Ribozyme mediated modification of α-lactalbumin gene expression

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ABSTRACT

Alpha-lactalbumin (α-lac) is a component of the enzyme complex lactose synthetase which plays a key role in the regulation of lactose synthesis and milk water secretion. Ribozyme mediated manipulation of α-lac expression should enable more precise elucidation of its physiological signficance and may lead to the development of transgenic animals.

Five ribozymes and controls targeting different regions of the α-lac gene have been synthesised and cloned. *In vitro* ribozyme analysis involved the incubation of *in vitro* transcribed α-lac and ribozyme RNA. The rate of ribozyme cleavage *in vitro* was influenced by temperature, $MgCl_2$ concentration, length of antisense flanking sequences and region of the a-lac gene targeted.

In vivo ribozyme analysis was carried out in the T7/vaccinia expression system using C127I mouse mammary cells. This system utilises the powerful bacteriophage T7 RNA polymerase which is expressed when cells are infected with a recombinant vaccinia virus. Gene constructs, cloned into vaccinia plasmids downstream from a T7 promoter are transfected into the cells and are transcribed and translated at very high levels. Using this system we have identified that ribozymes that targeted exposed stem loops and the 3' end of the gene down regulated the level of α-lac RNA to 10-20% of the controls. Similar reductions in expression of α-lac protein were observed for these key ribozymes, but in addition a full length antisense was equally as effective. The expression of these ribozymes in transgenic mice will enable evaluation of their effectiveness to influence lactose synthesis and milk water secretion.

Keywords Ribozymes, α-lactalbumin, gene expression, antisense RNA, milk protein, vaccinia virus, cell culture.

INTRODUCTION

Alpha-lactalbumin (α-lac) is one of the key regulating factors controlling lactose synthesis and, in turn, water secretion in milk (Kuhn *et al.*, 1980). It is synthesised in mammary epithelial cells and whilst on route to the apical membrane binds to β-1,4-galactosyltransferase forming the enzyme complex lactose synthetase. This complex in turn catalyses the synthesis of lactose from UDP-galactose and glucose. Lactose formed in the Golgi lumen is unable to permeate the Golgi membrane and as, the major osmole of milk, draws water into the lactose containing vesicles.

Manipulation of α-lac gene expression should enable more precise elucidation of its physiological significance in both lactose and overall milk synthesis and may lead to the development of transgenic animals with altered milk water secretion. The recent discovery and development of RNA enzymes (ribozymes) offers the potential for highly specific manipulation of α-lac gene expression.

Ribozymes (RZ) are small RNA molecules naturally found in plant and animal virus pathogens where they are involved in replication (Symons, 1989). They possess the property of highly specific self-catalysed cleavage. This cleavage reaction has a requirement for divalent metal ions and neutral or higher pH, and is normally an intra-molecular reaction, that is a single RNA molecule contains all the RNA-encoded functions required for cleavage. Detailed studies of ribozyme structure by Haseloff and Gerlach (1988) have identified the two components of a ribozyme; the substrate and enzyme domains, and have defined the necessary sequence in the substrate to enable an inter-molecular cleavage reaction to proceed against any targeted mRNA. In this paper we wish to describe the design of ribozyme targeted against the bovine α-lac gene and present results from *in vitro* and *in vivo* evaluations of ribozyme activity.

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MATERIALS AND METHODS

Ribozyme Design and Cloning Vectors

Ribozymes targeted against the α-lac gene were designed as two components: the highly conserved hammerhead and hairpin catalytic domain derived from satellite virus RNAs, and small 12 bp antisense flanking sequences, based on specific regions of the a-lac gene (Fig. 1). Start and stop codons, the polyadenylation signal and two 'apparently exposed' stem loop regions of the α-lac mRNA were chosen as the targeted sites. A control ribozyme targeting one of the five sites and containing a catalytically inactive cleavage domain was also made. All ribozymes were synthesised as single stranded DNA oligonucleotides and along with a cDNA clone for the bovine α-lac gene (Hurley and Schuler 1987) were cloned into an RNA transcription vector (bluescript sk+) and a mammalian expression vector (PTF 29+ vaccinia vector).

