Hybridization Arrest of Globin Synthesis in Rabbit Reticulocyte Lysates and Cells by Oligodeoxyribonucleoside Methylphosphonates†

Kathleen R. Blake, Akira Murakami, Sharon A. Spitz, Scott A. Glave, M. Parameswara Reddy,‡ Paul O. P. Ts'o, and Paul S. Miller*

Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland 21205

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ABSTRACT: Oligodeoxyribonucleoside methylphosphonates which are complementary to the 5′ end, the initiation codon regions, or the coding regions of rabbit globin mRNA were synthesized. These oligomers were shown to interact with their complementary mRNA binding sites by their ability to serve as primers for reverse transcriptase. In several cases, the priming efficiency of the oligomers was enhanced when the oligomer was preannealed with the mRNA. This behavior correlates with the predicted secondary structure of the mRNA and suggests that some oligomer binding sites occur in hydrogen-bonded stem regions of the mRNA. Methylphosphonate oligomers inhibit translation of globin mRNA in reticulocyte lysates. Inhibition is due to the interaction of the oligomers with mRNA. The extent of inhibition is affected by the sequence and chain length of the oligomer, the location of the oligomer binding site on the mRNA, and the secondary structure of the binding site. Oligomers which bind to the 5′ end and initiation codon regions of β-globin mRNA inhibit both α- and β-globin synthesis whereas oligomers which bind to the coding region of α-globin mRNA or the coding region of β-globin mRNA inhibit translation of their target mRNA in a specific manner. Oligodeoxyribonucleoside methylphosphonates inhibit globin synthesis in rabbit reticulocytes. The effects of various oligomers on cellular globin synthesis are similar to those in the lysate system and suggest that the conformation of globin mRNA is the same in both systems during translation.

Oligodeoxyribonucleoside methylphosphonates are a novel type of nonionic nucleic acid analogue which is resistant to hydrolysis by nucleases and is able to penetrate the membranes of living cells (Miller et al., 1981). These properties and their ability to form stable hydrogen-bonded complexes with complementary polynucleotides (Miller et al., 1979) enable these analogues to inhibit the function of cellular nucleic acids. We have previously shown that methylphosphonate oligomers which are complementary to the anticodon loops of transfer RNA inhibit in vitro aminoacylation of tRNA and protein synthesis in bacterial and mammalian cell-free systems (Miller et al., 1981). An oligomer complementary to the Shine/Dalgarno sequence of *Escherichia coli* 16S rRNA specifically inhibits bacterial but not mammalian protein synthesis both in a cell-free translating system and in *E. coli* (Jayaraman et al., 1981). In both examples, the oligomers produce an overall reduction in protein synthesis as a result of inhibiting either aminoacylation of tRNA or binding of ribosomes to bacterial mRNA.

Protein synthesis can be selectively inhibited during translation by forming hydrogen-bonded complexes between complementary nucleic acids and mRNA. Such hybridization arrest of translation is observed with DNA which is complementary to mRNA (Paterson et al., 1977) and with sequence-specific oligodeoxyribonucleotides (Stephenson & Zamecnik, 1978). In the preceding paper, we described the effects of sequence-specific oligodeoxyribonucleotides on translation of rabbit globin mRNA in rabbit reticulocyte lysates and wheat germ extracts (Blake et al., 1985). In this report, we describe the effects of oligodeoxyribonucleoside methylphosphonates on translation of rabbit globin mRNA. We show that sequence-specific methylphosphonate oligomers 4–12 nucleoside units in length inhibit globin synthesis at the level of translation in rabbit reticulocyte lysates and in rabbit reticulocytes.

Experimental Procedures

Rabbit globin mRNA, rabbit reticulocyte lysate, T_4 polynucleotide kinase, and avian myeloblastosis virus reverse transcriptase were purchased from Bethesda Research Laboratories, Inc. ^{35}S-labeled methionine (1000 Ci/mmol), [α-^{32}P]TTP (800 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from New England Nuclear, Inc., or Amersham, Inc. The syntheses and characterization of the oligodeoxyribonucleoside methylphosphonates were carried out by methods previously described (Miller et al., 1983; Murakami et al., 1985).

Characterization of Oligodeoxyribonucleoside Methylphosphonate: Globin mRNA Interactions. Oligodeoxyribonucleoside methylphosphonates were used as primers for reverse transcriptase. The reactions contained 0.05 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl)[1] (pH 8.3), 0.05 M potassium chloride, 0.005 M magnesium chloride, 0.01 M DTT, 50 μM each of the four deoxyribonucleoside triphosphates, 10–15 μCi of thymidine [α-^{32}P]triphosphate, 0.2 μg of rabbit globin mRNA, 40 μM oligodeoxyribonucleoside methylphosphonate, and 50 units of reverse transcriptase in a total volume of 10 μL. The reactions were

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‡ Present address: Beckman Instrument Co., Palo Alto, CA 94304.

