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A synthetic, chemically modified ribozyme eliminates amelogenin, the major translation product in developing mouse enamel *in vivo*

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Ribozymes are small RNA structures capable of cleaving RNA target molecules in a catalytic fashion. Designed ribozymes can be targeted to specific mRNAs, blocking their expression without affecting normal functions of other genes. Because of their specific and catalytic mode of action ribozymes are ideal agents for therapeutic interventions against malfunctioning or foreign gene products. Here we report successful experiments to 'knock out' a major translation product *in vivo* using synthesized, chemically modified ribozymes. The ribozymes, designed to cleave amelogenin mRNA, were injected close to developing mandibular molar teeth in newborn mice, resulting in a prolonged and specific arrest of amelogenin synthesis not caused by general toxicity. No carriers were required to assist cellular uptake. Amelogenins are highly conserved tissue-specific proteins that play a central role in mammalian enamel biomineralization. Ultrastructural analyses of *in vivo* ribozyme-treated teeth demonstrated their failure to develop normally mineralized enamel. These results demonstrate that synthesized ribozymes can be highly effective in achieving both timed and localized 'knock-out' of important gene products *in vivo*, and suggest new possibilities for suppression of gene expression for research and therapeutic purposes.

Keywords: 2′-*O*-allylribonucleotides/amelogenin/biomineralization/dental enamel/synthetic hammerhead ribozymes

Introduction

Interference with gene expression at the level of mRNA holds great promise for therapeutic interventions in cases of malfunctioning gene products and infectious diseases. Wagner (1994) recently summarized the available experience of using oligodeoxynucleotides for this purpose and listed the criteria that should be rigorously applied in the evaluation of such experiments. Designed hammerhead ribozymes represent an alternative technology with even greater potential advantages. Hammerhead ribozymes are small RNA structures capable of cleaving an RNA target molecule in a catalytic fashion in the presence of Mg^{2+}(Pyle, 1993). They bear a resemblance to enzymes, and contain a catalytic motif made up of three base paired stems and a core of highly conserved, non-complementary nucleotides essential for catalysis (Cech and Uhlenbeck, 1994). Their three-dimensional (3-D) structure and mode of action have recently been elucidated (Uhlenbeck, 1987; Pley *et al.*, 1994; Tuschl *et al.*, 1994). Hammerhead ribozyme activity can be targeted to specific mRNAs by choosing the sequences flanking the catalytic motif. The two hammerhead ribozymes used here were designed to block expression of amelogenin, the major translation product during mammalian tooth enamel matrix synthesis, in mice. The enamel is a unique tissue due to its hardness, which reflects a high degree of mineralization. The mineral component is mainly hydroxyapatite (HAp) in the form of closely packed ultramicroscopic crystals, larger than those in other mineralized mammalian tissues (bone, dentin and dental cementum). The crystals are organized by differential orientation into a basic pattern of prisms (rods) and interprism (interrod), which is common to all mammals. Amelogenins are the main product of ameloblasts (Termine *et al.*, 1980), the single layered, columnar epithelial cells lining the crown of the tooth anlage (Figure 1), and are expressed only in this organ (Chen *et al.*, 1994). Amelogenins are supposed to play a crucial role in mammalian enamel biomineralization, possibly by forming supramolecular structures that control the HAp crystal growth during enamel formation (Fincham *et al.*, 1994). The primary structure of amelogenin derived from cow, pig, rat, mouse and human demonstrates a high degree of sequence homology between these species (Brooks *et al.*, 1994).