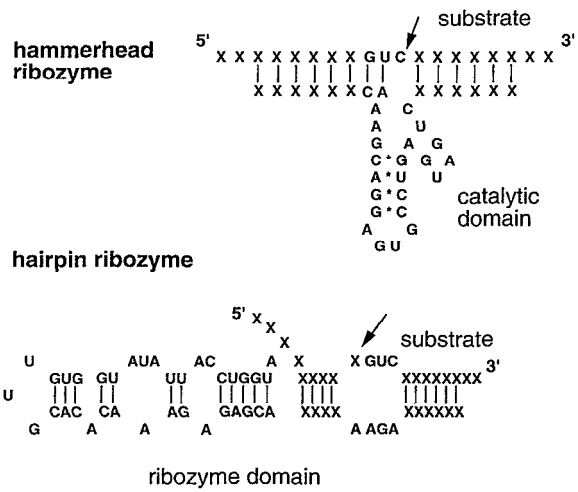

FIG 1 Hammerhead and Hairpin Ribozymes showing Conserved catalytic domains, predicted secondary structure and target binding conFiguration. Arrow indicates cleavage site.

In vitro Ribozyme Analysis

RNA transcripts for the α-lac gene and ribozymes were synthesised *in vitro* using standard techniques (refer Sambrook, *et al.*, 1989). The quality and size of these RNA transcripts was verified on 7M urea denaturing polyacryamide gel electrophoresis (PAGE).

In vitro ribozyme analysis were carried out by incubating α-lac and ribozyme RNA transcripts together in ribozyme buffer (50 mM Tris pH 8.0, 20 mM $MgCl_2$) at 37 or 50°C. Products of these reactions were analysed on 7M urea denaturing PAGE.

In vivo Ribozyme Analysis

In vivo experiments were carried out using the T7/vaccinia gene expression system (Fuerst, *et al.*, 1986) in cultured mouse mammary cells (C127I cells). This expression system is based on the powerful bacteriophage T7 RNA polymerase which is expressed by a recombinant vaccinia virus. α-lac and ribozyme genes were inserted into plasmids downstream from a T7 promoter and these plasmids were simultaneously introduced into the cells by conventional transfection. Selective high level expression of α-lac and ribozyme RNA is achieved by utilising the T7 polymerase to synthesize α-lac and ribozyme RNA off the transfected plasmids.

Routinely, *in vivo* experiments were carried out using 10cm dishes of 95% confluent C127I cells. Cells were infected with 10-30 pfu of virus and incubated for 1 hour. Plasmids were introduced into cells by $CaP0_4$ or Dotma transfection reagents. After 6-24 or 48 hours of further incubation cells were harvested for RNA or protein analysis. The analysis of RNA and protein levels were carried out by conventional northern and western analysis. Both techniques enable the specific identification of the α-lac RNA and protein respectively.

RESULTS AND DISCUSSION

In vitro Analysis

The cleavage activity of the five ribozymes targeted against different sites on the α-lac gene was evaluated in *in vitro* experiments as described above. Fig. 2a shows a typical analysis of ribozyme activity using one ribozyme to cleave the α-lac RNA into the two characteristic fragments.This reaction shows almost complete cleavage of the α-lac RNA in 120 min using a 10 fold excess of ribozyme. The rate of ribozyme cleavage was influenced by temperature, $MgCl_2$ concentration, length of RZ antisense flanking sequences

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Symons R.H. 1989. Self-cleavage of RNA in the replication of small pathogens of plants and animals. *Trends in Biochemical Science 14*: 445-450.