[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; EDTA, ethylenediaminetetraacetic acid; Cl_3CCOOH, trichloroacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; VSV, vesicular stomatitis virus; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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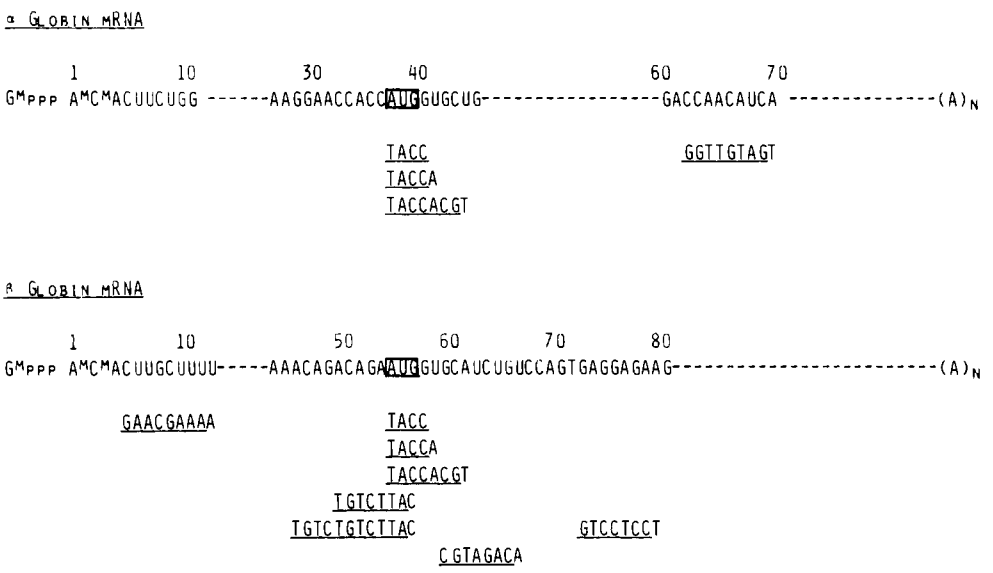

FIGURE 1: Partial nucleotide sequence of rabbit α-globin mRNA (Heindell et al., 1978) and rabbit β-globin mRNA (Efstratiadis et al., 1977). Below each mRNA are shown the sequences of the complementary oligodeoxyribonucleoside methylphosphonates. The underlined portion of each oligomer shows the position of the methylphosphonate internucleotide linkages.

incubated at 37 °C for 60 min and then stopped by addition of 0.5 μL of 0.5 M EDTA. The reaction mixture was then subjected to electrophoresis on a 10% polyacrylamide gel containing 7 M urea (Maniatis et al., 1982). The chain length of the transcript was determined as described by Murakami et al. (1985). In this procedure, the transcript was extracted from the gel (Lo et al., 1984) and was then treated with 25 μL of 1 M aqueous piperidine at 60 °C overnight. After lyophilization, the hydrolyzed transcript was subjected to polyacrylamide gel electrophoresis under denaturing conditions. Oligomers of known chain length were run as standards. The chain length was determined from plots of the log of the chain length of the standards vs. their mobility on the gel (Rickwood & Hames, 1982).

Translation of Globin mRNA in the Presence of Oligodeoxyribonucleoside Methylphosphonates. The translation reactions were carried out and assayed as previously described (Blake et al., 1985). The commercial rabbit reticulocyte lysate, which had been pretreated with micrococcal nuclease, contained 3.5 mM magnesium chloride, 50 μM EDTA, 25 mM potassium chloride, 5 mM DTT, 25 μM hemin, 50 μg/mL creatine kinase, 1 mM calcium chloride, 2 mM EGTA, and 70 mM sodium chloride. The reactions were run in sterile silanized glass test tubes and contained 25 mM HEPES (pH 7.2), 40 mM potassium chloride, 10 mM creatine phosphate, a 50 μM sample of each amino acid except methionine, 88.4 mM potassium acetate (pH 7.2), 4.2 μCi of [^{35}S]methionine, 0–300 μM oligodeoxyribonucleoside methylphosphonate, and 8.3 μL of reticulocyte lysate in a final volume of 25 μL. Reactions were initiated by addition of the reticulocyte lysate. Aliquots of the reaction mixture were removed at 30 and 60 min, and the reaction products were analyzed by polyacrylamide gel electrophoresis exactly as described in the preceding paper (Blake et al., 1985). Under these conditions, 0.4 μCi (400 fmol) of methionine was incorporated in a linear manner after 60 min of incubation in the absence of the methylphosphonate oligomer as determined by Cl_3CCOOH precipitation. After gel electrophoresis, between 2000 and 10 000 cpm were observed in each protein band.

Effects of Oligodeoxyribonucleoside Methylphosphonates on Globin Synthesis in Rabbit Reticulocytes. Reticulocytes were obtained on day 7 from an adult New Zealand white rabbit which had been injected subcutaneously for 5 consecutive days with 1.0–2.0 mL of a 1.2% 1-acetyl-2-phenylhydrazine solution neutralized in 1 M HEPES (pH 7.5). The cells were centrifuged at 1000 rpm for 5 min, washed 3 times with 5 volumes of Hank's buffered salt solution, and resuspended in an equal volume of Eagle's minimum essential medium lacking methionine. Fifty-microliter aliquots of the cell suspension were pipetted into sterile Eppendorf tubes. An aliquot of 1 mM oligonucleoside methylphosphonate was added to give a final concentration of 100 or 200 μM. Control reactions received an equivalent volume of sterile water. After the cells were preincubated with the oligomer for 0 or 1 h at 25 °C, [^{35}S]methionine was added to a final concentration of 40 μCi/mL. The cells were kept in suspension by continuous rotation. Aliquots were withdrawn at 0, 10, 20, 40, and 60 min, and after lysis in 0.1% SDS, the protein was precipitated in hot 20% Cl_3CCOOH. The precipitates were filtered on glass fibers, washed with 4 mL of 2 N hydrochloric acid and 4 mL of 95% ethanol, and counted in Betafluor. Incorporation of methionine was linear over the first 40 min. The translation products obtained at 60 min were analyzed by polyacrylamide gel electrophoresis as previously described for translation reactions in rabbit reticulocyte lysates (Blake et al., 1985) except 5-μL aliquots were diluted 1:10 in the gel loading buffer and the equivalent of 1 μL of original reaction mixture was loaded onto the gel. Approximately 0.3 μCi (300 fmol) of methionine was incorporated into Cl_3CCOOH-precipitable material after 60-min incubation in the absence of the oligomer.

