Specific Inhibition of Expression of a Human Collagen Gene (COL1A1) with Modified Antisense Oligonucleotides. The Most Effective Target Sites Are Clustered in Double-Stranded Regions of the Predicted Secondary Structure for the mRNA†

Alexey V. Laptev, Zichun Lu, Alain Colige, and Darwin J. Prockop*

Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Jefferson Medical College, Thomas Jefferson University, Philadelphia, Pennsylvania 19107

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ABSTRACT: A series of antisense oligonucleotides (ASOs) were synthesized and tested to define the best target sites within an RNA transcript of collagen for effective inhibition of expression. The test system consisted of mouse NIH 3T3 fibroblasts that were stably transfected with a human minigene for procollagen I so that the cells simultaneously synthesized full-length mouse proα1(I) chains and internally deleted human proα1(I) chains. The sequences of the transcripts from both genes were compared, and a series of 28 ASOs were designed to target sites in which there were at least two base differences within a 20-nucleotide sequence between the human and mouse transcripts. Six of the ASOs specifically decreased the levels of proα1(I) chain synthesized from the human gene without a decrease in the levels of proα1(I) chains from the mouse endogenous gene. The most effective ASOs reduced the intracellular levels of human proα1(I) chains relative to the mouse proα1(I) chains to 37–67% of the control values. Combined addition of two effective ASOs or a second administration of the same effective ASO did not produce any additive effect. The results did not support previous suggestions that the best target sites for ASOs were sequences around initiation codons for translation, at intron–exon boundaries, or in single-stranded loops in hairpin structures. Also, the results did not support previous suggestions that the most effective ASOs are those with the highest affinities for their target sequences. Instead, the most consistent pattern in the data was that the most effective ASOs were those targeted to sequences that were predicted to form clustered double-stranded structures in RNA transcripts.

ASOs[1] are widely used to inhibit expression of specific genes [for reviews, see Erickson and Izant (1992) and Wickstrom (1992)]. A major obstacle in employing ASOs is selection of the best target sites within nucleotide sequences for effective inhibition of expression. No useful guidelines are yet available. For example, several reports suggested that the most effective target sites are either the 5′-nontranslated sequences or the AUG start site for translation [see Chiang et al. (1991) and Sankar et al. (1989)]. However, many ASOs targeted to such sites were ineffective, and more internal sites were frequently found to be more effective [see Ricker and Kaji (1992)]. Stull et al. tried to develop a systematic approach for identifying target sites by calculating three thermodynamic indices: (a) an Sscore for the strength of the local mRNA sequences to form double-stranded secondary structures, (b) a Dscore for the strength of the binding of the ASO to the mRNA target expressed as the free energy change for duplex formation, and (c) a Cscore that corrected the Dscore for the strength of local mRNA sequences in forming double-stranded secondary structure, i.e., Cscore = Dscore − Sscore. They then compared the Dscores and Cscores with the efficacy of ASOs in five published reports. They found that the Dscore for heteroduplex formation was the best predictor of ASO efficacy and gave correlation coefficients that ranged from 0.44 to 0.99 with data from four of the five reports. In the fith report, however, neither the Dscores nor Cscores were able to predict efficacies of the ASOs.

Recently, Lima et al. (1992) explored the possibility that single-stranded loops of hairpin structures in RNA were the best target sites. They designed six ASOs to a 47-nt transcript of the activated *Ha-ras* gene that formed a stable hairpin structure. They found that two ASOs targeted to the single-stranded loop had about the same affinity for the transcript RNA as for the same sequence in a short oligonucleotide without secondary structure. In contrast, ASOs targeted to the double-stranded stem of the hairpin were less tightly bound with affinity constants that were 10^5–10^6-fold smaller. Therefore, their results supported the hypothesis that single-stranded loop structures were more promising targets for ASOs than double-stranded sequences. Also, Thierry et al. (1993) compared ASOs targeted to either the 5′-end of the coding region or to a single-stranded loop in mRNA from the multidrug resistance gene *mdr1*. Their results indicated that ASOs targeted to the single-stranded loop were more effective and specific than ASOs targeted to the 5′-end coding region.

Recently, we have reported use of ASOs in a test system of mouse fibroblasts that were transfected with an exogenous human gene for collagen so that the cells expressed both the normal gene for the full-length proα1(I) chain of mouse type I procollagen and a gene for an internally deleted proα1(I) chain of human type I procollagen (Colige et al., 1993). ASOs were targeted to sites that differed by 1-12 bp in the two transcripts and tested for inhibition of one transcript relative

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* To whom correspondence should be addressed. Telephone: (215) 955-4830. Fax: (215) 955-5393.

