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**Research Article**

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# Antisense Oligodeoxynucleotides Selectively Suppress Expression of the Mutant α2(I) Collagen Allele in Type IV Osteogenesis Imperfecta Fibroblasts

## A Molecular Approach to Therapeutics of Dominant Negative Disorders

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## Abstract

**We are investigating the use of antisense oligodeoxynucleotides to selectively suppress expression of the mutant type I collagen allele in osteogenesis imperfecta (OI). In this report, we target a human collagen mutation in its natural cellular context. We used cultured fibroblasts from a case of type IV OI, in which the mutant α2(I) allele produces mRNA with exon 16 deleted due to a point mutation in the splice donor site. Lipid-mediated transfection was used to deliver antisense, sense and missense phosphorothioates targeted to both the abnormal mRNA exon 15/17 junction and the nuclear level point mutation. Significant suppression of the mutant protein chain and mRNA was achieved with antisense oligonucleotide to both mRNA and nuclear levels. Mutant protein was suppressed to 44–47% and mutant α2(I) mRNA to 37–43% of their levels in control cells, indicating decreased mRNA as the basis for suppression.**

**Selectivity of mutant allele suppression was better with an mRNA target: suppression was sequence specific and normal mRNA was expressed at 79% of its level in untreated cells. With a nuclear target, significant suppression of mutant mRNA occurred not only with antisense and sense, but also with missense oligonucleotide, which suppressed mutant mRNA to 60% of its level in untreated cells.**

**We also investigated the time course of suppression of protein and mRNA in response to a 4 h transfection of antisense oligonucleotide. From 24–72 h after transfection, mutant protein was suppressed to ~ 50% of its untreated level and suppression of mutant message was significantly greater than that of normal message. The suppression achieved in these studies is insufficient for clinical intervention, but our results provide support for further development of antisense therapy as an approach to the treatment of dominant negative disorders. (*J. Clin. Invest.* 1996. 97: 448–454.) Key words: connective tissue • gene therapy • bone disease • connective tissue diseases • collagen diseases**

## Introduction

The gene therapy of dominant negative genetic disorders will require a fundamentally different approach than that appropriate for recessive genetic disorders. Recessive disorders of the enzymes of intermediary metabolism are, in essence, disorders of omission, characterized by an absent or inactive protein product. Clinical symptoms result from omission of a reaction and from the accumulation of the intermediary metabolites. The current therapeutic approach aims to replace the missing gene product in the crucial tissue(s) by an exogenous construct. This approach may restore adequate function to relieve clinical symptoms.

In contrast, dominant negative disorders, such as osteogenesis imperfecta (OI), are disorders of commission. In OI, the defective type I collagen chain is synthesized. In some mutations, there is substantial intracellular degradation of both mutant and normal chains. Most often, the defective chain is incorporated into collagen trimer, secreted from the cell and incorporated into higher order structures. The extracellular matrix containing the mutant collagen is then intrinsically defective (For reviews see references 1 and 2). This dominant negative situation will not be reversed by adding a normal collagen gene to the defective tissues.

The rational aim of molecular therapeutics for dominant negative disorders, such as OI, is to shut off expression of the mutant allele, creating a functionally null allele. For OI, the clinical consequence of a null allele is already known to be quite mild. OI type I is a quantitative defect in which patients have one null α1(I) allele and produce a reduced amount of otherwise normal collagen (3). Thus, in principle, selective suppression of the expression of the mutant collagen allele in connective tissues could modulate the clinically severe forms of OI into the biochemical equivalent of mild OI type I (4).

Antisense oligodeoxynucleotides represent a potential mechanism to selectively suppress production of the mutant allele. They have been widely used to suppress gene expression in culture, usually targeting total gene expression (5–7). The potential problems of poor oligonucleotide uptake and rapid intracellular degradation can be overcome by lipid-mediated transfection and phosphorothioate modifications of the oligonucleotides, respectively (8, 9). In a number of cases, oligonucleotides have been shown to be selective for a point mutation in the target gene (10, 11). However, the type I collagen genes, in which the mutations responsible for OI are located, have an unusually repetitious sequence, because they code for uninterrupted repeats of the triplet Gly-X-Y, in which X and Y are frequently proline and hydroxyproline, respectively. Prockop and co-workers used antisense oligonucleotides to selectively inhibit expression of a human COL1A1 minigene which was stably transfected into mouse 3T3 cells (12). Here, we have

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treated cultured fibroblasts from a patient with moderately severe OI type IV with antisense oligonucleotides to target the naturally occurring mutation in α2(I). Since the point mutation in this α2(I) gene occurs at a splice donor site (13) and causes splicing out of the adjacent exon, these cells allow us to independently target the structures present in the nucleus and mRNA of the mutant allele. We have investigated the selectivity and efficiency of antisense suppression of a human mutation in its natural structural context.