The murine amelogenin gene (AMEL) is located distally

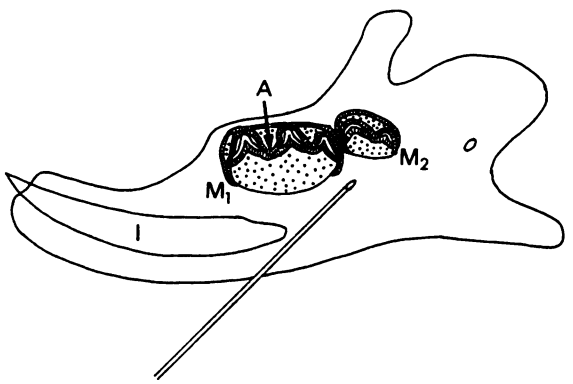

Fig. 1. Lingual view of right mandible of a newborn mouse showing incisor (I) and first (M_1) and second (M_2) molar tooth germs, M_1 where enamel formation has started, and M_2 where enamel formation is about to start (Cohn, 1957; Gaunt, 1964) (M_1 and M_2 depicted as if sectioned). From the tip of the cusps the ameloblasts (A) differentiate in the cervical direction to produce and mature enamel (Sasaki *et al.*, 1990). Needle position during injection is indicated.

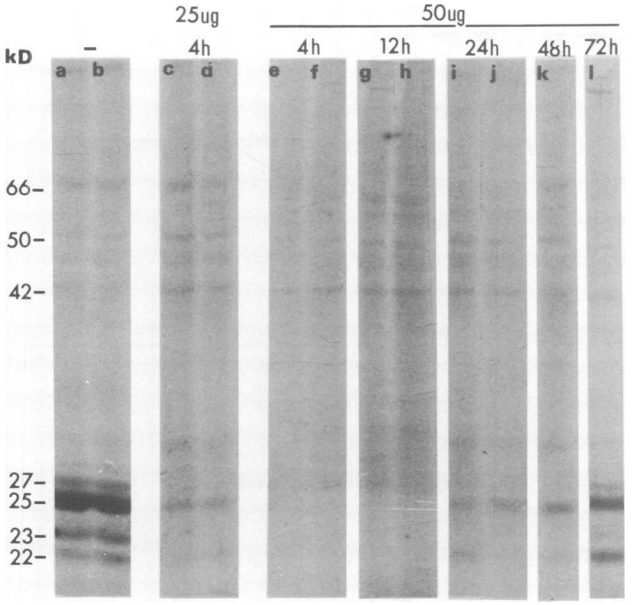

Fig. 2. Autoradiograph of [^{35}S]methionine labelled proteins from normal and ribozyme-treated first molar tooth buds from mandible of newborn mice. Lanes a and b contain proteins from tooth buds of untreated mice. Lanes c and d contain proteins from tooth buds isolated 4 h after injection of 25 μg of the AMEL ribozyme. Lanes e–l contain proteins from tooth buds isolated 4, 12, 24, 48 and 72 h after injection of 50 μg of the AMEL ribozyme. Each lane represents proteins from one half of a first molar tooth bud. The 22, 23, 25 and 27 kDa proteins were identified as amelogenins by protein sequencing. The disappearance of amelogenins indicated a total arrest of their synthesis. All other protein bands appeared unaffected by the ribozyme injection and served as internal controls for the specificity of the AMEL hammerhead ribozyme.

(0.73) on the X chromosome (Lau *et al.*, 1989) and its cDNA has been cloned (Snead *et al.*, 1985). The AMEL gene gives rise to four different polypeptides (Figure 2) caused by differential splicing (Lau *et al.*, 1992). Here we report successful *in vivo* experiments with synthetic hammerhead ribozymes, achieving a specific 'restricted knock out' of the AMEL gene. By local injections (Figure 1) of synthetic ribozyme constructions into newborn mice the major translation product of ameloblasts during the initial stage of enamel formation was eliminated. No carriers were required to assist uptake. The ribozyme effect was monitored both by direct measurement of target protein levels and by loss of biological function. A series of controls demonstrates that the blocking of AMEL expression was specific. Our experiments fulfill the criteria listed by Wagner (1994), and is the first reported study that clearly demonstrates the *in vivo* efficacy of synthesized, chemically modified hammerhead ribozymes.