RESULTS

Oligodeoxyribonucleoside Methylphosphonates Complementary to Rabbit Globin mRNA. The oligodeoxyribonucleoside methylphosphonates used in this study were synthesized by a polymer support procedure (Miller et al., 1983). Most of these oligomers terminate at the 5′-position with a 3′–5′-linked nucleoside phosphodiester unit rather than a 3′–5′-linked nucleoside methylphosphonate unit. They are symbolized as d-Np*NNNN*, the italicized letters indicating the position of the methylphosphonate linkages. As shown in Figure 1, the oligomers are complementary to either the 5′ end, the initiation codon region, or the coding region near the initiation codon of rabbit globin mRNA. Two additional oligomers, d-*ACAGACAT* and d-*TTTTTTT*, were also prepared. The former is not specifically complementary to either

Table I: Specificity of Duplex Formation between Oligodeoxyribonucleoside Methylphosphonates and Rabbit Globin mRNA

oligonucleoside methylphosphonates	binding site	duplex formation with globin mRNA having five or more contiguous base pairs[a]		size of DNA transcripts[b]	
		α-globin	β-globin	expected	observed
d-Ap*AAAGCAAG*	β 4–12	0	1 (9)	12	nd
			1 (5)		
d-Tp*GCACCAT*	α 37–44	1 (7)	1 (8)	44 α	63
	β 54–61	1 (6)	1 (6)	61 β	
		1 (5)	1 (5)		
d-Cp*ATTCTGT*	β 49–56	0	1 (8)	58	56
			4 (5)		
d-Cp*ATTCTGTCTGT*	β 45–56	0	1 (12)	56	55
			3 (5)		28
					17
d-Ap*CAGATGC*	β 59–66	0	1 (8)	66	64
			1 (7)		
d-Tp*GATGTTGG*	α 62–70	1 (9)	1 (6)	70	69
		2 (5)			57
d-Tp*CCTCCTG*	β 72–79	1 (5)	1 (5)	0	0
d-*ACAGACAT*	none	3 (5)	3 (5)	0	nd

[a] Number of duplexes having five or more contiguous base pairs found by a computer search which compares the sequence of the oligonucleotide methylphosphonate with the mRNA sequences. The numbers in parentheses show the number of contiguous base pairs in the duplex. [b] Size in nucleotide units of transcript obtained when the oligomer is used as a primer for reverse transcriptase catalyzed copying of globin mRNA. nd = no data.

α- or β-globin mRNA while the latter is complementary to the poly(A) tail of mRNA.

The complementarity between the phosphonate oligomers and the globin mRNA was examined by computer. In this search, the possibility of forming duplexes with five or more contiguous base pairs was determined. The results are shown in Table I. For example, d-Ap*AAAGCAAG* can form a full-length duplex (nine base pairs) with its expected β-mRNA binding site and a shorter duplex having five contiguous base pairs with a sequence in the coding region of β-globin mRNA. However, this oligomer cannot form any duplexes having five or more contiguous base pairs with α-globin mRNA.

The location of the mRNA binding sites of selected oligomers was characterized by using the oligomer as a primer for reverse transcriptase. The size of the resulting transcript was determined by polyacrylamide gel electrophoresis under denaturing conditions after the 5′-terminal methylphosphonate primer was removed by hydrolysis with 1 M aqueous piperidine (Murakami et al., 1985). Table I compares the expected size of the transcript with the size determined by this procedure. Each oligomer tested gave a transcript whose size was consistent with the predicted oligomer binding site on mRNA. One and two additional transcripts were observed respectively when d-Tp*GAGTTGG* and d-Cp*ATTCTGTCTGT* were used as primers.

Effects of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Translation of Globin mRNA. Rabbit globin mRNA was translated in a rabbit reticulocyte lysate at 37 °C in the presence and absence of the oligodeoxyribonucleoside methylphosphonates. The relative amounts of α- and β-globin synthesis were determined after the [^{35}S]methionine-labeled proteins were separated by polyacrylamide gel electrophoresis. The results of these experiments are shown in Figure 1 and Table II.

Oligomers which are complementary to the 5′ end, the initiation codon region, and the coding region of globin mRNA inhibit translation to various extents. As shown in Figure 2, inhibition by d-*CCAT* increases with increasing oligomer concentration and decreasing temperature. Inhibition by d-*CCAT*, d-Ap*CCAT*, and d-Tp*GCACCAT*, which share a common binding site on α- and β-globin mRNA, increases with increasing oligomer chain length and concentration.