## Methods

*Oligonucleotide synthesis.* Phosphorothioate oligodeoxynucleotides were synthesized by phosphoramidite chemistry with phosphorothioate modification uniformly along the backbone (Oligos, Etc., Inc., Wilsonville, OR). Gel electrophoresis of kinased phosphorothioates demonstrated that the oliogos were > 95% full length product.

*Treatment of cell cultures.* OI type IV dermal fibroblasts were grown in Dulbecco's Modified Eagle's Media (DME) containing 10% calf serum plus 2.0 mM glutamine. For oligonucleotide uptake experiments, OI type IV cells were plated at $2.6 \times 10^4$ cells/cm$^2$ in 23 mm$^2$ wells and allowed to grow for 20–22 h. Each oligo was 5′-labeled with T4 polynucleotide kinase and gel-purified. Labeled oligonucleotide ($1.5 \times 10^5$ cpm/well) was used to tag unlabeled oligonucleotide. The total concentration of oligonucleotide used was 0–1.5 μM. Oligonucleotide and lipid were premixed in serum-free DME for 15 min at room temperature. The micell mixture was then added to the cells. At the designated times, the media was removed and reserved. Cells were washed twice with PBS, then collected by scraping and sonication. Percent oligo uptake = total cts in cell layer/counts in cell layer + cts in media and was the average of triplicate wells. Aliquots of cell layer were examined on 15% PA-7M Urea sequencing gels to determine whether the oligonucleotide had been degraded.

To determine the suppressive effects of oligonucleotides on mutant α2(I) chain, OI type IV fibroblasts were plated at a density of $2.6 \times 10^4$ cells/cm$^2$ in triplicate 35-mm$^2$ wells (for protein analysis) or 60-mm$^2$ petri dishes (for RNA analysis) and allowed to grow for 20–22 h. They were then exposed to micells formed with 0.4 μM oligonucleotide and 10 μg/ml lipofectamine (GIBCO BRL, Inc., Gaithersburg, MD) for 4 h in serum-free media. After treatment, the media was removed, and the cells were washed with DME plus 10% FCS and 50 μg/ml ascorbic acid. The media was replaced with DME plus serum and ascorbic acid containing 325 μCi L[2,3,4,5-$^3$H] proline for protein analysis (14) and unlabeled media for RNA analysis (15). Cultures were harvested at 8 or 24 h after micell exposure began.

To determine the time course of mutant α2(I) suppression by transient exposure to antisense oligonucleotide, cells were plated and treated with antisense oligo to the mutant exon 15/17 junction, as above. After treatment, media with micells was replaced with fresh DME plus 10% FCS and 50 μg/ml ascorbic acid. 6 h before cells were harvested for collagen protein, 325 μCi/well of L[2,3,4,5-$^3$H] proline was added to each well. Cultures harvested for RNA analysis were unlabeled. Cultures were harvested in triplicate at 6, 12, 18, 24, 30, 36, 42, 48, 60, and 72 h.

*Protein analysis.* We harvested the combined media and cell layer of cultures for collagen protein analysis. Type I collagen was obtained by pepsin treatment of $^3$H-procollagen (14). The two α2(I) chains were partially separated by electrophoresis on a 5% PA-SDS-2 M urea gel. The α2(I) chains were cut from the gel, and digested in the gel slices with cyanogen bromide (CNBr). CNBr digestion of α2(I) chains was always > 95% complete. The products were electrophoresed on a 12% PA-SDS-2 M urea gel to separate the mutant and normal α2(I) CB 4 peptides (14). The peptides were quantitated by densitometry of radiographs in the linear range of exposure using a Microtek ScanMaker.

*RNA analysis.* Cell cultures were lysed with 4 M guanidinium isothiocyanate solution. Total RNA was prepared using the acid-phenol method (15). For quantitation of mutant and normal α2(I) mRNA remaining after oligo exposure, 2- and 5-μg aliquots of total RNA were slot blotted and probed with allele-specific probes. The probes were 3′-end labeled with terminal transferase and α-$^{32}$P-dGTP and gel purified. The probe for the mutant allele was complementary to the mutant exon 15/17 junction, but was not identical to the antisense oligonucleotide used in suppression experiments. It was a 20-mer with the sequence 5′-CAATTTCACCAGCAGGACCC-3′. For the normal allele, we used a 20-mer complementary to part of the exon 16 sequence, 5′-GGCACCTGGGAAGCCT GGAG-3′. In preliminary experiments, we verified that each probe did not cross react with the product of the other allele, by hybridizing each probe to mutant and normal subcloned cDNA and synthetic RNA transcript, made using T7 RNA polymerase and the OI patient's subcloned cDNA in a pGEM vector (16). Membranes were pre-hybridized for 4 h at 42°C in 6 × SSC, 0.5% SDS, 10× Denhart's solution, 50 μg/ml sheared E. coli DNA, and 0.05% NaPyrophosphate. Probes ($1–1.5 \times 10^6$ cpm/ml) were hybridized overnight at 45°C in 6× SSC, 10× Denhart's solution, 0.05% NaPyrophosphate, 20 μg/ml tRNA, and 10 μg/ml poly A. Membranes were first washed twice with 6× SSC/0.05% NaPyrophosphate for 30 min at room temperature. They were then washed twice with 3 M tetramethylammonium chloride (TMAC), 50 mM Tris pH 8.0, 2 mM EDTA, and 0.1% SDS for 10 min at 45°C. The mRNA hybridization signal was quantitated by densitometry of radiographs in the linear exposure range and expressed as a percent of the normal or mutant mRNA, respectively, present in a sham-treated control culture treated only with lipid.

