Use of an Antisense Oligonucleotide To Inhibit Expression of a Mutated Human Procollagen Gene (COL1A1) in Transfected Mouse 3T3 Cells†

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ABSTRACT: A series of antisense oligonucleotides were developed to inhibit specifically expression of a mutated exogenous gene for collagen without inhibiting expression of an endogenous gene for the same protein. The test system consisted of mouse NIH 3T3 cells that were stably transfected with an internally deleted construct of the human gene for the proα1(I) chain of type I procollagen [Olsen et al. (1991) *J. Biol. Chem. 266*, 1117]. The target site was a region at the 3′ end of exon 1 and the first few nucleotides of intron 1 of the exogenous human gene that differed in sequence by nine nucleotides from the sequence of the endogenous mouse gene. Expression of the two genes was assayed by Western blot with cross-reacting antibodies and by steady-state levels of mRNAs. None of the oligonucleotides were effective in concentrations up to 25 μM when administered without any carrier. However, when administered with 5 or 10 μg/mL lipofectin, one of the oligonucleotides in concentrations of 0.1–0.2 μM inhibited expression of the exogenous gene from 50% to 80% without significant inhibition of expression of the endogenous gene. Also, a missense version of the same oligonucleotide had no significant effect, and the inhibition observed with the most effective oligonucleotide was abolished by a single base change. Time course experiments indicated that, after a 4-h treatment, inhibition appeared at 8 h and persisted for at least 22 h. The results raised the possibility that the same oligonucleotide can be used to rescue the phenotype of fragile bones in transgenic mice expressing the same internally deleted collagen gene [Khillan et al. (1991) *J. Biol. Chem. 266*, 23373].

Over 100 different mutations in genes for fibrillar collagens have been shown to cause a series of genetic diseases (Byers, 1990; Sykes, 1990; Prockop, 1990; Kuivaniemi et al., 1991). Mutations in one of the two genes for type I procollagen (COL1A1 and COL1A2) cause osteogenesis imperfecta, mutations in the gene for type II procollagen (COL2A1) cause chondrodysplasias and related disorders, and mutations in the gene for type III procollagen (COL3A1) cause Ehlers–Danlos syndrome type IV and aortic aneurysms. With a few exceptions, most of the mutations produce disease phenotypes because they cause synthesis of structurally abnormal but partially functional proα chains of type I, type II, or type III procollagen. The partially functional proα chains associate with and become disulfide-linked to normal proα chains. As a result, they can have one of two major effects (Prockop, 1990). One is to prevent folding of three chains into a collagen triple helix and thereby cause degradation of both the abnormal and normal proα chains through a process referred to as procollagen suicide. The second effect is to produce minor changes in the conformation of the collagen triple helix and thereby generate mutated monomers that interfere with self-assembly of normal monomers synthesized by the same cells. Recently, the deleterious effect of expression of the mutated genes was demonstrated in transgenic mice that expressed mutated genes for type I procollagen and developed phenotypes resembling human osteogenesis imperfecta (Stacey et al., 1988; Khillan et al., 1991), and in transgenic mice that expressed mutated genes of type II procollagen and developed phenotypes resembling human chondrodysplasias (Vandenberg et al., 1991; Cheah et al., 1992; Garafalo et al., 1992).

Because many heritable diseases of collagen are caused by the protein products from mutated genes (Byers, 1990; Sykes, 1990; Prockop, 1990; Kuivaniemi et al., 1991), selective inhibition of expression of the mutated genes may provide a tool for therapy of the diseases. Recently, modified oligonucleotides that are antisense to specific RNAs have been used to inhibit the expression of a number of cellular and viral proteins [see Erickson and Izant (1992)]. Many hypotheses were proposed to explain the mechanisms by which antisense oligonucleotides can inhibit gene expression, and the specific mechanism involved may depend on the cell type studied, the RNA targeted, the specific site on the RNA targeted, and the chemical nature of the oligonucleotide (Chiang et al., 1991; Stein & Cohen, 1988). Here we have developed antisense oligonucleotides to inhibit specifically expression of an exogenous collagen gene stably transfected into mouse 3T3 cells (Olsen et al., 1991).