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[1] Abbreviations: ASO, antisense oligonucleotide.

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to the other. When administered with 5 or 10 μg/mL lipofectin, one of the ASOs in concentrations of 0.1–0.2 μM inhibited expression of the exogenous human gene for shortened proα1(I) chains from 50% to 80% without significant inhibition of expression of the endogenous mouse gene. Here we have used the same test system to identify additional target sites in the transcripts of human gene for proα1(I) chains that will specifically inhibit expression of the human gene without inhibition of expression of the homologous mouse gene.

MATERIALS AND METHODS

Oligonucleotide Synthesis. The ASOs were phosphorothioate oligonucleotides that were synthesized via phosphoramidite chemistry by sulfurization with tetraethylthiuram disulfide in acetonitrile (Vu & Hirschbein, 1991). The syntheses were performed for us by Dr. Kim de Riel, Temple University School of Medicine, Philadelphia, PA.

Sequencing of Mouse cDNA. A mouse clone (M314) containing a cDNA for proα1(I) chains was kindly provided by Dr. Mon-Li Chu of this department. The cDNA clone of 1.2 kb in a commercial plasmid (pBluescript II, SK+; Stratagene) was sequenced with the dideoxynucleotide method using the T3 and T7 promoter priming sites in the vector and four oligonucleotides whose sequence was based on the sequence of the human cDNA for proα1(I) chains. The four oligonucleotide primers were 5′-AGGTGATGTTCTGGGAG-3′ (Ac5; antisense in exon 51), 5′-AGCCAGCAGATCGAGAA-3′ (Ac6; sense in exon 49), 5′-TCACGGTCACGAACCACAT-3′ (Ac1; antisense in exon 49), and 5′-CTGGCAACCTCAAGAAG-3′ (Ac16; sense in exon 52). The new sequences obtained were deposited in GenBank under the accession number U03419.

Cell Cultures. Most of the experiments were carried out in stably transfected NIH 3T3 cells expressing an internally deleted version of the human COL1A1 gene (Olsen et al., 1991). The transfected NIH 3T3 cells were grown in DMEM containing 10% fetal calf serum and 400 μg/mL G-418 sulfate (Geneticin; GIBCO BRL; potency about 200 μg/mL). Several parallel experiments were carried out with embryonic fibroblasts from transgenic mice expressing the same internally deleted version of the human COL1A1 gene (Khillan et al., 1991). The cells were plated in 24-well plates (Falcon) at about 3×10^4 cells/well so as to obtain subconfluent cultures at the end of the experiment. From 20 to 24 h after plating, the cells were washed two times with prewarmed DMEM. Then 0.3 mL of DMEM containing 10 μg/mL lipofectin (GIBCO BRL) and 200 nM ASO were added to each well. The cells were incubated for 4 h, and then about 0.7 mL of DMEM containing 14% fetal calf serum previously heat-inactivated (56 °C for 1 h) and 400 μg/mL G-418 sulfate were added. After addition of the fetal calf serum, the cells were incubated at 37 °C for 16 h unless otherwise indicated. Fibroblasts from transgenic mice (Khillan et al., 1991) were used under the same conditions except that G-418 sulfate was not employed.

Protein Analysis. At the end of incubation with the ASOs, 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 X100, 10 mM EDTA, 0.5 unit/mL aprotinin (Sigma), and 3% β-mercaptoethanol in phosphate-buffered saline adjusted to pH 7.4. After 5 min of incubation at room temperature, the cell lysate was harvested and vigorously mixed and one-fourth volume of electrophoresis loading buffer was added (50% glycerol, 1% SDS, and 0.012% bromphenol blue in 0.6 M Tris-HCl buffer, pH 6.8). The lysate was then heated for 5 min at 94 °C, and 10 μL of the sample was separated by electrophoresis in SDS on a 7% polyacrylamide gel. Proteins were electrophoretically transferred to nitrocellulose filters (Schleicher and Schuell) and reacted with a rabbit polyconal antibody against a synthetic peptide with the same sequence as the last 21 amino acids of the proα1(I) chain of type I procollagen. It recognized both the human and mouse COOH-terminal propeptide of the proα1(I) chain (Olsen et al., 1991). The antibody was kindly provided by Dr. Larry Fisher, National Institutes of Health, Bethesda, MD. The proα1(I) chains were detected by reaction with a goat antirabbit antibody coupled to ^{125}I (Dupont-NEN) and subsequent autoradiography. The relative amounts of protein from the endogenous and exogenous COL1A1 genes were then assayed by using a laser densitometer (LKB, Ultroscan XL). A standard with mixtures of the two mRNAs indicated that the assay was linear with ratios of the human mini-proα1(I) chains relative to endogenous proα1(I) chains ranging from 0.5 to 2.0 (Sokolov et al., 1993).