## Results

*Selection of oligonucleotides.* The oligonucleotides used in these studies were designed for a particular case of type IV osteogenesis imperfecta. We have previously published (13) the detailed clinical and molecular description of this case. The fibroblasts are derived from a now 4-yr-old girl, whose moderately severe condition results from a $G^{+1} \rightarrow A$ change at the exon 16 splice donor site in one of her α2(I) collagen alleles. This results in splicing out of exon 16 in the mRNA made by this allele (Fig. 1).

One feature of this mutation is that the protein products of the mutant and normal α2(I) alleles have electrophoretically distinct cyanogen bromide peptide 4 (CNBr 4) products. The mRNAs can also be easily distinguished by the presence of either the sequence coding for exon 16 or the sequence of the novel exon 15/exon 17 junction.

A second feature of the collagen mutation in these cells is that the mutation has a different structure at the nuclear level, where it is a point mutation, and at the mRNA level, where it is a 54-bp deletion. This arrangement allows us to target oligonucleotides separately to each structure and compare their effectiveness. We used 20-mer phosphorothioates, targeting sets of antisense, sense and missense oligonucleotides to both the mutant exon 15/17 junction and the point mutation (Fig. 1). The 20-mer length of the phosphorothioates was chosen as a compromise between the length necessary to include sequence differences in the highly repetitive collagen structure and the risk of nonsequence specific effects associated with longer oligonucleotides. The nonsequence specific effects should also be minimized by the low concentration (0.4 μM) used with lipid transfection. For the mRNA level, the antisense oligonucleotide complementary to the mutant exon 15/17 junction has 16 of 20 nucleotides complementary to the normal exon 15/16 junction and 13 nt complementary to the exon 16/17 junction (17). The sense oligonucleotide has a sequence identical to the

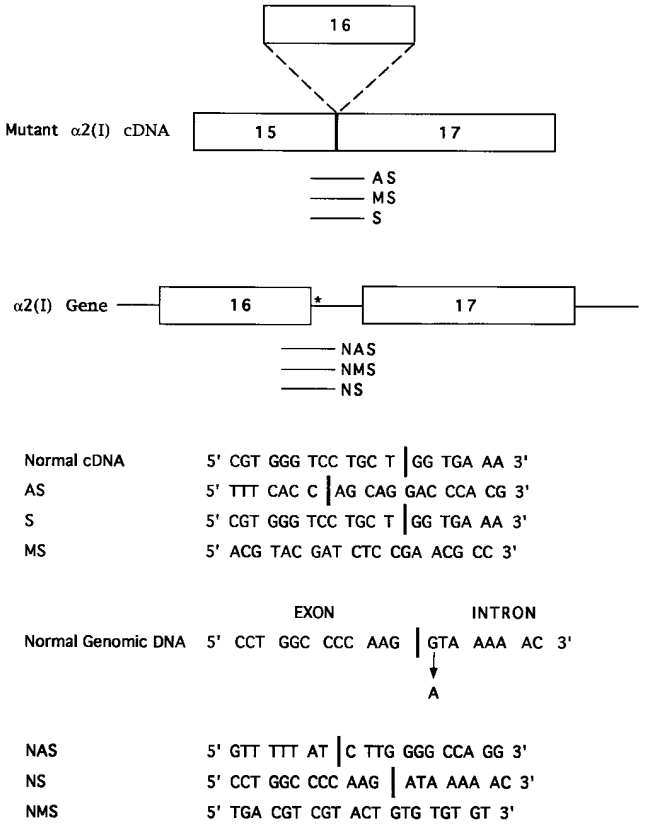



*Figure 1.* Schematic presentation of the sequence and location of phosphorothioates used for antisense experiments. There are two sets of antisense, sense and missense oligos: one set is targeted to the novel exon 15/17 junction at the mRNA level (*AS*, *S*, *MS*), the other is targeted to the point mutation at the nuclear level (*NAS*, *NS*, *NMS*). The vertical bar in the oligo sequence designates the junction of exon 15/17 (for the cDNA) or the exon 16/intron 16 junction (for nuclear structures). The asterisk indicates the point mutation ($G^{+1} \rightarrow A$) at the exon 16-splice donor site.

mutant mRNA exon 15/17 junction. The missense oligonucleotide has the same nucleotide composition as the antisense oligonucleotide, but in random order; it has only 6 nt complementary to any of the three exon junctions.