The control ribozyme with a catalytically inactive cleavage domain did not depress α-lac RNA below that observed in cells transfected with α-lac alone, suggesting the effects of antisense RNA were small. This observation provides evidence that the reduction in the level of α-lac RNA is likely to be due to ribozyme activity rather than antisense related effects on the α-lac RNA. Further evidence that RZ activity was responsible for the reduction in α-lac RNA levels comes from the identification of the cleavage fragments of a ribozyme reaction. In the cell expression system the cleavage fragments generated by a ribozyme reaction are unlikely to be as stable as the orginal α-lac transcripts and thus will be degraded rapidly and therefore more difficult to identify. Of the two cleavage fragments, the 5' fragment is known to fold into a structure that enhances its stability (Fuerst and Moss, 1989) and may be more easily detected. In Figure 3b this fragment has been observed in samples containing ribozymes but not in control α-lac or control ribozyme samples.

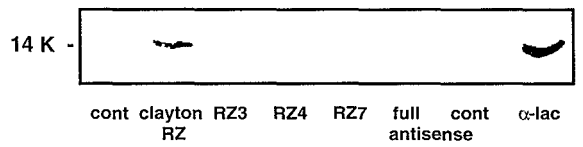

FIG 4 *In vivo* ribozyme analysis of effects of ribozymes on α-lac protein synthesis. Analysis by western blotting after 48 hours incubation of ribozymes in cells. Cont: no α-lac DNA transfected into cells, Clayton RZ: catalytically inactive ribozyme.

Analysis of the effects of ribozyme activity on α-lac protein synthesis was performed by western analysis of proteins harvested from cells after 48 hours incubation. Preliminary results show that with a 500 fold excess of RZs 3,4 and 7 the level of α-lac protein was reduced significantly (Fig 4). In addition, a full length antisense RNA to the α-lac gene was equally as effective as ribozymes in reducing the level of α-lac protein to below detectable levels (Fig 4). In comparison, the control 'claytons' ribozyme reduced the level of α-lac protein to only about 40-60 % of the control α-lac. At lower levels of ribozyme (100-250 fold excess) the relative effects of the full length antisense and ribozymes may differ given that ribozymes are able to act both by antisense and catalytic cleavage mechanisms. Cameron and Jennings (1990) have recently shown that ribozymes down regulate the level of chloramphenicol acetyltransferase protein synthesis in transfected Cos cells. In this case however, there was no antisense or catalytically inactive ribozyme controls.

CONCLUSIONS

The experiments reported here demonstrate that ribozymes have catalytic activity both *in vitro* and *in vivo* in cultured cells Ribozymes targeted to the more exposed regions of the α-lac gene or the 3' end showed the greatest level of ribozyme activity on the RNA. The level of protein synthesised in the cultured cells was equally reduced by the action of ribozymes, but a similar level of effectiveness was observed with a full length antisense RNA. These results suggest that ribozymes have the potential to interfere with α-lac expression and therefore possibly the synthesis of lactose in the mammary gland. The next stage of this study will concentrate on the expression of ribozymes in transgenic mice and cultured lactating bovine mammary cells

The expression and activity of ribozymes in transgenic animals remain to be clearly demonstrated. The results of this study suggested that very high levels of expression of ribozymes will be necessary to reduce the synthesis of a highly expressed protein like α-lac . This problem of expression of the ribozyme, along with its stability within the mammary cells are areas that will require considerable attention to overcome in the future.

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and region of the α-lac gene targeted (data not shown).

The identification of target sites within the gene that are cleaved rapidly was an important objective of the *in vitro* analysis. Large differences in the rate of cleavage of the five ribozymes was observed. Figure 2b shows cleavage analysis for the most active and inactive ribozymes. Ribozymes targeting the exposed stem loops (RZ3 and RZ8) showed the greatest rates of cleavage under 'near physiological conditons of 37°C and 5 mM $MgCl_2$ (Fig. 2b). The observation that exposed stem loops were more efficiently cleaved than other regions of the gene is supported by the observations of Fedor and Uhlenbeck (1990) who showed that cleavage activity varied 70 fold when different sites were targeted by ribozymes of the same design. The use of computer-aided RNA secondary structure predictions should help identify the more exposed regions of any RNA molecule and provide a starting point for ribozyme target sites.