Calculation of Secondary Structure of mRNA. The secondary structure of the RNA from the human minigene was calculated with two programs. The first calculation was with the FoldRNA program and the Squiggles graphic display provided by the Genetics Computer Group, Inc. (University Research Park, Madison, WI). The program made it possible to find the lowest-free energy secondary structure (Zuker & Stiegler, 1981). The second calculation was with the later program by Zuker (1989) that incorporates the following features: (a) a realization that non-base-paired nucleotides contribute sequence-dependent interactions that stabilize secondary structure, (b) a computer algorithm that allows incorporation of non-base-paired interactions in the prediction of secondary structure, and (c) information from several RNA secondary structures determined by phylogeny (Jaeger et al., 1989). To employ the procedure of Zuker (1989), the following was undertaken. The total sequence of 1870 nt was divided into three overlapping parts of 800 nt each. The results identified five segments of double-stranded structure, five regions containing one or more hairpin structures and one region in which two possible structures were equally probable. The overall structure appeared to consist of four major branches. Chimeric fragments that contained sequences from two or more branches were then formed. Folding of 16 such chimeric fragments repeatedly confirmed the presence of four major branches with the stable structures seen in the folding of the initial three overlapping fragments of 800 nt each. In addition, the folding of fragments that did not contain sequences for the most stable structures identified further regions likely to assume a double-stranded structure. Essentially the same results were obtained with the two computer programs (Zuker & Stiegler, 1981; Zuker, 1989).

RESULTS

Selection of Target Sites. To select target sites for ASOs, a comparison was made of the base sequences of the human minigene for proα1(I) chains and the mouse gene for proα1-(I) chains. The sequences of the human minigene were obtained from published data (D'Alessio et al., 1988; Westerhausen et al., 1991). The sequences for the corresponding 5′-half of the mouse gene were obtained from GenBank (Accession No. X54876; MUSCOL1A11; M. J. Breindl, 1990; S. I. Lorenzen, R. A. Rippe, A. Haller, D. A. Brenner, and M. Breindl, 1991). Because the 3′-half of the structure of the mouse COL1A1 gene was not available, a cDNA was prepared and sequences were defined for 1200 bp that included the last

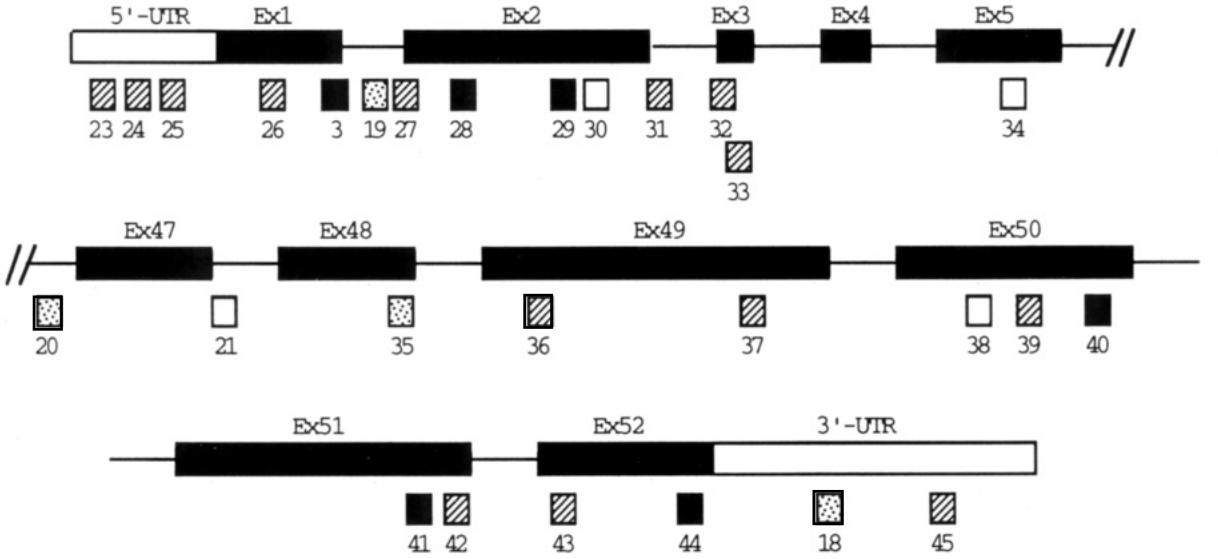

FIGURE 1: Target sites in the human minigene for proα1(I) chains. Symbols: shaded box, highly effective ASOs as defined in Table 1; striped box, moderately effective ASOs as defined by the data in Table 1; dotted box, ASOs that inhibited both human and mouse proα1(I) chains; open box, ineffective ASOs as defined by the data in Table 1.