At the nuclear level, the antisense oligonucleotide is complementary to the primary unspliced transcript of the mutant allele (13) and, thus, to the nontranscribed strand. The missense oligonucleotide is complementary to only 3 nt of the mutant splice donor site in the unspliced transcript.

*Uptake of oligonucleotides by fibroblasts.* Lipid-mediated transfection was used to facilitate entry of oligonucleotide into the cells. We optimized the lipid used, the concentration of lipid and oligonucleotide and the timing of the transfection for our cells. Kinased oligonucleotide complementary to the mutant exon 15/17 junction was used to tag unlabeled oligonucleotide for uptake experiments (see Methods). To determine the best combination of lipid and oligonucleotide, micells were formed with 3, 5, and 10 μg/ml lipofectin or with 5, 10, and 15 μg/ml lipofectamine, using 0–1.5 μM antisense oligonucleotide for each lipid concentration. Lipofectamine was always more effective (data not shown). The maximum transfection achieved uptake of 50% of the antisense oligo from the media into the intracellular layer, with 10 μg/ml lipofectamine and 0.4 μM oligonucleotide. With lipofection, the maximum uptake was ~10%. Higher concentrations of either component did not improve the results.

Using the optimum concentration of oligo and lipid, we compared the time course of transfection (Fig. 2) for lipofectamine (10 μg/ml) with each oligonucleotide (0.4 μM). Uptake of oligonucleotide from micells with lipofectamine was rapid. Uptake increased from 0–4 h, then appeared to level off. This may be due to saturation of cationic lipid uptake or to a balance between uptake and loss of 5′-end label. By 4 h, more than half (50–65%) of the total oligonucleotide recovered from the combined media plus cell layer was in the cell layer. In triplicate uptake experiments, we noted that the uptake of the oligonuclotides targeted to the nucleus was consistently greater than those targeted to mRNA (61.3±7.7% vs. 52.7± 4.0% at 4 h, $P = 0.01$), although these differences may be within the error of the experiment.

Furthermore, for oligonucleotide transfected with lipofectamine, gel electrophoresis of intracellular oligo demonstrated no internal cleavage of the phosphorothioates for 24 h after transfection (data not shown). Virtually no breakdown products were seen among the proportion of the oligo which retained its 5′-end label.

*Effect of oligonucleotide uptake on production of mutant α2(I) chain.* The cultured OI type IV fibroblasts were exposed to micells formed with 0.4 μM oligonucleotide and 10 μg/ml lipofectamine for 4 h (Table I; diagram). The control wells were treated with lipid alone. After treatment, the media was replaced with media containing [$^3$H]proline. Cultures were harvested at 8 and 24 h after micell treatment began. The collagen

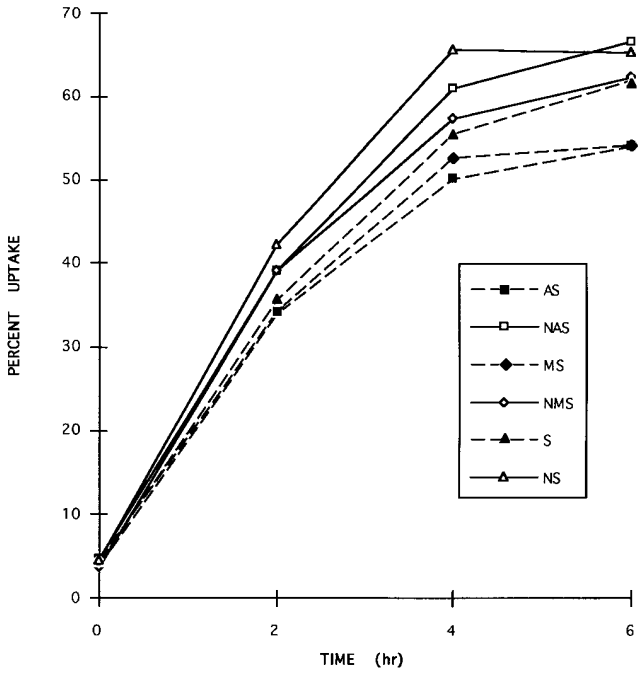
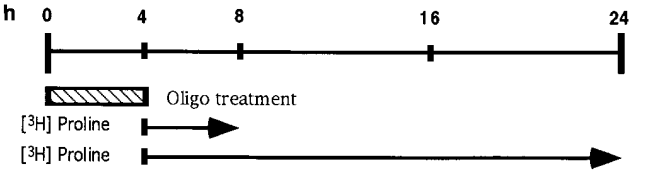



*Figure 2.* Time course of phosphorothioate transfection into fibroblasts in the presence of lipofectamine. Kinased and gel-purified oligo ($1.5 \times 10^5$ cpm/well) was used to tag unlabeled oligo (0.4 μM). Micells formed in serum-free media with 10 μg/ml lipofectamine were applied to cultured fibroblasts. Percent oligo uptake = total cts in cell layer/cts in cell layer + cts in media.