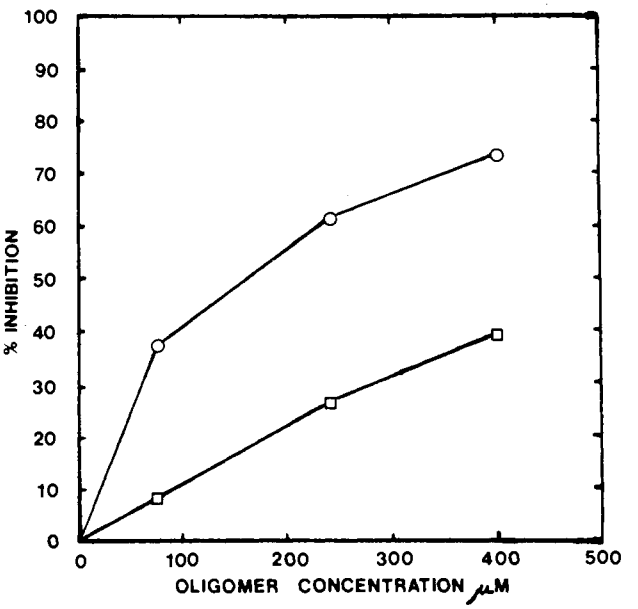

FIGURE 2: Effect of d-*CCAT* on globin protein synthesis in a rabbit reticulocyte lysate at 25 (○) and 37 °C (□). The percent inhibition was determined after precipitation of protein by Cl_3CCOOH.

Octamer d-Cp*ATTCTGT*, which is complementary to the initiation codon region of β-globin mRNA, is a very poor inhibitor even at high concentrations unless it is preannealed with the mRNA prior to the addition of the reticulocyte lysate. Although d-Ap*AAAGCAAG* is a good inhibitor of translation, its inhibitory effect is also enhanced by preannealing with mRNA before the start of translation.

Oligodeoxyribonucleoside methylphosphonates which are complementary to either the 5′ end or the initiation codon region of β-globin mRNA inhibit translation of both α- and β-globin mRNA to approximately the same extent (Table III). Octamer d-Ap*CAGATGC*, whose binding site is 3 nucleotides downstream from the initiation codon of β-globin mRNA, is a poor inhibitor at low concentrations and inhibits both α- and β-globin synthesis at 100 μM, whereas d-Tp*CCTCCTG*, which is partially complementary to the coding region of β-globin mRNA, 15 nucleotides downstream from -AUG-, inhibits β-globin synthesis in a selective manner. Selective inhibition

Table II: Inhibition of Rabbit Globin mRNA Translation by Oligodeoxyribonucleoside Methylphosphonates in a Rabbit Reticulocyte Lysate at 37 °C

oligomer	binding site	concn (μM)	% inhibition α	% inhibition β
5′ end				
d-Ap*AAAGCAAG*	β 4–12	100	56	51
		100[a]	70	70
initiation codon region				
d-*CCAT*	α 37–40	100	0	0
	β 54–57	200	28	42
d-Ap*CCAT*	α 37–41	100	23	36
	β 54–58	200	67	67
d-Tp*GCACCAT*	α 37–44	100	35	28
	β 54–61	200	73	70
d-Cp*ATTCTGT*	β 49–56	50	2	2
		100	0	7
		200	11	8
		100[a]	30	36
		200[a]	48	34
d-Cp*ATTCTGTCTGT*	β 45–56	25	17	14
		100	49	50
coding region				
d-Ap*CAGATGC*	β 59–66	25	5	−7
		50	5	4
		100	33	24
		100[a]	76	66
d-Tp*GATGTTGG*	α 62–70	25	16	8
		50	24	9
		100	38	11
d-Tp*CCTCCTG*	β 72–79	25	8	16
		100	15	36
other				
d-*ACAGACAT*	none	25	7	−14
		100	19	8
d-*TTTTTT*	poly(A) tail	300	0	0

[a] Oligomer preannealed to mRNA before translation.

Table III: Comparison of Inhibition of Globin mRNA Translation by Oligodeoxyribonucleoside Methylphosphonates and Oligodeoxyribonucleotides in a Rabbit Reticulocyte Lysate at 37 °C

oligomer sequence	binding site	concn (μM)	% inhibition: oligodeoxynucleoside methylphosphonate α	oligodeoxynucleoside methylphosphonate β	oligodeoxyribonucleotide[b] α	oligodeoxyribonucleotide[b] β
d-AAAAGCAAG	β 4–12	100	56	51	88	87
d-TGCACCAT	α 37–44	100	35	28		
	β 54–61					
d-GCACCATT	α 37–43	100			25	18
	β 53–60					
d-CATTCTGT	β 49–56	100	30[a]	36[a]	45	30
d-CATTCTGTCTGT	β 45–56	25	17	14	20	23
		100	49	50	61	29
d-ACAGATGC	β 59–66	100	33	24	8	20
d-TGATGTTGG	α 62–70	100	38	11	8	4

[a] Oligomer preannealed to mRNA before translation. [b] Blake et al. (1985).

of α-globin synthesis was also observed with d-Tp*GATGTTGG*, which is complementary to the coding region of α-globin mRNA.

Octamer d-*ACAGACAT*, which is partially complementary to the coding regions of α- and β-globin mRNA, is a weak inhibitor even at high concentration. The oligothymidine methylphosphonate d-*TTTTTT*, which is complementary to the poly(A) tail of mRNA, did not inhibit even at 300 μM.