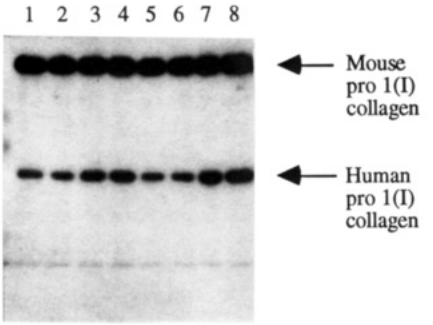

FIGURE 2: Western blot analysis of the levels of shortened proα1(I) chains and mouse full-length proα1(I) chains in transfected NIH 3T3 cells. Cells were incubated with ASOs as described in the text. Lanes 1 and 2: cells treated with AS3. Lanes 3 and 4: cells treated with a missense oligonucleotide (see Table 1). Lanes 5 and 6: cells treated with AS28. Lanes 7 and 8: control cells.

five exons (GenBank Accession No. U03419). To select target sequences for ASOs, the primary criterion was the presence of at least two base differences within a 20-nt span between the human and mouse genes. There were over 41 potential target sites when the sequences of 11 exons from the human minigene were compared to the corresponding sequences from mouse. ASOs were prepared to 28 of these sites. Most of the ASOs were directed to target sites in coding sequences, but three were to the 5′-nontranslated region, two to intron sequences, five to intron–exon boundaries, and two to the 3′-nontranslated region (Figure 1).

Tests of Effectiveness of the ASOs. Previous experiments (Colige et al., 1993) demonstrated that the most effective inhibition in the test system used here was obtained with ASOs in a concentration of about 0.2 μM in the presence of 10 μg/mL lipofectin and with an incubation time of 20 h. There was no specific inhibition with up to 25 μM of ASO when lipofectin was omitted. Also, there was a reduction in cell proliferation when concentrations of 0.4 μM ASO and 10 μg/mL lipofectin were employed. Similar results were obtained in preliminary experiments. Therefore, the concentrations of 0.2 μM oligonucleotide and 10 μg/mL lipofectin were used here. As indicated in Figures 1–3 and Table 1, the ASOs varied widely in their effectiveness as specific inhibitors of expression of the human minigene as measured by the intracellular levels of proα1(I) chains of human and mouse

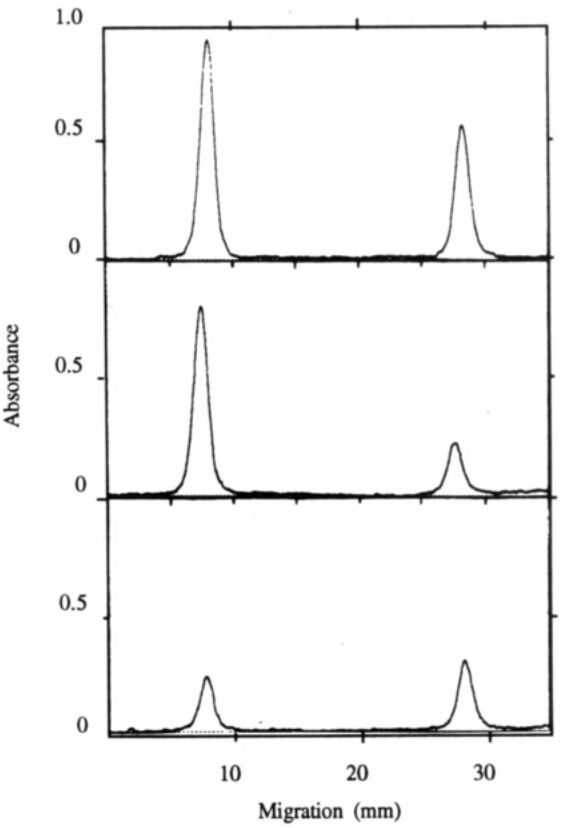

FIGURE 3: Densiometric tracings of Western blots. Top panel: results from cells incubated with missense oligonucleotide. Middle panel: cells incubated with AS28 that specifically inhibited expression of the human minigene for proα1(I) chains. Bottom panel: cells incubated with AS35 that inhibited expression of both genes.