MATERIALS AND METHODS

Oligonucleotide Synthesis. Phosphorothioate oligodeoxynucleotides were synthesized via phosphoramidite chemistry by sulfurization with tetraethylthiuram disulfide in acetonitrile (Vu & Hirschbein, 1991) by Dr. Kim De Riel, Temple University School of Medicine, Philadelphia, PA.

Treatment of Cell Cultures. NIH 3T3 cells stably expressing an internally deleted version of the human COL1A1 gene (Olsen et al., 1991) were grown in DMEM[1] containing 10% calf serum and 400 μg/mL Geneticin (GIBCO BRL).

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[1] Abbreviations: DMEM, Dulbecco's modified Eagle's medium; PCR, polymerase chain reaction.

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The cells were plated in 24-well plates (Falcon) at a concentration adjusted to obtain subconfluent cultures at the end of the experiment. Twenty hours later, the cells were washed two times with prewarmed DMEM, and 0.3 mL of DMEM containing the indicated concentration in lipofectin (GIBCO BRL) was added in each well. Oligonucleotides dissolved in distilled water were then added as a 20X stock solution and incubated for 4 h at 37 °C. About 0.7 mL of DMEM containing 14% calf serum previously heat inactivated at 56 °C for 1 h and 400 μg/mL Geneticin were added. The cells were then incubated at 37 °C for the additional times indicated.

Protein Analysis. At the end of incubation with the oligonucleotides, cells were washed two times in DMEM and solubilized in 0.1 mL of lysis buffer consisting of 1% SDS, 1% sodium deoxycholate, 0.1% Triton X-100, 10 mM EDTA, 0.5 unit of aprotinin/mL (Sigma), 3% β-mercaptoethanol, and PBS adjusted to pH 7.4. After 5-min incubation at room temperature, the cell lysate was harvested and strongly vortexed, and one-fourth volume of the sample loading buffer was added (0.6 M Tris-HCl buffer, pH 6.8, 50% glycerol, 1% SDS, 0.012% bromophenol blue). The lysate was then heated for 5 min at 94 °C, and 10 μL of the sample was electrophoresed on a 7.0% SDS–polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) and reacted with an antibody against a synthetic peptide corresponding to the last 21 amino acids of the human proα1(I) chain of type I procollagen. (The antibody was kindly provided by Dr. Larry Fisher, National Institutes of Health, Bethesda, MD.) The antibody recognized both the human and the mouse COOH-terminal propeptide of the proα1(I) chain (Olsen et al., 1991). The proα1(I) bands were detected by reaction with a goat anti-rabbit antibody coupled to ^{125}I (Du Pont-NEN) and subsequent autoradiography. Relative amounts of protein from the endogenous and exogenous COL1A1 genes were then assayed by using a laser densitometer (LKB, Ultroscan XL).

RNA Assay. For RNA assays, total cellular RNA was isolated from tissues using acidic guanidine thiocyanate–phenol–chloroform extraction (Chomczynski & Sacchi, 1987). The ratio of mRNAs from exogenous and endogenous genes was measured by a quantitative PCR assay. Primers for reverse transcription and polymerase chain reaction were designed to be complementary to the identical sequence in human and mouse proα1(I) mRNA (Mooslehner & Habers, 1988; Westerhausen et al., 1991). This provided the same efficiency of amplification for both mRNAs. Five micrograms of total cellular RNA was reverse transcribed in 20 μL of the reaction mixture using 200 pmol of the primer BS33 (5′-ACTAAGTTTGA-3′) and a preamplification system for first-strand cDNA synthesis (SuperScript, GIBCO BRL). After RNase H treatment, cDNA was amplified by PCR (GeneAmp, Perkin-Elmer Cetus) using primer BS31 (5′-TTGGCCCTGTCTGCCT-3′) and ^{32}P-labeled primer BS32 (5′-TGAATGCAAAGGAAAAAAAT-3′) at concentrations of 4 pmol/100 μL of reaction mixture. PCR conditions were 80 s at 94 °C, 1 min at 47 °C, and 20 s at 72 °C for 15 cycles. Amplified products from human proα1(I) mRNA and mouse proα1(I) mRNA were 176 and 177 bp long, respectively, and were distinguishable only after digestion with *Bst*NI. Ten microliters of PCR product was digested by 2 units of *Bst*NI for 1 h at 60 °C, denatured, and electrophoresed in 15% PAGE containing 6 M urea. The gel was fixed, dried, and exposed to X-ray film.