Results

Effect of the AMEL hammerhead ribozymes

N-terminal amino acid sequences of the 22, 23, 25 and 27 kDa protein bands appearing on SDS–PAGE of molar tooth extracts were revealed by Edman degradation and cyanogen bromide cleavage (Hewick *et al.*, 1981). The protein bands were identified as AMEL proteins when compared with previously reported murine amelogenin protein sequences (Fincham *et al.*, 1991) and were in accordance with the cloned murine AMEL cDNA sequence (Snead *et al.*, 1985). The half-life of amelogenins in molars of newborn mice was determined to be ~210 min in initial pulse–chase experiments where cycloheximide (8 μg/g body weight) and cold methionine (200 μg) were given 100 min after labelled methionine. Amelogenins were isolated and their content of [^{35}S]methionine determined after 100, 200, 300 and 400 min.

When compared with untreated siblings, all ribozyme-injected mice showed a marked decrease in the incorporation of radiolabelled methionine into the amelogenin bands of molar tooth extracts from the injected side after 4 h. Doses of 25 μg ribozyme resulted in a nearly 90% decrease of [^{35}S]methionine in the amelogenin bands. At 50 μg per animal a complete arrest of amelogenin synthesis on the side of injection resulted (Figure 2). This complete arrest of AMEL gene expression lasted ~24 h (Figure 3a). After 3 days amelogenin synthesis was still nearly 50% inhibited. It took 90 h to restore its synthesis to the normal level (100%). After 100 h a brief overexpression (120%) of the AMEL gene was observed. Amelogenin synthesis was back to normal before 120 h and then stabilized at this level.

To see what influence the 3′ terminal phosphorothioate protection of the internucleotide linkages had on ribozyme function and stability, an AMEL hammerhead ribozyme not carrying this modification was applied. Except for a slightly higher and longer lasting efficacy and a slightly more rapid breakdown of the unsulfurized ribozyme, the two AMEL hammerhead ribozymes acted similarly (Figure 3b).

Inhibition of amelogenin synthesis was observed in both the first and the second molar on the side of the injection. In the corresponding contralateral molars a reduction of labelled amelogenins of ~25% was observed at 12 h.

The AMEL hammerhead ribozymes had full activity against all AMEL splice products as none of the four amelogenins appeared in the electrophoresis gels from teeth of ribozyme-treated mice (Figure 2). No other proteins in these gels were affected by the ribozyme injections.

Effect of control injections

Three control oligomers were also designed in order to isolate the ribozyme effects from other oligoribonucleotide effects. One control was an inactive version of the above chemically modified ribozymes in which 'G12' in the conserved hammerhead motif (Haseloff and Gerlach, 1988) was replaced by an 'A', leading to loss of catalytic activity. The second control was a straight antisense oligo(2′-*O*-allylribonucleotide) designed against the same AMEL mRNA region as the ribozymes and applied to distinguish between the ribozyme effect and an ordinary antisense effect. The third control was a randomized oligomer of 18 bases applied for detection of unspecified or toxic actions of the synthetic ribozymes. A fourth control containing only 5 μl sterile saline was included to see whether the injection trauma itself influenced enamel formation.

Injections with the mutated ribozyme gave an immediate inhibition of AMEL expression of >80% (Figure 3c). After 12 h, however, the inhibition was only 40%, and normal levels of amelogenin synthesis were restored within