type I procollagen. Moreover, there was considerable variation in the values obtained with the same ASO. The variability was not reduced by careful control of the density of plating of the cells or by passage number of cells prepared from frozen stocks (not shown). At least six of the ASOs, however, specifically decreased the level of proα1(I) chains synthesized from the minigene without any appreciable decrease in the level of proα1(I) chains from the mouse endogenous gene. These six ASOs consistently gave values that ranged between 37% and 67% of control (Table 1). The next 14 ASOs gave

Table 1: Target Sites and Inhibitory Activity of ASOs

ASO no.	code	location	target sequence	ratio of human/mouse proα1(I) chains (% of control)[b]
			A. Effective ASOs	
1	AS41	Ex51	A T C C G C G C C G A G G G C A A C A C * * A * A * G * * * A * * * * * * *	37 ± 4 (12)
2	AS28	Ex2	G T A C C A T G A C C G A G A C G T G T * G T * * C C A * T G * T * * G A C * *	50 ± 18 (36)
3	AS44	Ex52	G C T T C G A C G T T G G C C C T G T C * A C * A * * * A * * * * * * * * * *	63 ± 9 (12)
			B. Moderately Effective ASOs	
7	AS24	5′UTR	C G C G G A G T G T G A G G C C A C * * * * * * * * * * [ ] * * * * * * *	71 ± 21 (16)
8	AS45	3′UTR	T A T C T T T G A C C A A C C G A A C A * * C * A A A * * * * * * T * * * * G	75 ± 11 (12)
9	AS42	Ex51	A C A G C G T C A C T G T C G A T G G C * * * * * A G G G T * * * G * * C * * *	79 ± 10 (12)
10	AS43	Ex52	G C C T G G G G C A A G A C A G T G A T T A * T * * * * * * * * * * * C * * C	79 ± 12 (12)
11	AS23	5′UTR	C T C C T C G G G G T C G G A G C A * * * * * * * * * * A O * * * * * * *	80 ± 18 (16)
12	AS33	Ex3	A C C G A C C A A G A A A C C A C C G G C C * * A A * * T C * * * * G A T G T A * * A	80 ± 20 (28)
13	AS27	In1–Ex2	g c c t g t c c c a g T C C C A C C A A T c a t * t g t t * * * * * * * * T G A * G *	81 ± 25 (16)
			C. Noneffective or Nonspecific ASOs	
21	AS30	Ex2	C C C C G G C G C C G A A G T C C C C T * * * A A * C * * C * * A G A * G G	99 ± 20 (24)
22	AS19	In1	g c a a g g a t a c t c t a t a t c g c g c t t * c t a c a g * * * * * * g g t a t t g	102 ± 10 (16)[c]
23	AS38	Ex50	T C T G C A A C A T G G A G A C T G G T G A * * * * * * * * * * * * * * A * * A C	103 ± 15 (12)
24	AS18	3′UTR	G A A C C C C C T C A A A A G C C A A A A A A G * * T T T * * C * * * * * * * * A * * * * T *	105 ± 7 (16)[c]
25	AS34	Ex5	C G G A C C C C C C G G A C C C C C C T * * T * * T * * T * * T * * * * * T	106 ± 15 (20)
26	AS20	In46	t g a a c t g c c t t t t t a t c t g t c c c c t g a a c t g c * * * * c * * * * * t * *	108 ± 14 (16)[c]
27	AS21	Ex47–In47	C G A g t a a g t c a t g c c t t c t c t * * G * * * * * * t g c a t * * * * c a *	110 ± 15 (16)
28	AS35	Ex48	C T G G T G A T G C T G G T C C T G T T * * * * * * * * C A G C * * C * * * * C *	112 ± 20 (12)[c]

[a] Exon sequences in capitals and intron sequences in lower case. Human sequence is in top line, and mouse sequence is in second line. Symbols: *, identical base; O, single base deletion; [ ], deletion of two or more bases. [b] Mean ± standard deviation. Cells were incubated with the ASOs for a total of 20 h. In each experiment, duplicate samples of cells were treated with a missense oligonucleotide (Colige et al., 1993) and an ASO. The missense oligonucleotide had a scrambled sequence of ASO3, i.e., ATC-CTGCTTCGTTCTGGCTC. The value for the ratio of human mini-proα1(I) chains to mouse proα1(I) chains was calculated for each sample. The value for each test sample was divided by each of two controls to obtain two values of percent control for each sample. Number in parentheses is total number of values used to calculate the mean and the standard deviation. [c] ASOs that decreased the levels of both human and mouse proα1(I) chains relative to fibronectin and, therefore, did not specifically inhibit expression of one proα1(I) chain relative to the other (see lower panel in Figure 3).