Table I: Design of Modified Oligonucleotides

A. DNA Sequence at the Exon1/Intron 1 Junction[a]

```
        200        210        220
         .          .          .
5'  CAAGTCGAGGGCCAAGACGAAGACAgt  3'
3'  GTTCAGCTCCCGGTTCTGCTTCTGTca  5'        Exogenous
    |  |  |        | |||||||||
5'  CTCCTGACGCATGGCCAAGAAGACAgt  3'
3'  GAGGACTGCGTACCGGTTCTTCTGTca  5'        Endogenous
         .          .          .
        170        180        190
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B. Phosphorothioate oligodeoxynucleotides

Code name	Sequence (5′→ 3′)	Target
AS3	ACTGTCTTCGTCTTGGCCCT	Exo (224 to 205)
MS3[b]	ATCCTGCTTCGTTCTGGCTC	Missense of AS3
S3[c]	AGGGCCAAGACGAAGACAGT	Exo (205 to 224)
AS7[d]	ACTGTATTCGTCTTGGCCCT	Exo (224 to 205)
AS8	TGTCTTCGTCTTGGCCCTCG	Exo (222 to 203)
AS9	TCTTCGTCTTGGCCCTCGAC	Exo (220 to 201)
AS10	ACTGTCTTCGTCTTG	Exo (224 to 210)
AS11	GTCTTGGCCCTCGACTTG	Exo (215 to 198)
AS12	ACTGTCTTCTTGGCCATGCG	Endo (195 to 176)
AS14[e]	ACTGTATTCTTGGCCATGCG	Endo (195 to 176)
AS15[e]	ACTGTCTACTTGGCCATGCG	Endo (195 to 176)
AS16[d]	ACTGTCTACGTCTTGGCCCT	Exo (224 to 205)

[a] Bases from exon 1 are in capital letters, and bases from intron 1 are in lower-case letters. Vertical bars indicate identity between exogenous (human) and endogenous (mouse) COL1A1 genes. For both exogenous and endogenous genes, the adenine at the start of transcription was counted as position +1. [b] MS3 contains the same content in A, C, G, and T as AS3 but in a random order. [c] S3 is the sense version of AS3. [d] Same sequence as AS3, except for one mismatch (underlined base). [e] Same sequence as AS12, except for one mismatch (underlined base).