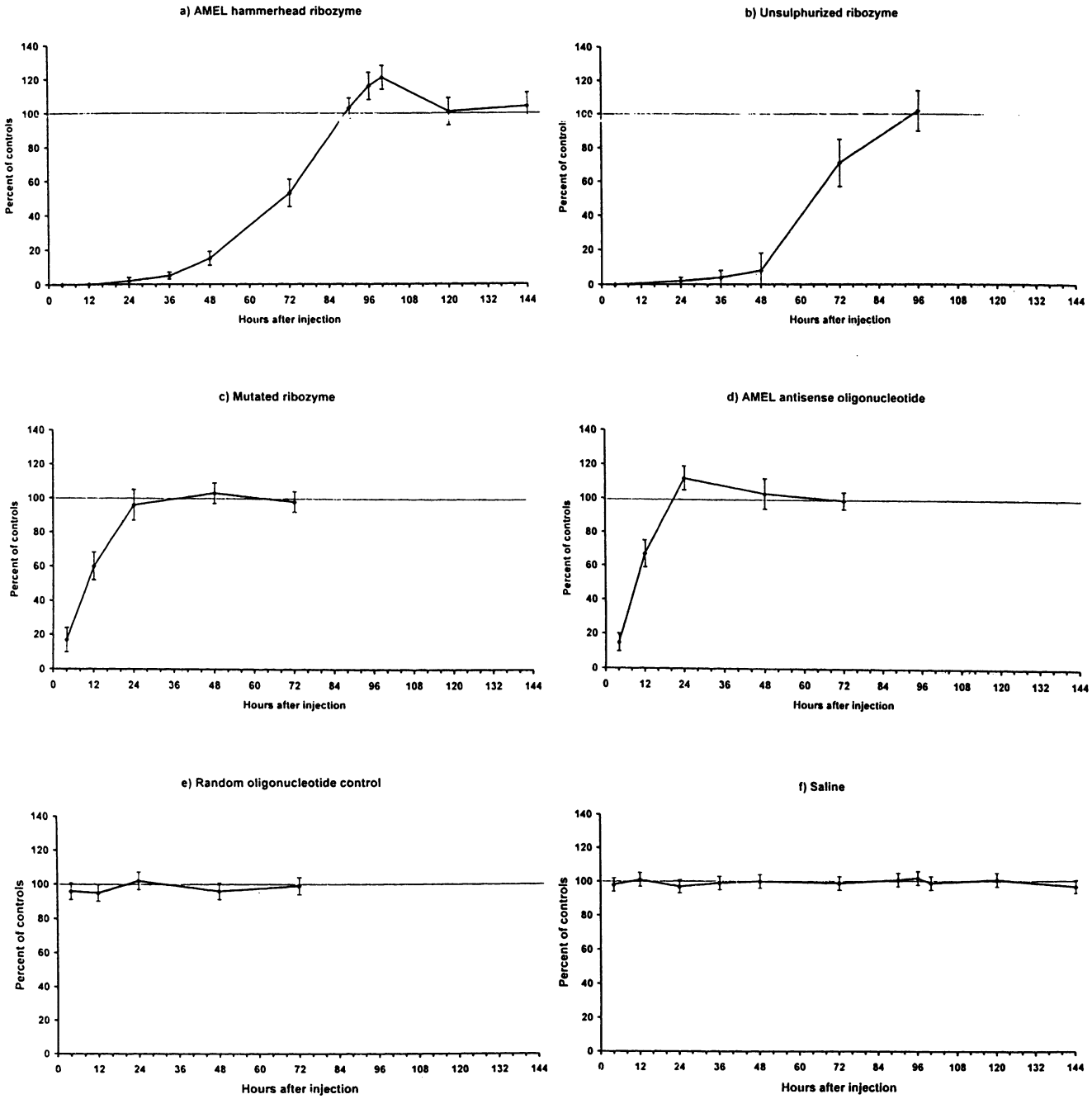

Fig. 3. Effect of injection of AMEL hammerhead ribozymes on [^{35}S]methionine incorporation into amelogenins in newborn mice. Ribozymes and various control oligonucleotides were injected into separate newborn (day 1) mice. Saline injected and untreated litter mates served as controls. The [^{35}S]methionine incorporated into tooth buds was quantitated in a PhosphorImager (Molecular Dynamics). Values are in percent of [^{35}S]methionine incorporated into amelogenins of the corresponding teeth of untreated litter mates, normalized for the size of the available free methionine pool in each tooth bud. These control values are indicated by the 100% line (n = 16 at each time point). (**a**) AMEL hammerhead ribozyme. Each value is the mean ± SD of four experiments, each with five mice (n = 20). (**b**) AMEL hammerhead ribozyme without 3′ phosphorothioate protected internucleotide linkages. Each value is the mean ± SD of one experiment with five mice (n = 5). (**c**) Mutated ribozyme (G12→A). Each value is the mean ± SD of two experiments, each with five mice (n = 10). (**d**) AMEL antisense oligonucleotide (n = 10). (**e**) Random oligonucleotide controls (n = 8). (**f**) Saline controls (n = 8).

