alternatively it may reflect the secondary structure in this region of the molecule. The latter explanation is less probable given the observation that RZ1 cleaved the substrate transcript *in vitro*. The low catalytic rate of RZ1 *in vitro* (Table I) is likely to be a contributing factor. At site 442 the hairpin ribozyme reduced α-lac mRNA to a greater extent than did the hammerhead ribozyme. This may reflect the number of flanking bases on each ribozyme, which results from the different design parameters of the two types of ribozyme (12/12 bp versus 12/4 bp for the hammerhead and hairpin ribozymes respectively).

The most effective ribozyme target site identified in this work (cleaved by RZ # 5) lies downstream of the termination codon and ~10 bp upstream of the polyadenylation signal (nucleotide 694). It is well established (Jackson and Standart, 1990) that 3′ untranslated sequences can exert a stabilizing effect on mRNA and are also involved in the regulation of translation. Whether the 3′ untranslated region might constitute a generally applicable site for ribozyme targeting of mRNA is not clear and further work would be required to determine whether this 'rule' for α-lac applies generally to other mRNAs.

In this work we have distinguished effects due to true catalytic activity from those that may be due to antisense action by engineering a catalytically inactive form (RZ4c) of the otherwise active RZ # 4. A similar approach has been adopted by Saxena and Ackerman (1990) and Steinecke *et al.* (1992) who used a catalytically inactive ribozyme targeted to transcripts of the α-sarcin domain of 28S RNA or the neomycin phosphotransferase gene respectively. Although antisense effects of RZ4c were evident when α-lac levels were examined by Western analysis (Figure 4), only minor effects were detected by Northern analysis. In plant protoplasts, Steinecke *et al.* (1992) observed a larger

antisense effect (46%) than that found in this study (~25%). This may reflect the generally greater effects of antisense on gene expression in plants compared with animal cells (Stout and Caskey 1987; van der Krol *et al.*, 1988). Thus the substantial effects of the ribozymes studied here are almost certainly due to transcript cleavage although we could not detect cleavage products *in vivo*. However, in some cases (data not shown), low levels of putative cleavage fragments were observed on some gels. Our failure to detect cleavage products routinely, probably results from the intracellular instability of the cleavage fragments (Cameron and Jennings, 1989).

Finally, the results obtained from the immunofluorescent detection of α-lac in individual cells to which ribozymes were delivered, indicates that Northern analysis of the total cellular mRNA may have provided an underestimate of the efficacy of the individual ribozymes studied in this work. The results presented in Figure 5 indicate that although RZ3 reduced the level of protein by ~80% overall, a subset of cells nevertheless continued to fluoresce as strongly as did the controls. Further work will be required to provide a definitive explanation for this phenomenon but it is clear that for some of the ribozymes studied here, protein synthesis in some cells was essentially totally repressed.

Materials and methods

Viruses and cells

C127I mouse mammary cells and CV1 monkey kidney cells were obtained from the American Type Culture Collection. Both C127I and CV1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. Recombinant vaccinia virus (vvTF7-3) incorporating the bacteriophage T7 gene 1 (Fuerst *et al.*, 1986) was supplied by B.Moss. Virus was propagated in CV1 and C127I cells using standard techniques (Fuerst *et al.*, 1986).

Plasmids and cloning

Plasmid pTF29 was constructed by inserting an additional 29 bp polylinker sequence that incorporated a *Kpn*I, *Eco*RV, *Pst*I and *Sal*I multi-cloning site at the *Bam*HI site of pTF7-5 (Fuerst *et al.*, 1987). Plasmid pTM1 contained an additional 579 bp of the encephalomyocarditis virus genome, which improves the efficiency of translation of uncapped RNA (Elroy-Stein *et al.*, 1989).

Bovine α-lac cDNA (Hurley and Schuler, 1987) was modified by PCR to remove CG tailing and introduce *Nco*I and *Bam*HI restriction sites at the 5' and 3' termini respectively (Sambrook *et al.*, 1989). The full length α-lac PCR product was digested with *Nco*I and *Bam*HI and ligated with *Nco*I and *Bam*HI digested and dephosphorylated pTM1 plasmid DNA to yield pTM1α-lac.