RESULTS

Initial Tests of Modified Oligonucleotides. To develop antisense oligonucleotides, the test system employed consisted of mouse NIH 3T3 cells that were stably transfected with an internally deleted construct (Olsen et al., 1991) of the human gene for the proα1(I) chains of type I procollagen (COL1A1). In initial experiments, a series of modified oligonucleotides were synthesized using a region at the 3′ end of exon 1 and the first two nucleotides of intron 1 of the exogenous gene as a target (Table I). The target site was selected because the human gene contained 27 nucleotides in exon 1 that were not found in the corresponding endogenous mouse gene (Habers et al., 1984). Also, previous reports indicated that mRNA splice sites were a good target for antisense nucleic acids (Kole et al., 1991; Munroe, 1988). None of the oligonucleotides were effective in inhibiting expression of either the exogenous or the endogenous gene in concentrations up to 25 μM when the oligonucleotide was administered without any carrier (data not shown). However, when administered with 10 μg/mL lipofectin in order to increase the uptake of nucleic acid (Chiang et al., 1991), several of the oligonucleotides designed as antisense inhibitors of the exogenous gene were effective. The oligonucleotide that appeared to be most effective, AS3, reduced the relative expression of the exogenous gene to about 43% of the control (Figure 1 and Table II). The missense oligonucleotide MS3 reduced expression to about 81% of the control, and the sense oligonucleotide S3 reduced expression to about 74% of the control. However, the small degrees of inhibition seen with MS3 and S3 were not consistently observed

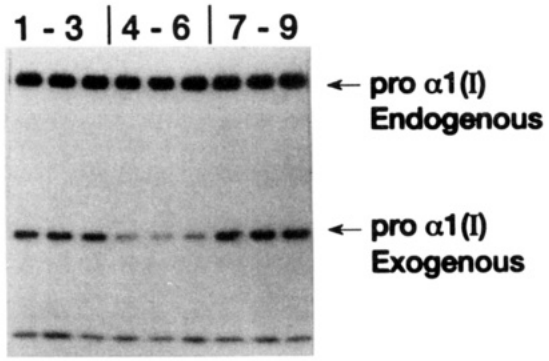

FIGURE 1: Western blot assays of expression of the endogenous gene and the exogenous gene for proα1(I) chains (COL1A1). The cells were incubated with a 0.2 μM quantity of the oligonucleotides in serum-free medium containing 10 μg/mL lipofectin for 4 h. Heat-denatured calf serum was then added to achieve a final concentration of 10%, and the cells were incubated for an additional 16 h. Extracts of cells were separated by polyacrylamide gel electrophoresis in SDS and blotted onto nitocellulose filters. The filters were then incubated with an antibody that reacted with both the mouse proα1(I) chain synthesized from the endogenous gene and the human proα1(I) chain synthesized from the exogenous, internally deleted human gene (Olsen et al., 1991). Lanes: 1–3, cells treated in triplicate with missense oligonucleotide MS3; 4–6, cells treated in triplicate with the antisense oligonucleotide AS3; 7–9, triplicate samples of control cells not treated with oligonucleotides.

in all experiments. The relative effectiveness of the oligonucleotides was more apparent when the values were compared to the values seen with the missense oligonucleotide (MS3). On this basis, AS3 was the most effective oligonucleotide and reduced the expression of the exogenous gene to 53% of the control. As also indicated in Table II, altering a single nucleotide in AS3 at one site had little effect (see AS7), but a single nucleotide change at another site decreased the effectiveness of the oligonucleotide (see AS16).

As a control for general toxicity of the oligonucleotides, their effects on expression of the fibronectin gene were examined with antibodies to fibronectin and Western blot assays similar to those shown in Figure 1. No significant changes (not shown) in the ratio of fibronectin to the mouse proα1(I) chains were seen with the same oligonucleotides and under the same conditions as in Table II.

In additional experiments, two antisense oligonucleotides (AS12 and AS15) were designed to complement RNA transcripts of the endogenous mouse gene. Both AS12 and AS15 appeared to decrease expression of both genes (Table III), but the effects were difficult to assess because of the difficulty of adequately controlling for cell number, and both AS12 and AS15 had similar effects on expression of fibronectin (not shown). Both AS12 and AS15, however, consistently decreased the ratio of proα1(I) chains from the endogenous gene relative to proα1(I) chains from the exogenous gene. Therefore, expression of the endogenous gene was selectively inhibited. An oligonucleotide that differed by one base from one of the inhibitory oligonucleotides was less effective (compare AS14 and AS12).

Inhibition of mRNAs. To verify the effects of the oligonucleotides, the mRNAs from cells were transcribed into single-stranded cDNAs using an oligonucleotide that primed both the mRNA for the human and mouse proα1(I) chain. The single-stranded cDNA was then amplified by PCR using a single set of primers with one of the primers labeled with ^{32}P. The antisense oligonucleotide AS3 selectively decreased the steady-state level of mRNA for proα1(I) chains from the exogenous gene to about 50% of the control value (Figure 2). In the same experiments the relative expression at the protein level was also decreased by about 50% (not shown).