*Table I. Percent of Mutant α2(I) Chain in Fibroblast Cultures Treated with Phosphorothioates as Compared with Untreated Cultures*

| | 8 h | 24 h |
|---|---|---|
| mRNA | | |
| Sense | 91.0±29.1 | 93.7±7.7 |
| Missense | 79.0±20.4 | 76.7±10.1 |
| Antisense | 56.0±25.9 | 44.3±11.7* |
| Nuclear | | |
| Sense | 92.7±6.9 | 78.3±15.0 |
| Missense | 93.7±5.0 | 96.7±3.4 |
| Antisense | 77.0±28.7 | 46.7±12.9* |




The diagram shows the experimental plan: after 4 h of oligonucleotide treatment (*shaded bar*), the media was replaced with fresh media containing $^3$H-proline and harvested at 8 or 24 h after transfection began. An asterisk designates statistically significant ($P < 0.01$) suppression of mutant protein in treated, as compared to untreated, cultures. The data are the mean±SD of densitometry analysis of CB4 peptides from triplicate cultures.

protein thus consists of all collagen accumulated during the labeling interval.

Mutant and normal peptides were separated by electrophoresis of cyanogen bromide peptides of the α2(I) chain and quantitated by densitometry. An example of one such electrophoresis is shown (Fig. 3 *A*). Table I shows the percent of mutant peptide in oligonucleotide-treated as compared to sham-treated triplicate cultures. There was no significant suppression of mutant α2(I) chain at 8 h after micell exposure began. Cultures treated with either of the antisense oligonucleotides and harvested at 24 h after beginning micell exposure showed significant suppression of the mutant chain. Suppression was comparable, 44.3±11.7% and 46.7±12.9%, respectively, with the antisense oligonucleotides to either the mutant mRNA or nuclear structure.

There was also nonsignificant suppression of the mutant chain by missense oligonucleotide to the mutant exon 15/17 junction and by sense oligonucleotide to the nuclear point mutation. In addition, antisense suppression is rather variable among the cultures and the standard deviations of some triplicate cultures are not small. Other investigators have attributed these variations in suppression to differences in the responsiveness of cells depending on density and cell cycle (12).

*Effect of oligonucleotide uptake on production of mutant and normal α2(I) mRNA.* We examined the levels of mutant and normal α2(I) mRNA after oligonucleotide treatment to determine whether suppression of the mutant α2(I) chain reflected decreased mRNA. Total RNA was slot blotted and probed with oligonucleotides which had been demonstrated to be allele-specific: for the normal allele the probe is derived from exon 16 sequences and for the mutant allele the probe is complemenatry to the exon 15/17 junction. Fig. 3 *B* shows an example of one such pair of slot blots, indicating decreased mutant α2(I) mRNA in cells treated with each antisense oligonucleotide. The level of normal mRNA is unchanged in these antisense treated cells as compared to the control cells.