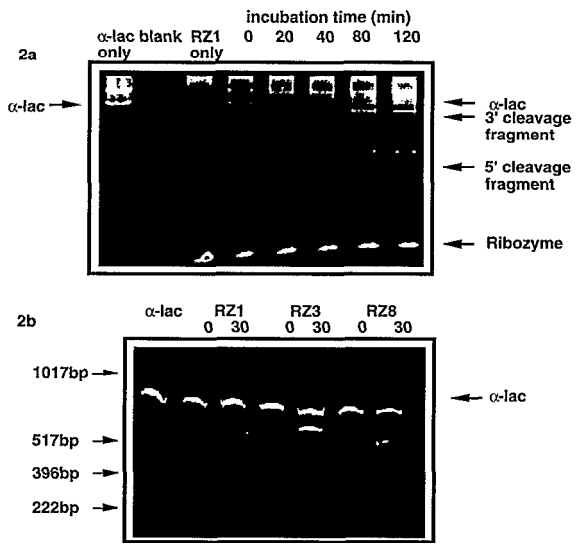

FIG 2 *In vitro* ribozyme analysis. 2a, *In vitro* cleavage of α-lac when incubated with ribozyme 1 over a 120 minute time course at 37°C. 2b, Comparative *in vitro* analysis of α-lac incubated with ribozyme 1, 3 and 8 for 30 minutes at 37°C.

In vivo Analysis

In vivo experiments involved the T7/vaccinia expression system. This system expressed the α-lac and ribozyme RNA molecules in the cytoplasm of the cell. Analysis of the RNA levels by northern blotting indicated high level transcription of the α-lac and ribozyme RNA when transfected singly, and substantial reduction of target α-lac transcripts where α-lac and ribozyme plasmids were transfected together. When a 200 fold excess of ribozyme was used the level of α-lac RNA transcripts was reduced to as little as 10-20% of the level in controls (Fig. 3a). As with the *in vitro* experiments, ribozymes that targeted the exposed stem loops (RZ 3,7 and 8) reduced the level of α-lac RNA more than other ribozymes. Further to this, RZ4 which targeted the 3' polyadenylation signal exhibit a level of ribozyme activity comparable to RZ 3,7 and 8. A recent study using antisense RNA has similarly identified that the 3' end of a gene may be a particularly potent site for targeting ribozymes or antisense RNA (Strickland, *et al.*,1988). RZ1 and 2, which targeted sites at the 5' end or start of the gene show a very low level of ribozyme activity. RZs 3 and 7 contained hammerhead and hairpin catalytic domains respectively and target the same site within the α-lac gene The hammerhead catalytic domain appeared to show greater ribozyme activity than the hairpin catalytic domain at this site (Fig. 3a).

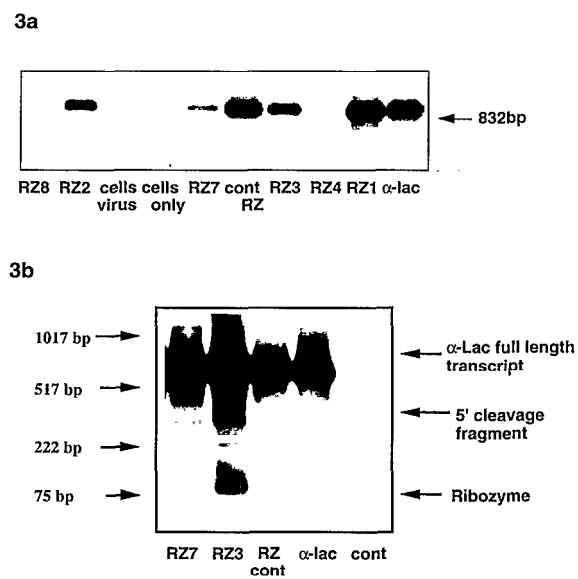

FIG 3 *In vivo* ribozyme analysis (northern blot analysis). 3a Comparative analysis of ribozymes (RZ) targeting different sites with α-lac gene. 3b *In vivo* ribozyme analysis showing 5' cleavage products. RZ cont: catalytically inactive claytons ribozyme, Cont: no α-lac DNA transfected into cells.