Time Course for the Effects of Antisense Oligonucleotide. For reasons that were not apparent, the steady-state ratio of proα1(I) chains from the exogenous and endogenous genes increased with time after cell cultures were initiated (Figure 3). After exposure of the cells to the oligonucleotide and lipofectin in serum-free medium for 4 h, maximal inhibition with AS3 was observed in about 20 h (Figure 3). The inhibition began after 8 h and persisted for at least 20 h. Reexposure of the cells after 24 h to the oligonucleotide and lipofectin in serum-free medium did not increase the degree of inhibition (not shown).

Effects of Varying the Concentrations of Lipofectin and the Oligonucleotides. Preliminary experiments demonstrated a marked reduction in cell proliferation when mixtures of 10 μg/mL lipofectin and 0.4 μM oligonucleotides were added to the cell cultures. Therefore, lower concentrations of both components were used to define optimal conditions for inhibiting expression of the exogenous gene. The greatest inhibition was obtained with 5 μg/mL lipofectin and a 0.1 μM quantity of the effective oligonucleotide AS3 (Table IV). With these conditions, expression of the exogenous gene was specifically reduced to 22% of the MS3 value. Less inhibition was observed with 5 μg/mL lipofectin and higher concentrations of oligonucleotide, possibly because saturation of the cationic lipid with oligonucleotide prevented fusion with cell membranes (Chiang et al., 1991).

DISCUSSION

Synthetic oligonucleotides modified to prevent intracellular degradation have been shown to be effective in selectively inhibiting expression of a variety of genes (Chiang et al., 1991; Stein & Cohen, 1988; Chang et al., 1991). For example, Chang et al. (1991) reported selective inhibition of a *ras* p21 gene that differed from a normal gene by a single base.

Selective inhibition of expression of a mutated procollagen gene is of interest because the deleterious effects of most mutations in collagen genes are explained by the synthesis of structurally abnormal but partially functional proα chains that either cause degradation or interfere with the biological function of normal proα chains (Byers, 1990; Sykes, 1990; Prockop, 1990; Kuivaniemi et al., 1991). The results here demonstrate that selective inhibition of an exogenous COL1A1 gene can be obtained with modified antisense oligonucleotides. In the test system, the exogenous gene consisted of a construct of the human COL1A1 gene. The target sequence of the most effective oligonucleotide was 20 nucleotides that differed by nine nucleotides between the human gene and the endogenous mouse gene. The inhibitions observed were specific in that from 50% to 80% inhibition of the exogenous gene was obtained with less than 10% inhibition of expression of the endogenous collagen gene or the fibronectin gene. Missense or sense versions of the same oligonucleotide had essentially no effect. Also, the inhibition observed with the most effective oligonucleotide was reduced by introducing a single base change. In the test system, lipofectin was necessary for inhibition by the antisense oligonucleotide. Similar requirements for lipofectin in such experiments have been reported by others (Chiang et al., 1991). Selective inhibition of expression of the exogenous gene was consistently observed in all the experiments carried out here. However, the degree of inhibition varied from experiment to experiment, apparently because of small differences in the responsiveness of the cells.