24 h. In contrast to earlier *in vitro* experiments with antisense oligo(2′-*O*-allylribonucleotide)s (Johansson *et al.*, 1994), the straight antisense oligoribonucleotide injections produced an effect much like that of the mutated ribozyme, initially inhibiting AMEL expression by 80% (Figure 3d), after 12 h inhibition was only 30%, and normal amelogenin synthesis levels were restored before 24 h. Random oligonucleotide injections or saline injections had essentially no influence on the monitored protein synthesis, and the quantity of incorporated [^{35}S]methionine in these animals did not differ significantly from that of their untreated siblings (Figure 3e and f).

Control injections of 5 µl 0.5% Trypan Blue in saline were used to mark the area of diffusion of the injected fluid. The dye could easily be demonstrated in the mandibular molar tooth buds within 30 min after injection.

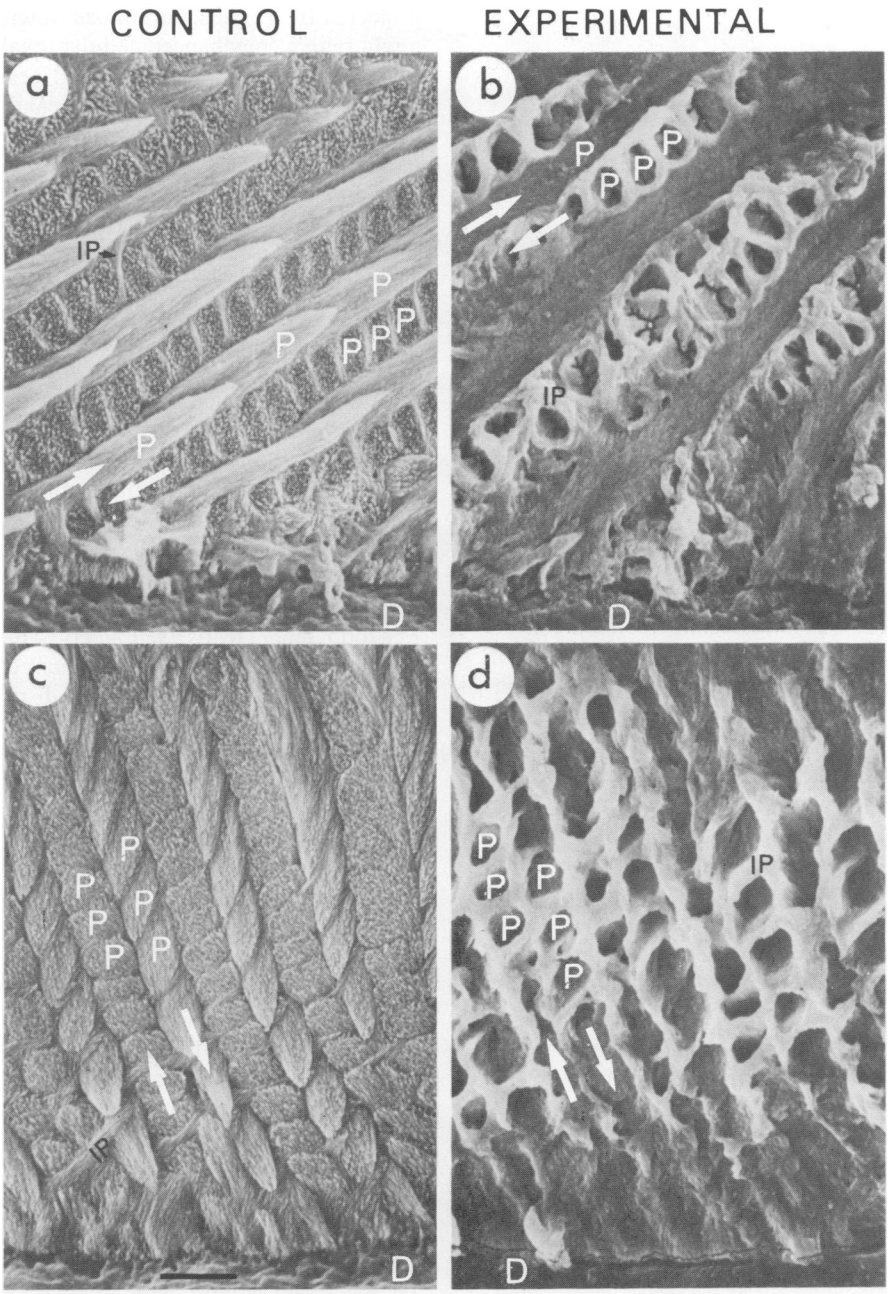

Fig. 4. SEM micrographs of mouse molar inner enamel abutting on dentin (D). (**a** and **c**) Normal control enamel with structure similar to rat enamel (Risnes, 1979a,b). (**b** and **d**) Corresponding regions of enamel affected by the AMEL hammerhead ribozymes. Prisms (P) of adjacent prism rows are oppositely oriented (unlabelled arrows). The interprism (IP) separates individual prisms. The affected enamel is severely hypomineralized with an accumulation of organic material at the prism periphery–interprism and with no visible HAp crystals within cross-cut prism domains. Magnification bar, 5 μm.

Scanning electron microscopy observations

To look at the effect of the ribozyme on enamel formation, randomly selected mice injected with active ribozyme on day 1 after birth were sacrificed at 5 weeks of age, when their molar teeth were fully developed. Mice injected with the randomized oligonucleotide or saline only and untreated siblings were included as controls. The mandibular molars from these mice were prepared for scanning electron microscopy (SEM) (Risnes, 1985). Molars from ribozyme-treated mice revealed severely disturbed enamel with a pronounced hypomineralization and accumulation of organic material at the prism periphery–interprism, whereas cross-cut prisms appeared as empty holes with no visible HAp crystals present (Figure 4). The general design of the prism pattern and the enamel thickness appeared unaffected. Molars from untreated, saline injected or random oligonucleotide-treated mice showed no such changes. Regions with normal enamel were also observed in teeth from ribozyme-treated animals. Second molars were more severely affected than first molars. No differences were detected in eruption-time or macroscopic tooth morphology between ribozyme-treated, randomized oligonucleotide-treated, saline injected or untreated mice.

Discussion

The AMEL hammerhead ribozyme motif (Haseloff and Gerlach, 1988) was flanked by two AMEL mRNA binding regions (6 and 7 bp, respectively), chosen to optimize the catalytic activity of the ribozyme without losing specificity (Goodchild and Kohli, 1991). Its target was the GUC sequence of the mouse AMEL gene in which the G is at base 86 of the mRNA (Snead *et al.*, 1985). The proposed hammerhead ribozymes were chemically modified (Paolella *et al.*, 1992; Sproat *et al.*, 1994) to contain mostly 2′-*O*-allylribonucleotides and only five ribonucleotides required for catalysis, *viz.* G5, A6, G8, G12 and A15, using the standard numbering system (Hertel *et al.*, 1992), so as to have long term stability *in vivo*. The allylation of U4 is essential to block RNase A attack at this position. In addition, one of the two otherwise identical AMEL hammerhead ribozymes, the mutated ribozyme and the antisense oligoribonucleotide, carried two sulfurized 3′-proximal internucleotide linkages to reduce 3′-exonuclease attack further.

Global 2′-*O*-allylation of oligoribonucleotides confers nuclease resistance, chemical stability, in hybridization high specificity for RNA over DNA and minimal non-specific binding (Iribarren *et al.*, 1990; Lamond and Sproat, 1993), all of which are essential requirements for *in vivo* experiments. Cellular uptake may also be facilitated because of the increased hydrophobicity imparted by 2′-*O*-allylation.

The ribozymes used here totally inhibited expression of the AMEL gene in newborn mice without affecting normal functions of other genes. Apparently, no other proteins were affected by the ribozyme injections (Figure 2), thus constituting a valuable internal control for the specificity of the ribozymes.