Ribozyme sequences were synthesized as oligonucleotides using automated phosphoramidite chemistry. The 56- and 68mer hammerhead and hairpin ribozyme oligonucleotides were phosphorylated using [α-^{32}P]ATP, purified by electrophoresis on a 12% polyacrylamide gel and ligated to *Kpn*I and *Sal*I or *Kpn*I and *Eco*RI digested Bluescript SK+ (Sambrook *et al.*, 1989). Second strand synthesis was performed by incubating ligated plasmid with M-MLV reverse transcriptase or Klenow DNA polymerase I. Full length ribozyme inserts were subsequently subcloned into *Kpn*I and *Sal*I digested pTF29 plasmid (*Kpn*I and *Pst*I digested pTF29 plasmid for RZ#2). The sequences of all ribozyme constructs were confirmed by dideoxy sequencing methods using an Applied Biosystems model 373A automated sequencer.

In vitro transcription and in vitro cleavage reactions

Ribozyme and α-lac plasmids were linearized using *Sal*I. Transcription reactions were performed in the presence of [α-^{32}P]UTP using T7 RNA polymerase (Promega) as recommended by the supplier. The transcription reactions were then incubated with DNase I, extracted with phenol–chloroform and the RNA recovered by ethanol precipitation.

For *in vitro* cleavage reactions, α-lac and ribozyme transcripts were incubated in 50 mM Tris–HCl pH 8.0, 20 mM $MgCl_2$ at 37°C. The products of the reaction were resolved by electrophoresis on a 5% polyacrylamide–7 M urea gel and detected by autoradiography.

For determination of K_m and k_{cat} values, steady-state rates of cleavage were measured (as described above) at six substrate concentrations (0.006–0.22 μM) for each ribozyme. K_m and V_{max} values were determined from Lineweaver–Burk plots using linear regression analysis.

Transcription of α-lac mRNA and ribozymes in C127I cells

C127I mouse cells were grown to sub-confluence (~95%) in 10 cm plastic dishes and infected with recombinant vaccinia virus (vvTF7-3) at a multiplicity of 10–20 p.f.u/cell. The virus was adsorbed for 1 h at 37°C and the inoculum then rinsed from the cells by two consecutive washes with DMEM. Three millilitres of DMEM were then added that contained a total of 10–50 μg of varying ratios of α-lac and ribozyme plasmid DNA that had been complexed with 50 μg of a cationic liposome reagent (Dotap Boeringher-Mannheim). After 5–18 h at 37°C, a further 3 ml of DMEM containing 20% fetal calf serum were added to the cells. Cells were harvested at 24–48 h post-virus infection for RNA analysis or 48 h for immunoblotting and immunofluorescence.

RNA extraction and Northern analysis

Transfected cells were washed with PBS and total cellular RNA was extracted using a modified version of the guanidinium–phenol method (Chomczynski and Sacchi, 1987). Cells were lysed with 4 M guanidinium and extracted once with water saturated phenol:chloroform (10:1) and twice with phenol:chloroform (1:1) followed by isopropanol precipitation. RNA samples were fractionated on a 2.2 M formaldehyde–agarose gel and transferred to Hybond N+ nylon membrane. Northern hybridization was carried out with ^{32}P-labelled probes using the method described by Church and Gilbert (1984) as modified by Westneat *et al.* (1988). α-lac cDNA sequences were labelled by standard random-priming techniques using Klenow DNA polymerase I (Sambrook *et al.*, 1989).

Immunoblotting and immunofluorescence

For immunoblotting, transfected cells were washed in PBS, followed by lysis in an equal volume of SDS Laemmli application buffer containing 2-mercaptoenthanol, and fractionation on a 15% SDS–polyacrylamide gel (Laemmli, 1970). Proteins were transferred to nitrocellulose by standard electroblotting techniques (Towbin *et al.*, 1979). Membranes were blocked with 0.2% Tween 20 and incubated with a rabbit anti-human α-lac polyclonal antisera (Sigma Chemical Company) followed by donkey anti-rabbit Ig and streptavidin-biotinylated horseradish peroxidase complex (Amersham). α-lac protein was detected using the enhanced chemiluminesence method (Amersham) followed by autoradiography.

For immunofluorescence, cells were grown within tissue plates on glass microscope coverslips and infected with vaccinia virus (vvTF7-3) followed by transfection with plasmid DNA as described above. At 48 h after virus infection, coverslips were removed from tissue culture plates, the cells were washed with PBS, fixed in 10% fresh paraformaldehyde and permeabilized with 0.2% Triton X-100. The cells were then blocked with non-immune goat serum followed by reaction with a rabbit anti-human α-lac antiserum. A fluorescein isothiocyanate-labelled goat antiserum prepared against rabbit IgG (Calbiochem) was then applied. After washing with PBS, cells were covered with 20% glycerol in PBS and visualized by fluorescence microscopy.

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