Of special note is that, in the presence of lipofectin, the concentrations of oligonucleotide required for effective in-

Table II: Effects of Antisense Oligonucleotides against Expression of the Exogenous Gene (COL1A1)

oligonucleotide	COL1A1 expression[a]		ratios		
	endogenous	exogenous	exo/endo	% of control[b]	% of MS3 value[b]
control	10.5 ± 0.2	4.4 ± 0.2	0.42 ± 0.03	100	123
AS3	9.0 ± 0.8	1.7 ± 0.2	0.18 ± 0.01	43[d]	53[d]
MS3	10.7 ± 1.4	3.6 ± 0.2	0.34 ± 0.05	81	100
S3	11.4 ± 1.7	3.5 ± 0.2	0.31 ± 0.06	74	91
AS7[c]	11.3 ± 1.3	2.5 ± 0.2	0.22 ± 0.04	52[d]	65
AS8	8.1 ± 0.3	1.5 ± 0.1	0.19 ± 0.01	45[d]	54[d]
AS9	12.1 ± 1.1	3.0 ± 0.2	0.25 ± 0.06	60[e]	74
AS10	14.0 ± 2.9	4.2 ± 0.7	0.31 ± 0.06	74	91
AS11	10.3 ± 0.9	2.4 ± 0.2	0.23 ± 0.01	55[d]	68[e]
AS16[c]	10.1 ± 1.1	3.0 ± 0.2	0.30 ± 0.01	71[d]	88

[a] Expression assayed in arbitrary units by densitometry of Western blots (see Figure 1). Values are mean ± standard deviation ($n = 3$). [b] To correct for variability in cell number among samples, effects were evaluated from the ratio of protein from the exogenous to the endogenous gene versus the untreated control or cells treated with missense oligonucleotide (MS3). [c] Differ by one nucleotide from AS3 (Table I). [d] p value < 0.001. [e] p value < 0.01.

Table III: Effects of Antisense Oligonucleotides against Expression of the Endogenous Gene

oligonucleotide	COL1A1 expression		ratio	
	endogenous	exogenous	endo/exo	% of control[a]
control	13.5 ± 2.4	6.2 ± 1.3	2.18 ± 0.07	100
AS12	2.2 ± 0.1	1.5 ± 0.4	1.46 ± 0.27[c]	67[d]
control	15.2 ± 0.8	4.2 ± 0.2	3.60 ± 0.24	100
AS14[b]	6.7 ± 1.5	2.6 ± 0.2	2.48 ± 0.54	69
AS15[b]	6.1 ± 0.1	3.0 ± 0.3	2.03 ± 0.16	56[c]

[a] Effects evaluated from the ratios as indicated in Table II. [b] Differs by one nucleotide from AS12 (see Table I). [c] p value < 0.001. [d] p value < 0.01.

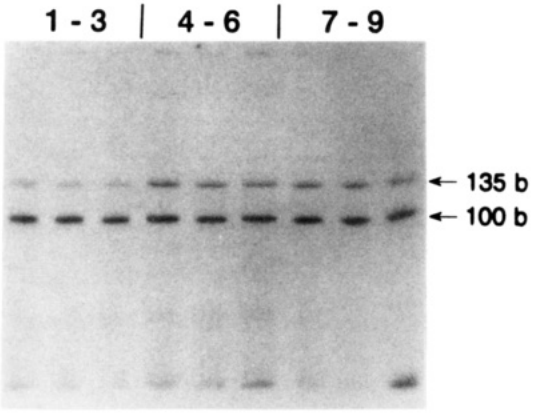

FIGURE 2: Assay of the steady-state levels of mRNAs from the exogenous and endogenous genes. Cells were treated with the oligonucleotide and lipofectin as in Figure 1. RNAs from the cells were then converted to cDNAs with reverse transcriptase and a specific oligonucleotide primer for both the human and mouse proα1(I) chains. The single-stranded cDNAs were then amplified by a single set of PCR primers. One primer (BS32) was labeled with ^{32}P. Products of 176 and 177 bp were obtained from human and mouse cDNAs, respectively, but cleavage with *Bst*NI and subsequent denaturation generated a labeled fragment of 135 bases from the human (exogenous) and 100 bases from the mouse (endogenous) cDNAs. Lanes: 1–3, cells treated in triplicate with 0.2 μM AS3 and 10 μg/mL lipofectin; 4–6, cells treated in triplicate with 0.2 μM MS3 and 10 μg/mL lipofectin; 7–9, cells treated in triplicate with 10 μg/mL lipofectin alone. Densitometry of the film (not shown) demonstrated that AS3 decreased the level of the human mRNA to 50% of the value obtained with MS3. There was no effect on the level of the mouse mRNA.