The fact that the control dye was detectable in the tooth buds within 30 min, the observed rapid loss of AMEL expression and the fact that amelogenins are produced within ameloblasts only (Chen *et al.*, 1994), provide strong evidence that the modified oligoribonucleotides effectively reach and penetrate these cells, as has been demonstrated for oligodeoxynucleotides when applied to cultured tooth organs (Diekwisch *et al.*, 1993).

The mutated ribozyme and the antisense oligoribonucleotide showed similar effects during the first 12 h. This suggests that the amelogenin-specific hybridizing arms of the mutated ribozyme make it act like an ordinary antisense oligoribonucleotide inhibiting amelogenin synthesis for a short period, possibly by temporary blockage of ribosome progression along mRNA and that the hammerhead motif itself does not affect the specific binding of its flanking regions to amelogenin mRNAs.

Injections containing the randomized oligonucleotide had essentially no influence on AMEL expression (Figure 3d), nor did they produce any structural defects in the mature enamel. This indicates that the observed ribozyme effect was not due to a non-specific or toxic influence of oligonucleotides on AMEL expression. Neither did mice injected with saline show any significant difference from their untreated siblings, indicating that the injections themselves did not affect protein synthesis or function in developing enamel.

The slightly increased efficacy of the ribozyme not protected by 3′ phosphorothioate internucleotide linkages might reflect an enhanced cellular uptake of this oligomer. The apparent slightly more rapid cessation of its effect may be due to a more rapid breakdown caused by an increased sensitivity to 3′-exonuclease attack on this ribozyme.

The difference in occurrence of enamel defects among first and second molars reflects the different developmental stages of these teeth at the time of ribozyme injection. The fact that the second molar was more severely affected than the first molar indicates that amelogenin plays a key role at a very early stage in enamel development and may be less important at later stages. Areas with normal enamel formation within each affected tooth represent early (occlusal) and late (cervical) enamel matrix synthesis unaffected by the time-limited ribozyme effect applied here.

Designed hammerhead ribozymes are promising agents for blocking specific gene expression. Chemical modifications enhance their stability, potency and ability to enter cells. As the 3-D structures of the hammerhead motifs are revealed, even more effective ribozymes can be developed. The tooth model lends itself nicely to the study of ribozyme function *in vivo* since the biological consequences of the ribozyme effect are permanently displayed in the mature enamel. We report the first demonstration of *in vivo* efficacy of synthesized, chemically modified ribozymes. Only a few cases of specific gene inhibition caused by antisense oligodeoxynucleotides have been rigorously demonstrated in cultured cells before (Wagner, 1994). Our experiments clearly illustrate the advantages of combining the catalytic activity of a hammerhead motif with the specificity of antisense oligomers in a chemically modified oligoribonucleotide to obtain both enhanced and prolonged specific blocking of gene expression *in vivo*. The strategy used in this model could be highly effective in achieving both timed and localized 'knock-outs' of important gene products *in vivo*, and suggests new possibilities for suppression of gene expression for research and therapeutic purposes.

Materials and methods

Oligoribonucleotide synthesis

The AMEL hammerhead ribozyme (5′-UGUUGACUgaUgAGGCCGUUAGGCCgAAaCAGC***PS***A***PS***C), the unsulfurized ribozyme (5′-UGUUGACUgaUgAGGCCGUUAGGC CgAAaCAGCAC), the mutated ribozyme (5′-UGUUGACUgaUgAGGCCGUUAGGCCaAAaCAGC***P-S***A***PS***C), the straight antisense oligo(2′-*O*-allylribonucleotide)(5′-UGUUGAGACAGC***PS***A***PS***C) and the 18mer randomized oligonucleotide (scrambled, unmodified) were synthesized on solid-phase using phosphoramidite chemistry (capital letters are 2′-*O*-allylribonucleotides and lower case letters are ribonucleotides, phosphorothioate protected internucleotide linkages are denoted ***PS***, underlined sequences are complementary to the AMEL target sequence). The ribonucleotides carried standard 2′-*O*-*tert*-butyldimethylsilyl protection, which was subsequently removed by treatment of the partially deprotected oligomer with neat triethylamine trihydrofluoride (Gasparutto *et al.*, 1992). The oligonucleotides were purified by HPLC before injection.