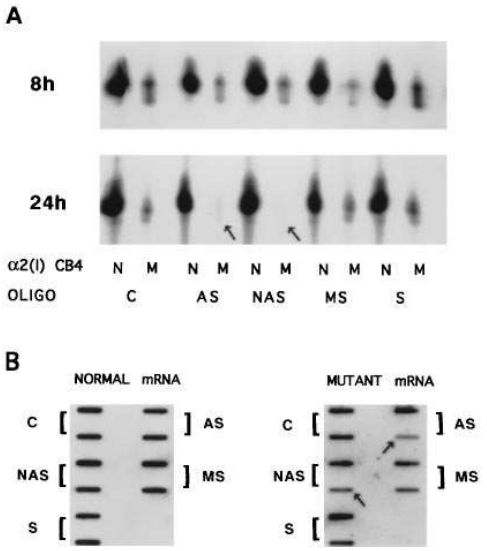



*Figure 3.* Analysis of antisense suppression of collagen protein and mRNA. (*A*) Analysis of collagen protein by examination of α1(I)CB4 cyanogen bromide peptides. The OI fibroblasts were treated with the designated oligos (*C*, lipid treated control; *AS*, antisense to mRNA; *NAS*, antisense to nuclear point mutation; *MS*, missense to mRNA; *S*, sense to mutant nRNA). Cultures were harvested 8 and 24 h after transfection. The α1(I) and α2(I) collagen chains were first separated on a 5% PA-SDS-Urea gel (data not shown). The normal (*N*) and mutant (*M*) α2(I) bands were cut separately from the gel. The gel slices were treated with CNBr and the products were electrophoresed on a 12% PA-SDS-urea gel to fully resolve the normal and mutant CB4 peptides. Mutant CB4 peptide was suppressed in antisense treated cultures (*arrows*). (*B*) Analysis of collagen mRNA by Northern slot-blot hybridization. Total RNA was prepared from cell cultures 24 h after transfection with oligos. For cultures treated with each oligo, 2 and 5 μg total RNA were blotted on the membrane and hybridized with radiolabeled probes specific for the normal and mutant α2(I) mRNAs. Arrows indicate the mutant mRNA which is suppressed by antisense treatment. In the same cells, the level of normal α2(I) mRNA is comparable to controls. The brackets to the side of each pair of slots indicate the oligo with which the cells were treated.

Table II shows the composite RNA results from slot blots of triplicate cultures harvested at 24 h. Both antisense oligonucleotides achieve suppression of the mutant message to ~40% of its level in control cells. Thus, suppression of mutant α2(I) protein can be explained entirely in terms of decreased mRNA levels. With both antisense oligonucleotides, suppression of the mutant, as compared to the normal, α2(I) message is significant. However, the suppression is relative, in that the normal α2(I) mRNA is decreased to ~80% of its level in control cells.

There was also suppression of the mutant α2(I) mRNA after transfection of two of the control oligonucleotides. The sense oligonucleotide to the nuclear level point mutation suppressed both the mutant (58% of control levels) and normal (75% of control levels) α2(I) mRNA. The missense oligonucleotide also suppressed both α2(I) mRNAs to a moderate degree, 66 and 88% of control levels of mutant and normal mRNA, respectively. Surprisingly, given the random order of nucleotides in the missense oligonucleotide, suppression of the alleles was statistically distinct.

*Table II. Percent Normal and Mutant mRNA in Phosphorothioate-treated OI Type IV Cells 24 h after OligoTreatment*

| | Normal | Mutant |
|---|---|---|
| Control mRNA | 100 | 100 |
| Sense | 84.3±24.3 | 77.0±2.6 |
| Missense | 79.0±7.0 | 86.7±33.9 |
| Antisense | 79.0±18.0 | 43.3±18.6* |
| Nuclear | | |
| Sense | 74.8±4.2 | 57.8±7.1* |
| Missense | 88.0±2.9 | 66.3±4.6* |
| Antisense | 81.0±7.0 | 37.0±22.0* |

Triplicate cultures were treated with each oligo, as in Table I and Fig. 3 *B*, except that no $^3$H-proline was added. The data are the mean±SD of densitometry analysis of Northern slot blots. An asterisk designates significant suppression of mutant, as compared to normal, mRNA in the treated cultures.

*Time course of protein and mRNA suppression in response to transient exposure of cells to micells of lipid-antisense oligonucleotide.* We investigated the time course of transient lipid-mediated antisense suppression using the antisense oligonucleotide to the mutant exon 15/17 junction. In this experiment, parallel cultures were exposed to micells with oligonucleotide for 4 h. Then the media with micells was removed, and replaced with fresh media. To examine the collagen protein synthesized at various times post-transfection, the cells were exposed to [$^3$H]proline only for the 6 h before the cultures were harvested. To examine collagen mRNA, unlabeled cells were lysed at the same time points that cultures were harvested for protein.

We selected the 72-h limit of our time course after examining the growth curves (data not shown) of the treated and untreated OI type IV fibroblasts under our culture condition. Cells appeared to be quite synchronized in their growth, doubling during 12-h intervals. Treated cells grew slightly more slowly than control cells during the first doubling, but, after 48 h, growth of treated and untreated cultures was indistinguishable. After 96 h, both treated and untreated cells were no longer doubling, under our culture conditions. Thus, until and including the 72-h time point we know that both treated and untreated cells are dividing and that, after that time point, they are limited by culture conditions, rather than by antisense treatment.

Fig. 4 shows the effect of 4 h of antisense oligonucleotide exposure on normal and mutant mRNA over the following 72 h. Each mRNA is plotted as compared with its level in untreated control cells from the patient, at that time point. From 24 through 72 h, the suppression of the mutant message is significantly greater than that of the normal message (*P* values range from $P = 0.01$ to 0.05). Mutant α2(I) mRNA is suppressed to as low as 15–20% of the levels of mutant mRNA in control cells. However, it is clear that the suppression of the mutant α2(I) mRNA is only relatively selective, with suppression of normal α2(I) mRNA to ~ 25–80% of its level in control cells.

Over the same time course, the suppression of the mutant α2(I) protein chain is more consistent than mRNA suppression. From 12–72 h after the 4-h transfection, the mutant protein chain was suppressed to ~ 50% of its level in control cells (Fig. 5).