Table 2: Specificity of Inhibition by ASOs and Differences in Affinities to Human and Mouse Target Sequences

ASO no.	ASO code	ratio of human proα1(I)/ fibronectin (% of control)[a]	ratio of human/ mouse proα1(I) (% of control)	ΔΔG[c] (kcal/mol)
		A. Effective ASOs		
1	AS41	38	37	−27.0
2	AS28	38	50	−22.3
3	AS44	38	62	−25.3
4	AS29	63	69	−29.1
5	AS3	75	56	−14.8
6	AS40	42	66	−22.7
		B. Moderately Effective ASOs		
7	AS24	57	63	−7.0
9	AS42	73	78	−26.0
10	AS43	73	79	−12.2
11	AS23	89	70	−11.8
12	AS33	88	85	−34.1
13	AS27	77	73	−10.4
14	AS32	88	80	−23.0
15	AS26	87	66	−11.4
16	AS39	76	83	−19.4
17	AS31	92	88	−32.2
18	AS37	80	92	−30.5
19	AS36	96	91	−19.8
20	AS25	110	91	−22.2
		C. Noneffective or Nonspecific ASOs		
21	AS30	155	99	−33.9
22	AS19	86[b]	109[b]	
23	AS38	116	104	−12.0
24	AS18	83[b]	106[b]	−23.1
25	AS34	125	110	−22.8
26	AS20	82[b]	105[b]	
27	AS21	113	116	
28	AS35	59[b]	138[b]	−23.9

[a] Values are means of duplicate samples from one or two experiments. [b] ASOs that decreased the levels of both human and mouse proα1(I) chains relative to fibronectin and, therefore, did not specifically inhibit expression of one proα1(I) chain relative to the other (see lower panel in Figure 3). [c] ΔΔG values calculated as ΔG for ASO-human sequence double-strand − ΔG for ASO-mouse sequence double-strand (see text).

lesser degrees of inhibition, and occasionally no inhibition was observed. Four of the ASOs decreased the intracellular levels of both the proα1(I) chains from the human minigene and the proα1(I) chains from the endogenous mouse gene to about the same extent (lower panel in Figure 3). Therefore, these ASOs (AS18, AS19, AS20, and AS35) were regarded as nonspecific in their effects (Table 1). Four additional ASOs produced no effect on either the human or mouse proα1(I) chains.

The specificity of the ASOs was further tested by assaying their effects on the intracellular levels of the proα1(I) chains relative to the intracellular levels of fibronectin. The same ASOs that produced specific inhibition of the minigene based on the ratio of the two proα1(I) chains (Table 1) were also specific as assayed by the ratio of human proα1(I) chains to fibronectin (Table 2).

Effects of Combining Two ASOs. In further experiments, attempts were made to determine whether combining two ASOs produced greater inhibition than administration of one alone. As indicated in Table 3, no consistent additive effective was observed by combined additions of AS3 and AS28. In other experiments, the effectiveness of two additions of the same ASO were tested. No consistent effect was observed (Table 3). In still other experiments, the effectiveness of the ASOs in fibroblasts from transgenic mice expressing the same minigene was examined. The results were essentially the same as with the stably transfected NIH 3T3 cells.

Table 3: Factors Affecting Inhibitory Activity of Antisense Oligonucleotides

cell type	ASO (nM) AS3	ASO (nM) AS28	time of incubation (h)	ratio of human/ mouse proα1(I) chains[a] (% control)
NIH 3T3	200		20	66
	150	50	20	63
	100	100	20	67
	50	150	20	55
	200	200	20	62
		200	20	65
	200		20	63
	200		30	100
	400[b]		30	53
		200	20	56
		200	30	72
		400[b]	30	49
mouse fibroblasts	50		20	62
	100		20	51
	200		20	62
		50	20	85
		100	20	78
		200	20	60

[a] Values are means of duplicates. [b] 200 nM added at zero time and 200 nM additional at 10 h.

Table 4: Correlation between Structure of Target Sequence and Inhibitory Activity of ASOs

target sequence	no. of ASOs	nonspecific inhibition[a]	specific inhibition[b] none	specific inhibition[b] intermediate	specific inhibition[b] effective
double-stranded mRNA	19	1	1	12	5
single-stranded mRNA	6	1	2	2	1
introns	4[c]	2	1	1	0

[a] Approximately equal decreases in the level of human and mouse proα1(I) chains. [b] Specific inhibition of the ASOs was defined as indicated in Table 1 and text. [c] Number includes AS21 and AS31 that were targeted to intron–exon boundaries but more than half to the intron sequences (see Figure 1 and Table 1).