The methylphosphonate oligomers were also tested for their ability to inhibit translation in a wheat germ extract (data not shown). Oligomers d-*CCAT*, d-Ap*CATT*, d-Tp*GCACCAT*, and d-Cp*ATTCTGTCTGT* inhibited both α- and β-globin synthesis by 7%, 45%, 45%, and 32%, respectively, at a concentration of 100 μM. The other oligomers tested, d-Cp*ATTCTGT*, d-Ap*CAGATGC*, and d-Tp*GATGTTGG*, did not inhibit even at high concentrations or after preannealing with mRNA. Inhibition by d-Cp*ATTCTGTCTGT* did not increase after preannealing with mRNA.

Table IV: Effects of Oligodeoxyribonucleoside Methylphosphonates on Globin Synthesis in Rabbit Reticulocytes at 25 °C

oligomer	concn (μM)	% inhibition α	% inhibition β
d-Tp*GCACCAT*	200	42	29
d-Cp*ATTCTGT*	200	0	0
d-Cp*ATTCTGTCTGT*	200	21	17
d-Tp*GATGTTGG*	100	59	59

Effects of Oligodeoxyribonucleoside Methylphosphonates on Globin Synthesis in Rabbit Reticulocytes. Rabbit reticulocytes were preincubated for 1 h with the oligomers and then assayed for incorporation of [^{35}S]methionine. The levels of α- and β-globin synthesis in the presence and absence of the oligomers were determined by gel electrophoresis. As shown in Table IV, globin synthesis was inhibited by each oligomer except d-Cp*ATTCTGT*. Preincubation of the cells with the oligomer appeared to increase the inhibitory effect. In the absence of preincubation, 100 μM d-Tp*GCACCAT* inhibited globin synthesis by only 17%.

Discussion

The oligodeoxyribonucleoside methylphosphonates used in this study were designed to bind to three distinct regions of globin mRNA: the 5′-terminal nucleotides near the 7-methylguanine cap structure of β-globin mRNA, the initiation

codon regions of α- and β-globin mRNA, and the coding regions of α- and β-globin mRNA near the -AUG- codon (Figure 1). The interaction of these oligomers with rabbit globin mRNA was first studied. Computer comparison of the oligomer sequences with globin mRNA showed that each oligomer should specifically form only one full-length duplex with its targeted mRNA (Table I).

In addition to fully base-paired duplexes, partial duplexes having five or more contiguous base pairs were also considered. As discussed in the previous paper (Blake et al., 1985), we felt that partial duplexes with contiguous base-paired regions would be more stable than those in which base-paired regions were separated by nonpaired base regions. In most cases, partial duplex formation can occur only within other regions of the targeted mRNA and not with the nontargeted mRNA; for example, oligomers complementary to the 5′ end or the initiation codon regions of β-globin mRNA can form partial duplexes only with β-globin mRNA. An exception is the α-mRNA-targeted nonamer d-Tp*GATGTTGG*, which could form a duplex having five contiguous base pairs with nucleotides 529–534 in the noncoding region of β-globin mRNA in addition to a full-length duplex with α-globin mRNA. A similar situation can occur with d-Tp*CCTCCTG*. This octamer can form a partial duplex with nucleotides 72–79 in the coding region of β-globin mRNA; in addition to the five contiguous base pairs which include three G-C base pairs, an A-T base pair can form between the 5′-terminal thymidine of the oligomer and adenosine 79 of β-globin mRNA (Figure 1). This octamer could also form a partial duplex with nucleotides 128–132 located within the coding region of α-globin mRNA.

The oligonucleoside methylphosphonates bind to rabbit globin mRNA as demonstrated by their ability to hybridize to globin mRNA in agarose gels (Murakami et al., 1985). Reverse transcriptase was used to confirm that the methylphosphonate oligomers interact with their complementary mRNA binding sites. Oligodeoxyribonucleoside methylphosphonates serve as primers for reverse transcriptase, and the chain length of the resulting cDNA transcript defines the binding site of the oligomer on mRNA (Murakami et al., 1985).

As shown in Table I, each of the methylphosphonate oligomers gives a unique major transcript whose chain length is consistent with the oligomer binding to its complementary site on its targeted mRNA. Both d-Cp*ATTCTGTCTGT* and d-Tp*GATGTTGG* give shorter transcripts in addition to the expected transcript. Additional oligomer binding sites on either α- or β-globin mRNA which would be consistent with the length of these extra transcripts were not found. Therefore, it is possible that the shorter transcripts arise from premature termination of transcription by the transcriptase. The nature of these transcripts is under further investigation.

Several observations suggest that mRNA secondary structure may attenuate the ability of the methylphosphonate oligomers to bind and thus affect their efficiency as primers for reverse transcriptase. Octamer d-Tp*GCACCAT*, which is complementary to both α and β mRNA, gives a major transcript 63 nucleotides in length. This chain length is consistent with priming from nucleotides 54–61 of β-globin mRNA (see Figure 1). A second shorter transcript is observed at 10-fold higher mRNA concentration. The estimated chain length of this transcript is consistent with oligomer priming from nucleotides 37–44 of α-globin mRNA. This behavior can be explained by the model for the secondary structure of the 5′ ends of rabbit globin mRNA proposed by Pavlakis et al. (1980). According to this model, the binding site for d-Tp*GCACCAT* occurs in a single-stranded loop region on β-globin mRNA and in a partially hydrogen-bonded stem region of α-globin mRNA. This stem structure may prevent d-Tp*GCACCAT* from forming a complex of sufficient stability with α-globin mRNA to allow it to serve as an efficient primer. A similar situation occurs with d-Cp*ATTCTGT* and d-Cp*ATTCTGTCTGT*. The efficiency of the priming reaction increases when the oligomers are first preannealed to the globin mRNA. According to the model of Pavlakis et al. (1980), the binding sites for these oligomers occur in a partially hydrogen-bonded stem which forms part of the initiation codon region of β-globin mRNA. In contrast to this behavior, the priming ability of d-Tp*GATGTTGG* is not affected by preannealing. In this case, the oligomer binding site is located in a large single-stranded loop in the coding region.