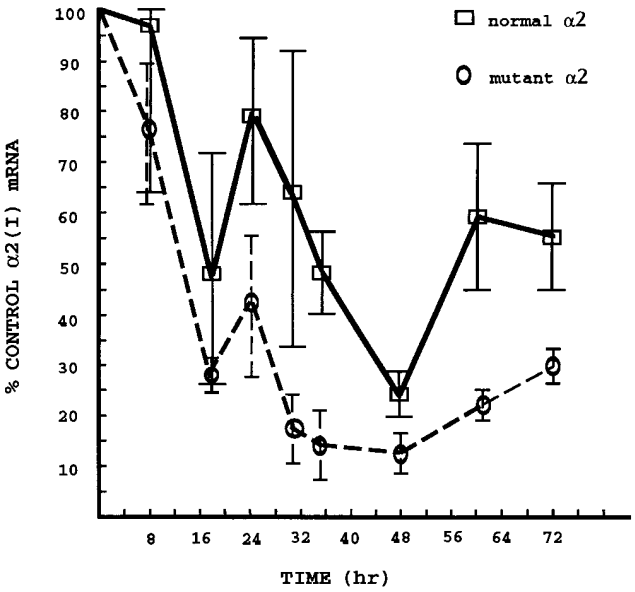



*Figure 4.* Time course of antisense suppression of α2(I) mRNA. Triplicate cultures were treated as described in Materials and Methods. Total RNA harvested at the indicated times was analyzed by Northern slot blot. The normal (*open square*, *solid line*) and mutant (*open circle*, *dashed line*) α2(I) mRNA detected in treated cultures was compared with the amount present in parallel untreated cultures. Data presented are the mean±SD of densitometry analysis of triplicate cultures.

## Discussion

This paper reports the use of antisense oligonucleotides to achieve allele specific suppression of mutant collagen protein and mRNA in cultured OI Type IV fibroblasts. We have demonstrated that 20-mer phosphorothioates can discriminate a novel exon junction at the cytoplasmic level. In time course experiments with antisense oligonucleotide to the mutant mRNA exon 15/17 junction, the mutant protein chain was suppressed to ~ 50% of its level in untreated OI cells from 12 to 72 h after a 4 h transfection. The mutant α2(I) mRNA was suppressed to 15–50% of the levels of mutant mRNA in untreated OI cells during this same time period. Thus, although there are antisense mechanisms which are RNase H-independent (18), RNase H-dependent mechanisms (19) can fully account for the suppression demonstrated in these cultured cells.

The specificity of antisense suppression is crucial to potential therapeutic approaches. We targeted antisense oligonucleotides at both a nuclear level point mutation and at the mRNA level mutant exon junction. It is a formidable challenge to achieve specificity of oligonucleotide annealing in this system. In addition to the similarity of mutant and normal target sites which differ by only one (nuclear mutation) or 4–7 (mRNA) nucleotides, the collagen genes are highly repetitious and the oligos must discriminate among over 300 similar 9-nt sequences in each of the α1(I) and α2(I) cDNAs. Furthermore, these oligonucleotides must recognize the target site in its naturally occurring location. Prockop and co-workers used antisense oligonucleotides to target a human α1(I) minigene which