Correlation between Binding Affinities and the Effectiveness of the ASOs. To examine whether the effectiveness of the ASOs were related to the affinity of the ASOs for their target sites, values were calculated for the free energy of binding to the target sequences in the mouse proα1(I) RNA versus the target sequences in the human proα1(I) RNA. To carry out the calculation, the sequence of the ASO was first linked to five unspecified bases and then to the target human sequence so that a double-stranded stem–loop structure was formed. The ΔG for the double-stranded hairpin structure was then estimated with the FoldRNA program and then refined with the MFold program (Zuker & Stiegler, 1981). A similar calculation was made with the same ASO and the corresponding mouse sequence. The two values were then subtracted to give the differences in free energies of binding, i.e., ΔΔG = ΔG for ASO-human sequence double-strand − ΔG for ASO-mouse sequence double-strand. There was no apparent correlation between ΔΔG values and the values for specific inhibition by the ASOs (Table 2). For example, the mean ΔΔG value for the six most effective ASOs was −23.5 kcal/mol ± 4.99 SD. The mean ΔΔG value for the five ASOs without any specific inhibition was −23.1 kcal/mol ± 7.76 SD.

Correlation with Secondary Structure of the mRNA. To examine whether the effectiveness of ASOs was related to secondary structure, the secondary structure of the mRNA from the human minigene was calculated by using the FoldRNA program and refined with the mFold program (Zuker, 1989). As indicated in Figure 4, the mRNA had a