The effects of oligodeoxyribonucleoside methylphosphonates on translation of globin mRNA in a rabbit reticulocyte lysate were investigated (Figure 2 and Table II). These experiments show that the oligomers inhibit translation as a result of their binding to globin mRNA. The following observations in combination with the results of the reverse transcriptase experiments described above are consistent with this conclusion: inhibition increases as the concentrations and chain lengths of the oligomers increase for the series d-*CCAT*, d-Ap*CCAT*, and d-Tp*GCACCAT*; inhibition is greater at 25 °C than at 37 °C; inhibition by d-Ap*AAAGCAAG*, d-Cp*ATTCTGT*, or d-Ap*CAGATGC* increases when the oligomer is preannealed to the mRNA; and no inhibition occurs in the presence of d-*TTTTTT*, which is complementary to the poly(rA) tail, even at very high oligomer concentrations. The latter observation shows that inhibition is not due to nonspecific binding of methylphosphonate oligomers to other components in the translating system.

Octamer d-*ACAGACAT* can potentially form complexes which have five contiguous base pairs with α- and β-globin mRNA (see Table I). Two of the complexes would occur in the coding region of each mRNA. This oligomer had low inhibitory activity even at high concentration when tested in the lysate system. This result suggests that partial binding involving five or less contiguous base pairs does not significantly contribute to the inhibitory effects. This octamer, which is complementary to the initiation codon region of vesicular stomatitis virus N protein mRNA, was shown to selectively inhibit virus protein synthesis in VSV-infected mouse L cells (Miller et al., 1984). In a recent series of experiments which will be published elsewhere, we have shown that 100 μM d-Ap*ACAGACAT* specifically inhibits translation of VSV N protein mRNA to the extent of 47% but does not inhibit translation of M or NS protein mRNA when these three mRNAs are translated simultaneously in the rabbit reticulocyte lysate system. This result further suggests that specific interactions between the oligodeoxyribonucleoside methylphosphonates and their target mRNAs are required for inhibition of translation.

Inhibition of mRNA translation by the oligonucleoside methylphosphonates is affected by mRNA secondary structure as shown by the preannealing experiments. The binding sites for d-Ap*AAAGCAAG*, d-Cp*ATTCTGT*, and d-Ap*CAGATGC* whose inhibitory effects are enhanced by preannealing occur in hydrogen-bonded stem regions in β-globin mRNA (Pavlakis et al., 1980). Preannealing opens up this secondary structure and allows the oligomers to bind to the mRNA. Although d-Cp*ATTCTGT* and d-Cp*ATTCTGTCTGT* share a common binding site on β-globin mRNA, the dodecamer inhibits

translation without being preannealed while the octamer requires preannealing in order to inhibit. This result is consistent with the expected binding interactions of longer vs. shorter oligomers to complementary polynucleotides because the binding constant of the dodecamer should be significantly higher than that of the octamer. Therefore, the dodecamer can more effectively compete with the secondary structure of the binding region.

In addition to the effects of mRNA secondary structure, the region of the mRNA to which the oligonucleoside methylphosphonate binds influences its effectiveness as an inhibitor of mRNA translation. The results in Table II suggest that oligomers complementary to the 5′ end and initiation codon regions are somewhat better inhibitors than oligomers which bind to the coding regions. Thus, it appears that the initiation step of translation is more sensitive to oligomer–mRNA binding than the elongation step of translation.

Oligodeoxyribonucleoside methylphosphonates have inhibitory effects similar to those of oligodeoxyribonucleotides in the reticulocyte lysate except when complementarity involves the coding region of mRNA (Table III). In this case, the methylphosphonate oligomers are more effective inhibitors. The recent studies of Liebhaber et al. (1984) suggest that a helix-destabilizing activity found in rabbit reticulocyte lysates can disrupt secondary structure in the coding region of mRNA and thus prevent hybridization arrest of translation by cDNAs complementary only to this region excluding the AUG initiation codon. Our results suggest that this activity does not efficiently displace oligomers with a noncharged methylphosphonate backbone. Possibly the displacement reaction depends upon interaction between the protein and the negatively charged sugar–phosphate backbones of the mRNA–cDNA complex. It does appear, however, that phosphodiester oligomers are better able to bind to sites involved in secondary structure than are the methylphosphonate oligomers. Thus, d-Cp*ATTCTGT* inhibits effectively only after preannealing with mRNA while the corresponding diester, d-CATTCTGT, does not require preannealing.