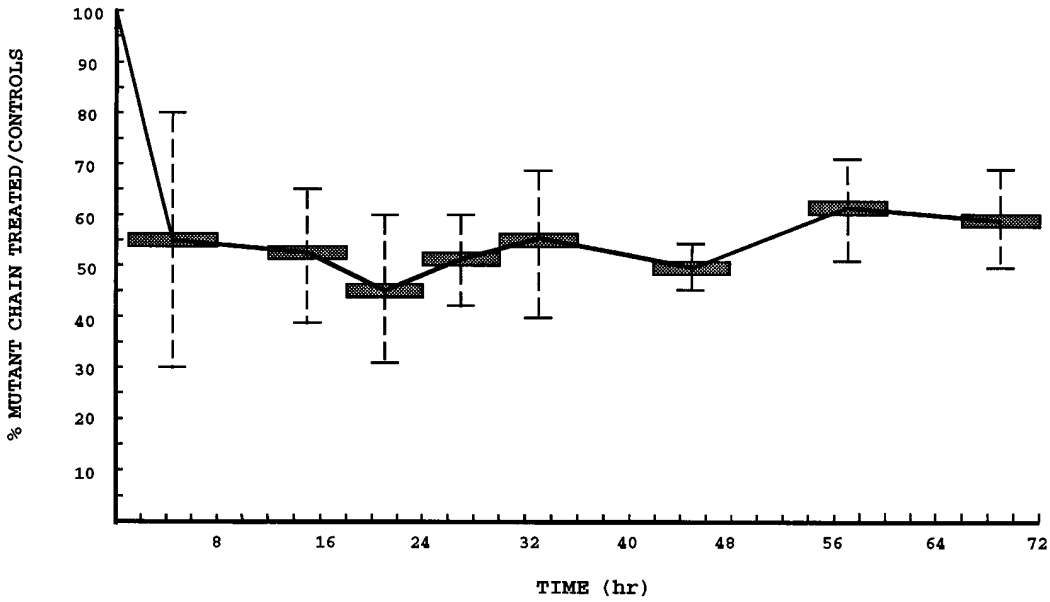



*Figure 5.* Time course of antisense suppression of α2(I) protein chain. Triplicate cultures were treated and harvested as in Methods. The amount of mutant collagen CB4 peptide was determined as in Fig. 3 *A* and quantitated by densitometry. The amount of mutant CB4 peptide detected was compared to the amount present in parallel untreated cultures. The shaded bar indicates the time interval over which the cultures were labeled. The data are the mean±SD of triplicate cultures.

had been stably transfected into mouse NIH 3T3 cells (12), demonstrating that a specific human target could be discriminated when removed from its normally occurring structural context and expressed in a mouse background. We have targeted a naturally-occurring human collagen mutation, demonstrating that allele specificity can be achieved in a normal cellular context. Although the statistical specificity achieved in these investigations is encouraging, it is still relatively, rather than absolutely, specific. This is seen most clearly at the mRNA level. When the antisense oligonucleotides suppress the mutant mRNA to 37–43% of its level in untreated OI cells, the normal mRNA is also suppressed to $\sim$ 80% of its untreated level. In a therapeutic situation, manipulation of oligonucleotide length and alignment with respect to the mutation might decrease the suppression of normal mRNA.

The importance of oligonucleotide sequence is highlighted by the nonspecific effects which were obtained with the two control nucleotides to the nuclear target. The sense and missense oligos to the nuclear target each suppresses mutant mRNA production to more than half of the extent achieved by the antisense oligo. Moreover, these controls affect the mutant and normal mRNA in a statistically distinct way. Given these two factors, about half of the apparent allele-specific suppression obtained with the antisense oligo to the nuclear target must be attributed to non-specific effects. The reason that the nuclear and cytoplasmic results are different is not clear. Possible explanations include the nuclear level per se, the kind of mutation being targeted (a point mutation), or the sequence surrounding this particular mutation. Fluorescent oligos have been shown to be released from endosomes by lipofectin and to localize in the nucleus (20). Furthermore, an oligo antisense to an intron can cause suppression (20). Thus, entry into the nucleus and RNase H-dependent suppression should both be possible, suggesting that the lack of specificity resides in the sequences surrounding this point mutation and that it will be possible to target other mutations at the nuclear level.

We note that the allele-specific effects of oligonucleotide to mRNA occur only with the antisense oligo, as would be necessary for a therapeutic situation. This may be because the targeting process is inherently more specific at the mRNA level, or it may reflect more fortuitous choice of target sequence at the mRNA level in these experiments. Indeed, other possibilities for improving efficiency and selectivity of antisense oligonucleotides include moving the oligo several nucleotides off center of the target nucleotide or varying the length of the oligonucleotide (21).

The data presented in this report are encouraging about the ability of antisense oligonucleotides to recognize a target in its natural context and discriminate the target sequence from similar sequences. Clearly, multiple practical issues lie between such a demonstration in cultured cells and therapeutic clinical use. One primary goal is to increase the specificity of binding. In part, successful specificity is likely to be related to the length of the antisense oligomer. The shortest sequence that is likely to be unique within an RNA pool of $2 \times 10^7$ bases is 13 nucleotides (19). If partially matched oligomers can hybridize effectively, then longer oligos will have decreased specificity. This is complicated by the unusual repetitive nature of the collagen sequences. Ribozymes represent a possible way to increase the efficiency of target degradation (22), but their specificity may not be comparable to oligomers in the collagen context. A second goal is the increased efficiency of suppression. We have demonstrated $\sim$ 50% suppression of mutant chain. This level of inhibition, though encouraging, is not sufficient for therapeutic trials.

Furthermore, some of the important issues for antisense therapy in dominant negative disorders are quite distinct from those in antisense treatment of HIV or neoplasms. First, domi-

nant genetic disorders will require chronic, essentially life-long suppression of the mutant allele. Second, successful therapy of OI must be delivered to the resident osteoblasts of the skeleton. Pre-osteoblast stem cells must be isolated, enriched, modified genetically and returned to the skeletal system to take up residence in matrix lacunae. Osteocytes are not a rapidly exchanging population; mechanisms must be developed to facilitate the exchange of modified cells for mutant cells. Finally, since almost all OI collagen mutations described to date are unique, treatment must be individualized. Although these issues pose considerable challenges to therapeutic intervention, antisense approaches are currently the most promising for treatment of dominant negative disorders and merit further development.

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