Experimental design

Oligoribonucleotides were dissolved in sterile saline to a final concentration of 10 μg/μl each, and 50 μg of each delivered by submandibular injection into live newborn BALB/C (albino) mice without addition of liposomes or other vehicles to enhance cellular uptake. On the first day after birth each animal received an injection of ribozyme or control oligonucleotides on the lingual side of the right mandibular molar area. Other controls received 5 μl saline. A reference group of untreated

siblings of the experimental animals was included to monitor the normal AMEL expression in these mice.

Injections of 5 µl 0.5% Trypan Blue in saline were used to mark the area of diffusion. All injections were carried out using a Hamilton syringe with a 0.3 mm needle at the rate of ~1 µl/s. At 0, 8, 20, 32, 44, 68, 86, 92, 96, 116 or 140 h after the mandibular injections experimental animals and a reference group received a 4 h pulse of 20 µCi [^{35}S]methionine (Amersham) given i.p. The mice were then sacrificed and their mandibular molar tooth buds were dissected out. Each tooth bud was rinsed in sterile saline and boiled in 50 µl 2× SDS–PAGE sample buffer (0.4 g SDS, 1.0 g 2-mercaptoethanol, 0.02 g bromophenol blue and 4.4 g glycerol in 10 ml 0.125 M Tris–HCl, pH 6.8) for 5 min. Half of each sample was then submitted to electrophoresis on 12% SDS–polyacrylamide gels at 80 mA overnight. The gels were dried and placed in a PhosphorImager (Molecular Dynamics) to detect radioactive methionine incorporated into the proteins present. Subsequently, the gels were submitted to ordinary autoradiography for 30 days as a visual control. Proteins in the other half of each sample were precipitated with 0.6 N perchloric acid (PCA) and the supernatant was cleared by centrifugation. The free radiolabelled methionine in the supernatant was measured in a Packard Tricarb Scintillation counter and used to normalize the values from the PhosphorImager so that individual differences in the availability of radiolabelled methionine were adjusted for.

Scanning electron microscopy

Randomly selected mice injected once with the AMEL hammerhead ribozyme on day 1 after birth were sacrificed at 5 weeks of age, when their molar teeth were fully developed and erupted. Mice injected with the randomized 18mer oligonucleotide and with saline only, and untreated siblings were included as controls. The mandibular molars from these mice were dissected out, taking care not to damage enamel structures, and prepared for scanning electron microscopy (SEM) by sectioning and grinding (Risnes, 1985), etching three times for 10 s in 0.1% nitric acid and sputter-coating with gold–palladium. A Phillips 515 SEM was operated at 15 kV.

Protein sequencing

Amelogenin protein bands separated by SDS–PAGE were transferred onto a poly(vinylidene difluoride) membrane by the semi-dry 'sandwich' electroblotting technique (Matsudaira, 1987). The membrane was stained with Coomassie Blue and the four amelogenin bands cut out, separated and submitted to Edman degradation and *in situ* cyanogen bromide cleavage (Hewick *et al.*, 1981).

N-terminal amino acid sequences were analyzed with an Applied Biosystems 477A instrument coupled to a 120A analyzer. After a sufficient number of Edman degradation cycles, the remaining filter-bound polypeptides were cleaved *in situ* with CNBr. The filters with the adhering polypeptides were placed in Eppendorf tubes, 30 µl CNBr solution (0.2 g/ml 70% formic acid) was applied to the filters, and an extra 30 µl was placed in the bottom of each tube, below the filters, to keep them moist. Nitrogen gas was introduced, after which the tubes were sealed and incubated for 24 h in the dark. Following this treatment, the filters were dried under vacuum and reapplied to the sequencer.

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