The specificity of inhibition by the oligonucleoside methylphosphonates is also affected by their binding position on the mRNA. The methylphosphonate oligomers which are complementary to the 5′ end and the initiation codon region of β-globin mRNA, d-Ap*AAAGCAAG*, d-Cp*ATTCTGT*, and d-Cp*ATTCTGTCTGT*, inhibit both α- and β-globin synthesis. These oligomers would be expected to inhibit the initiation step of translation of β-globin mRNA. Oligomers d-Tp*GATGTTGG* and d-Tp*CCTCCTG*, which bind to the coding regions of α- and β-globin mRNA, respectively, inhibit α-globin and β-globin synthesis in a selective manner. Because the binding sites for these oligomers are located approximately 20 nucleotides downstream from the initiation codons, they would be expected to inhibit the elongation step of translation. These observations are consistent with our previous conclusion that translation of α- and β-globin mRNA is coordinated in the reticulocyte lysate (Blake et al., 1985). That is, inhibition of the initiation step of β-globin mRNA translation results in inhibition of α-globin synthesis as well. It appears that translation of α- and β-globin mRNA is less tightly coupled once the initiation phase is completed. The effect of d-Ap*CAGATGC* on translation is also consistent with this conclusion. The binding site for this oligomer is three nucleotides downstream from -AUG- in the coding region of β-globin mRNA. However, this region is most likely still part of the ribosomal binding site (Legon, 1976), and oligomers bound in this region could perturb the initiation step of translation. Thus, at high concentrations, d-Ap*CAGATGC* inhibits both α- and β-globin synthesis.

The methylphosphonate oligomers are generally less effective inhibitors of translation in wheat germ extracts (Blake et al., 1985) than the phosphodiester oligomers. The reason for this difference is not known. It does not appear to be due to the inability of the methylphosphonate oligomers to bind adequately to mRNA at 25 °C, the temperature of the wheat germ reaction. In the reticulocyte lysate, the methylphosphonate oligomers tested at 25 °C inhibit as well as or better than those tested at 37 °C.

The effects of oligonucleoside methylphosphonates on globin synthesis in rabbit reticulocytes were studied. On the basis of our previous experiments on the uptake of oligonucleoside methylphosphonates by mammalian cells in culture (Miller et al., 1981), the oligomers used in this study were expected to enter the reticulocytes intact. As shown in Table IV, oligodeoxyribonucleoside methylphosphonates are effective inhibitors of globin protein synthesis. For the oligomers d-Tp*GCACCAT*, d-Cp*ATTCTGT*, and d-Cp*ATTCTGTCTGT*, the inhibitory effects are similar to those in the lysate. It is interesting to note that d-Cp*ATTCTGT* has no inhibitory effect in the reticulocytes. This oligomer only inhibits in the lysate after it is preannealed to the mRNA. Assuming inhibition in the cells is due to oligomer binding to mRNA, this observation suggests that during translation the globin mRNA has the same conformation in both the reticulocytes and lysate. In contrast to its effect in the lysate, d-Tp*GATGTTGG* inhibits both α- and β-globin synthesis in the reticulocyte. This effect could be due to a tighter coordination of translation of α- and β-globin mRNA in the reticulocytes vs. that found in the lysate.

Our studies show that oligodeoxyribonucleoside methylphosphonates are effective inhibitors of globin synthesis in both reticulocyte lysates and intact cells. Because the binding of methylphosphonate oligomers is sensitive to secondary structure, it appears that they may be used to probe double-stranded regions of actively translating mRNA in cells. Preliminary results indicate inhibition of protein synthesis by methylphosphonate oligomers can be extended to other mammalian cellular systems including mouse L cells infected with vesicular stomatitis virus (Miller et al., 1984). The results of our present study suggest that by suitable choice of oligomer sequence and binding site it will be possible to control translation of specific mRNAs. Therefore, this technique should provide a valuable tool for studying and/or controlling gene expression in mammalian cells.

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Registry No. d-Ap*AAAGCAAG*, 98014-33-6; d-Tp*GCACCAT*, 96791-04-7; d-Cp*ATTCTGT*, 98014-34-7; d-Cp*ATTCTGTCTGT*, 98014-35-8; d-Ap*CAGATGC*, 98049-92-4; d-Tp*GATGTTGG*, 98014-36-9; d-Tp*CCTCCTG*, 98014-37-0; d-*ACAGACAT*, 98049-93-5.

REFERENCES

Blake, K. R., Murakami, A., & Miller, P. S. (1985) *Biochemistry* (preceding paper in this issue).

Efstratiadis, A., Kafatos, F. C., & Maniatis, T. (1977) *Cell (Cambridge, Mass.) 10*, 571–585.

Heindell, H. C., Liu, A., Paddock, G. V., Stunicka, G. M., & Salser, W. A. (1978) *Cell (Cambridge, Mass.) 15*, 43–54.

Jayaraman, K., McParland, K. B., Miller, P. S., & Ts'o, P. O. P. (1981) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1537–1541.

Legon, S. (1976) *J. Mol. Biol. 106*, 37–53.