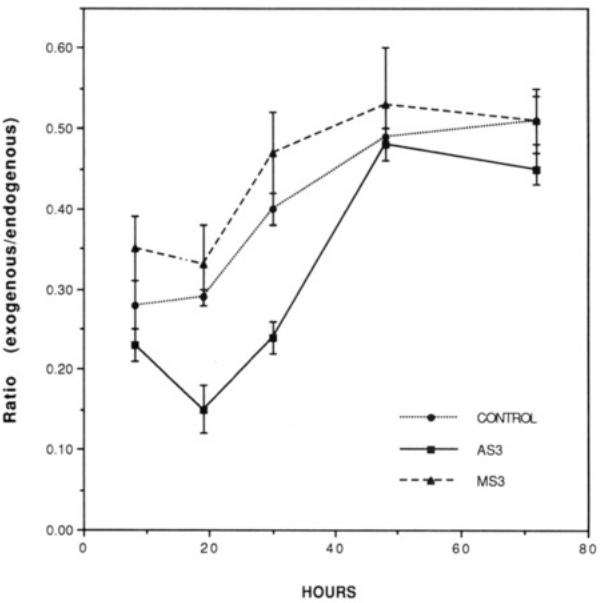

FIGURE 3: Time course for the specific inhibition of expression of the exogenous COL1A1 gene. Conditions for treating the cells were as in Figure 1. Cells were removed at the times indicated, and expression of the genes was assayed by Western blotting (see Figure 1). Values are mean ● standard deviation ($n = 3$).

Table IV: Effects of Varying Concentrations of Lipofectin and Oligonucleotides AS3 and MS3

lipofectin (μg/mL)	oligonucleotide (μM)	expression ratio (exo/endo)		% of MS3 value[a]
		AS3	MS3	
2.5	0	0.45 ± 0.05		
2.5	0.05	0.31 ± 0.01	0.48 ± 0.00	76[b]
2.5	0.1	0.30 ± 0.00	0.41 ± 0.05	65
2.5	0.2	0.42 ± 0.00	0.46 ± 0.05	91
2.5	0.4	0.49 ± 0.06	0.49 ± 0.03	100
5	0	0.44 ± 0.01		
5	0.05	0.24 ± 0.03	0.47 ± 0.01	51[c]
5	0.1	0.11 ± 0.01	0.50 ± 0.00	22[b]
5	0.2	0.19 ± 0.05	0.39 ± 0.02	49
5	0.4	0.24 ± 0.05	0.44 ± 0.06	54
10	0	0.46 ± 0.09		
10	0.05	0.35 ± 0.05	0.44 ± 0.07	79
10	0.1	0.30 ± 0.01	0.39 ± 0.04	77
10	0.2	0.14 ± 0.02	0.36 ± 0.04	39[c]
10	0.4	0.27 ± 0.02	0.62 ± 0.00	44[b]

[a] Effects evaluated from the ratios as indicated in Table II. All conditions were tested in duplicate. [b] p value < 0.001. [c] p value < 0.01.

hibition were as little as 0.1 μM. Since phosphorothioate oligonucleotides cause no observable toxicity or mortality when injected in mice at concentrations as high as 15 μM (Agrawal et al., 1991), the results raise the possibility that the same oligonucleotides can be used to rescue the phenotype of fragile bones in transgenic mice expressing the same internally deleted gene (Khillan et al., 1991). For in vivo experiments, lipofectin

cannot be used since it is ineffective in the presence of serum proteins. However, it may be possible to employ one or more of several strategies currently being developed such as coupling the oligonucleotides to lipophilic compounds or incorporating them into liposomes [see Erickson and Izant (1992) and Wickstrom (1992)]. If such experiments are successful, it may be feasible to use similar approaches to treat selective patients with osteogenesis imperfecta and related diseases in whom expression of a mutated collagen allele can be shown to be more deleterious than inactivation of the allele [see Byers (1990), Prockop (1990), and Kuivaniemi et al. (1991)].

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