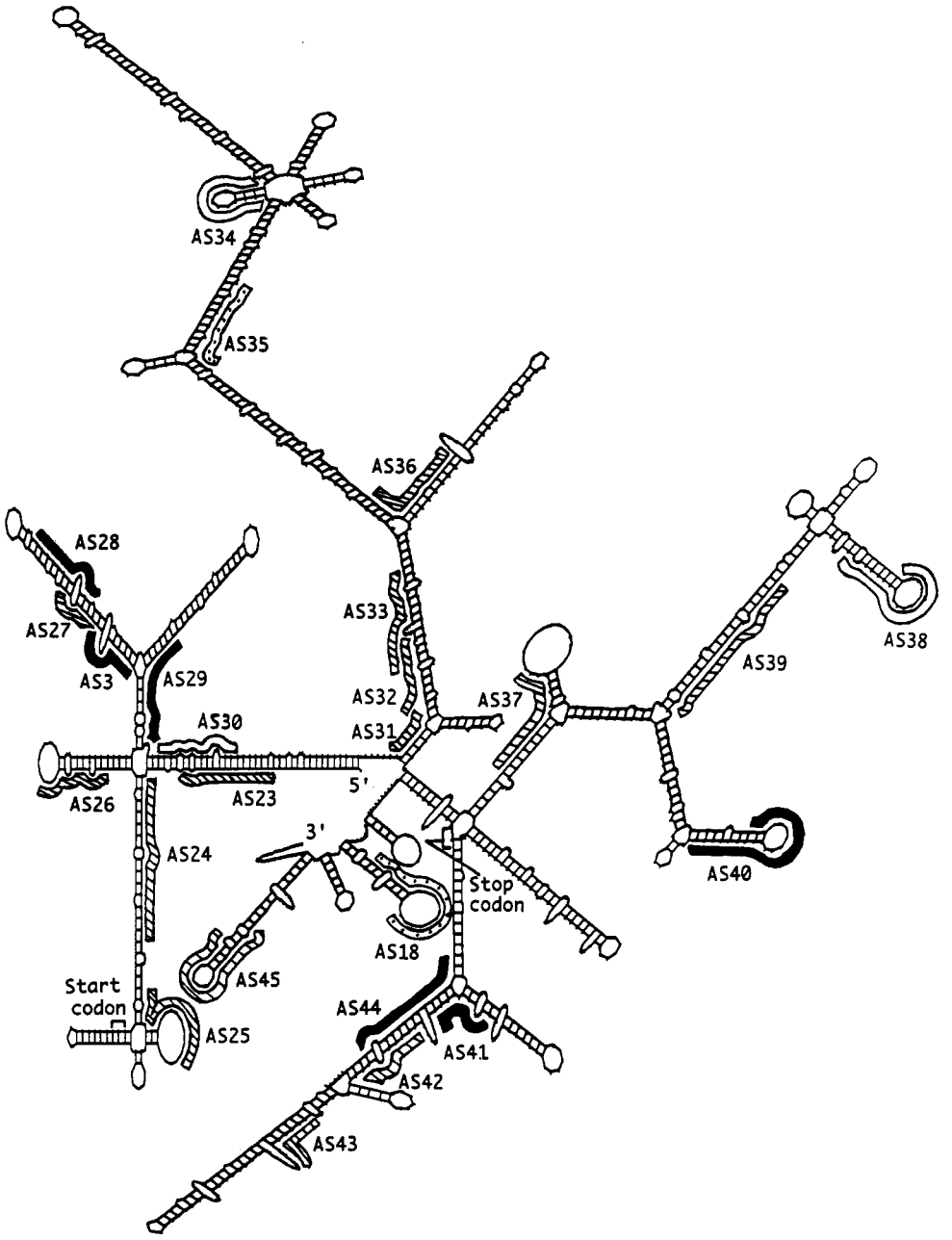

FIGURE 4: Calculated secondary structure of mRNA encoded by the human minigene for proα1(I) chains. Symbols: shaded box, highly effective ASOs as defined by the data in Table 1; striped box, moderately effective ASOs as defined by the data in Table 1; dotted box, ASOs that inhibited both human and mouse proα1(I) chains; open box, ineffective ASOs.

complex secondary structure. As indicated in Figure 4 and Table 4, five of the six most effective ASOs were directed toward target sites in sequences predicted to be double-stranded. Conversely, only two of 19 targeted to double-stranded structures were without a specific effect. In contrast, three of six targeted to sequences predicted to be single-stranded had no specific effect.

DISCUSSION

The test system of transfected fibroblasts employed here provided a simple but highly specific assay for inhibition of a target gene by ASOs. The human proα1(I) minigene had a sequence that was highly homologous to the mouse endogenous proα1(I) gene, and specific effects on expression of either gene were readily assayed by the intracellular levels of protein synthesized from each gene in the same sample. ASOs that decreased the level of proα1(I) chains from the minigene without decreasing the level of proα1(I) chains from the endogenous gene obviously had highly specific effects (Colige et al., 1993). Previous observations demonstrated that the values for the ratios of the two proα1(I) chains directly reflected the intracellular levels of the two mRNAs (Colige et al., 1993). The specificity of the assay was further established here by demonstrating that ASOs that decreased the ratio of the human proα1(I) chains relative to the mouse proα1(I) chains also decreased the ratio of the human proα1-(I) chains relative to fibronectin, another matrix protein synthesized by the same cells. As expected, a few of the ASOs produced nonspecific inhibition of both proα1(I) genes. At least six, however, were highly specific.

Although ASOs have now been tested in a variety of systems, previous observations did not provide effective rules for selecting nucleotide sequences in RNA transcripts as targets for ASOs. In most previous reports (Erickson & Izant, 1992;

Wickstrom, 1992; Chiang et al., 1991; Sankar et al., 1989; Ricker & Kaji, 1992; Stull et al., 1992; Lima et al., 1992; Thierry et al., 1993), five or fewer target sites were compared within specific genes or mRNAs. These limited comparisons suggested three general sites as being the most effective: (a) sequences around initiation codons for translation, (b) intron–exon boundaries, and (c) single-stranded loops in hairpin structures. The data here in which 28 different target sites were tested are inconsistent with all three of these suggestions. Specifically, (a) none of the three ASOs targeted to the 5′-nontranslated region were among the most effective (Table 1), (b) only one of five ASOs targeted to intron–exon boundaries ranked among the most effective, and (c) only one of six ASOs targeted to single-stranded sequences was among the most effective and three of the six did not demonstrate any specific inhibition (Table 4). In contrast, five of 19 targeted to double-stranded structures were among the most effective, and 12 additional ASOs from the 19 showed an intermediate level of effectiveness (Table 4). There was no apparent correlation with the calculated affinities of the ASOs to the human and mouse target sequences (Table 2). Instead, the most consistent pattern was that ASOs targeted to double-stranded structures were generally more effective (Figure 4 and Table 4). The effectiveness of ASOs targeted to double-stranded mRNA structures is consistent with the unexpected observations with sense oligonucleotides. In our previous work (Colige et al., 1993), we observed that a sense oligonucleotide reduced the ratio of human to mouse proα1(I) chains to 74% of the control value. More recently, Thierry et al. (1993) found one sense oligonucleotide was partially effective in inhibiting expression, *mdr1*. Inhibition by such sense oligonucleotides might well be explained by their targeting the complementary strand in a double helix. At the same time, it was apparent that the preferential targeting of double-stranded structures by ASOs is counterintuitive.

One of the further observations here was that combined addition of two effective ASOs did not produce an additive inhibition of expression (Table 3). Also, a second addition of the same ASO at a later time did not produce greater addition. Both of the observations may be explained by saturation of the cellular mechanisms for uptake of ASOs. The failure to reduce levels of expression below 50–80% of control levels may reflect an intrinsic limit to the effectiveness of the ASOs or some incidental feature of the experiment conditions such as nonspecific toxicity from the liopfectin carrier.

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