Liebhaber, S. A., Cash, F. E., & Shakin, S. H. (1984) *J. Biol. Chem. 259*, 15597–15602.
Lo, K.-M., Jones, S. S., Hackett, N. R., & Khorana, H. G. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 2285–2289.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, p 184, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Miller, P. S., Yano, J., Yano, E., Carroll, C., Jayaraman, K., & Ts'o, P. O. P. (1979) *Biochemistry 18*, 5134–5143.
Miller, P. S., McParland, K. B., Jayaraman, K., & Ts'o, P. O. P. (1981) *Biochemistry 20*, 1874–1880.
Miller, P. S., Agris, C. H., Murakami, A., Reddy, M. P., Spitz, S. A., & Ts'o, P. O. P. (1983) *Nucleic Acids Res. 11*, 6225–6242.
Miller, P., Agris, C., Aurelian, L., Blake, K., Kelly, T., Murakami, A., Reddy, M. P., Spitz, S., Ts'o, P., & Wides, R. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, Abstr. 1811.
Murakami, A., Blake, K. R., & Miller, P. S. (1985) *Biochemistry 24*, 4041–4046.
Paterson, B. M., Roberts, B. E., & Kuff, E. L. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4370–4374.
Pavlakis, G. H., Lockhard, R. E., Vamvakopoulos, N., Riser, L., RajBhandary, U. L., & Vournakis, J. N. (1980) *Cell (Cambridge, Mass.) 19*, 91–102.
Rickwood, D., & Hames, B. D. (1982) *Gel Electrophoresis of Nucleic Acids: A Practical Approach*, p 52, IRL Press Limited, Oxford.
Stephenson, M. L., & Zamecnik, P. C. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 285–288.

Aldehyde and Ketone Substrate Analogues Inhibit the Collagenase of *Clostridium histolyticum*†

Damian Grobelny*,‡ and Richard E. Galardy*
Department of Biochemistry and Sanders-Brown Research Center on Aging, The University of Kentucky, Lexington, Kentucky 40536
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ABSTRACT: The collagenase from *Clostridium histolyticum* is a mixture of several collagenases, all of which are zinc metalloproteases. This enzyme catalyzes the cleavage of the X–Gly peptide bond in the repeating sequence of collagen: -Gly-Pro-X-Gly-Pro-X-. Thus the S_3, S_2, and S_1 subsites on the enzyme appear to be occupied by the sequence -Gly-Pro-X- and the S_1', S_2', and S_3' subsites also by -Gly-Pro-X-. Short peptides up to and including N^{α}-acyltetrapeptides containing the repeat sequence do not detectably inhibit the enzyme (IC_{50} > 10 mM). However, peptide aldehydes of the form aminoacyl-X-glycinal, presumably occupying the S_1, S_2, ..., S_n subsites, are inhibitors. The most potent of these was Pro_6-Gly-Pro-glycinal, with an IC_{50} of 340 ± 70 μM. The single peptide aldehyde investigated, which could occupy the S_1' and S_2' subsites, 4-oxobutanoyl-L-proline, did not inhibit collagenase (IC_{50} > 20 mM). The peptide ketone 5-benzamido-4-oxo-6-phenylhexanoyl-Pro-Ala (XXV), which could occupy the S_1–S_3' subsites, inhibits collagenase with an IC_{50} of 120 ± 50 μM, over 80-fold more potently than its parent peptide analogue benzoyl-Phe-Gly-Pro-Ala (XXIII). The alcohol analogue of XXV, 5-benzamido-4-hydroxy-6-phenylhexanoyl-Pro-Ala (XXVI), is over 60-fold less potent with an IC_{50} of 8 ± 2 mM. Extending the peptide ketone XXV to occupy the S_2–S_3' subsites gave 5-(N^{α}-carbobenzoxy-L-prolinamido)-4-oxo-6-phenylhexanoyl-Pro-Ala (XXVII). Surprisingly, XXVII had an IC_{50} of only 5.2 ± 2 mM. Neither XXV nor XXVII exhibited the kinetic characteristic of a slow-binding inhibitor. We conclude that 5-benzamido-4-oxo-6-phenylhexanoyl-Pro-Ala (XXV) does not inhibit collagenase by simply forming an enzyme–inhibitor complex similar to the enzyme–substrate Michaelis complex. It must bind to the enzyme by a different mechanism, but not necessarily as an analogue of the transition state in the enzyme-catalyzed hydrolysis of substrate.

The collagenase from *Clostridium histolyticum* (EC 3.4.24.3) is a zinc metalloprotease also known as clostridiopeptidase A and collagenase A (Seifter & Harper, 1971). This collagenase makes a large number of cleavages in native triple-helical collagen, usually at the X–glycine bond in the sequence X-glycyl-L-prolyl-X, where X is frequently alanine or hydroxyproline but can be any amino acid. Synthetic oligopeptides are cleaved with similar specificity. The triple-helical region of native soluble collagen is relatively resistant to nearly every protease except the collagenases (Burleigh, 1977). This enzyme is now known to be a mixture of collagenases (Lwebuga-Mukasa et al., 1976; Sugasawara & Harper, 1984) and has been separated into six individual enzymes (Bond & Van Wart, 1984a,b). These six enzymes fall into two classes, class I (α and β collagenases) having high activity against collagen and lower activity against several small peptide substrates and class II (γ, δ, ε, and ζ collagenases) having the opposite trend of activity (Bond & Van Wart, 1984a,b).

Compounds such as cysteine that are nonspecific inhibitors of all zinc metalloproteases inhibit collagenase (Seifter & Harper, 1971). N^{α}-(Benzyloxycarbonyl)tripeptides, and -tetrapeptides, -pentapeptides containing the sequence glycyl-L-proline have been reported to inhibit the enzyme with K_i's as low as 4 mM (Yagisawa et al., 1965). Similar peptides with a C-terminal chloromethyl ketone group show some

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* Authors to whom correspondence should be addressed.
‡ Present address: The Institute of Organic and Polymer Technology, Technical University of Wroclaw, Wroclaw, Poland.

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