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Inhibition of Vesicular Stomatitis Virus Protein Synthesis and Infection by Sequence-Specific Oligodeoxyribonucleoside Methylphosphonates†

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ABSTRACT: Oligodeoxyribonucleoside methylphosphonates which have sequences complementary to the initiation codon regions of N, NS, and G vesicular stomatitis virus (VSV) mRNAs were tested for their ability to inhibit translation of VSV mRNA in a cell-free system and in VSV-infected mouse L cells. In a rabbit reticulocyte lysate cell-free system, the oligomers complementary to N (oligomer I) and NS (oligomer II) mRNAs inhibited translation of VSV N and NS mRNAs whereas oligomer III had only a slight inhibitory effect on N protein synthesis. At 100 and 150 μM, oligomer I specifically inhibited N protein synthesis in the lysate. In contrast, at 150 μM, oligomer II inhibited both N and NS protein synthesis. This reduced specificity of inhibition may be due to the formation of partial duplexes between oligomer II and VSV N mRNA. The oligomers had little or no inhibitory effects on the synthesis of globin mRNA in the same lysate system. Oligomers I–III specifically inhibited the synthesis of all five viral proteins in VSV-infected cells in a concentration-dependent manner. The oligomers had no effects on cellular protein synthesis in uninfected cells nor on cell growth. An oligothymidylate which forms only weak duplexes with poly(rA) had just a slight effect on VSV protein synthesis and yield of virus. Oligomers I–III have extensive partial complementarity with the coding regions of L mRNA. The nonspecific inhibition of viral protein synthesis in infected cells may reflect the role of N, NS, and/or L proteins in the replication and transcription of viral RNA or result from duplex formation between the oligomers and complementary, plus-strand viral RNA. The results of this study indicate that inhibition of viral protein synthesis in a cell-free lysate and in infected cells is primarily due to the interaction of oligomers I–III with complementary VSV mRNAs. Oligomers I–III also significantly inhibited VSV production in a manner corresponding to their effects on VSV protein synthesis. These results demonstrate that oligonucleoside methylphosphonates can be used to study viral gene expression and to control virus production.

Hybridization arrest of nucleic acid function is a promising technique for studying and possibly controlling cellular and viral gene expression. A number of laboratories have demonstrated that cDNAs, antisense mRNAs, and complementary oligodeoxyribonucleotides may be used to selectively inhibit translation and/or processing of mRNA in cell-free systems and in living cells (Paterson et al., 1977; Stephenson & Zamecnik, 1978; Izant & Weintraub, 1984; Melton, 1985; Ellison et al., 1985; Blake et al., 1985a; Toulme et al., 1986). Our laboratory has been studying the possibility of using complementary nonionic oligonucleotide analogues to selectively inhibit mRNA function. In previous reports, we have described methods to synthesize and characterize oligodeoxyribonucleoside methylphosphonates of specific sequence and methods to study their interaction with messenger RNA (Miller et al., 1983a, 1986; Murakami et al., 1985). These nonionic nucleic acid analogues contain a neutral methylphosphonate linkage in place of the phosphodiester internucleotide bond normally found in oligonucleotides. Methylphosphonate oligomers bind to complementary nucleic acids according to the base-pairing rule, are taken up by mammalian cells in culture, and are resistant to hydrolysis by cellular nucleases (Miller et al., 1981).

Recently we have shown that oligodeoxyribonucleoside methylphosphonates with sequences complementary to the 5′ end, the initiation codon regions, and coding regions of rabbit globin mRNA inhibit translation of the mRNA by a hybridization arrest mechanism in a reticulocyte cell-free system

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and in intact reticulocyte cells (Blake et al., 1985b). In addition, preliminary studies show a methylphosphonate oligomer complementary to the donor splice junction of SV40–large T antigen pre-mRNA inhibits the synthesis of large T antigen (Miller et al., 1983b). We have also demonstrated that a methylphosphonate oligomer complementary to the acceptor splice junctions of Herpes simplex virus (HSV)[1] type 1 immediate early pre-mRNAs 4 and 5 inhibits virus protein synthesis, virus DNA replication, and virus yield in HSV-infected human or monkey cells in a selective manner (Smith et al., 1986).

In this paper, we extend our studies of hybridization arrest of translation by sequence-specific oligonucleoside methylphosphonates to vesicular stomatitis virus (VSV). VSV is an enveloped Rhabdovirus containing a single strand of RNA as its genome which is approximately 11 000 bases in length and codes for 5 viral proteins (Emerson, 1976). These include G protein (glycoprotein) (M_r 62 354), M (matrix) protein (M_r 26 064), N (nucleocapsid) protein (M_r 47 355), NS (nonstructural) protein (M_r 25 110), and L (large) protein (M_r 241 012) (Rose & Gallione, 1981; Gallione et al., 1981; Schubert et al., 1984). We have prepared methylphosphonate oligomers complementary to the initiation codon regions of N, NS, and G protein mRNAs of VSV. These oligomers also have extensive partial complementarity with the coding region of L protein mRNA. The effects of these oligomers on VSV protein synthesis in a rabbit reticulocyte lysate translation system and in VSV-infected mouse L929 cells as well as their effects on virus production were studied. The results of these studies were compared with the effects of methylphosphonate oligomers on mouse L cell protein synthesis and plating efficiency in order to ascertain the specificity of the oligomers on their inhibition of viral gene expression.

Experimental Procedures

The syntheses and characterization of oligodeoxyribonucleoside methylphosphonates were carried out by methods previously described (Miller et al., 1986; Murakami et al., 1985). [^{35}S]Methionine (>800 Ci/mmol) and [^{3}H]leucine (130–190 Ci/mmol) were obtained from New England Nuclear, Inc. Actinomycin D was obtained from Calbiochem, NP 40 from Sigma Chemical Co., and oligo(dT)–cellulose type 3 from Collaborative Research. SDS was obtained from BDH chemicals. All other chemicals were reagent or electrophoresis grade.

Cells and Virus. Monolayers of mouse L929 cells were cultivated in either 75 cm^2 flasks (Falcon, Inc.) or 150 cm^2 flasks (Costar) using ERM supplemented with 10% fetal calf serum (Biolabs) and 60 μg/mL gentamycin (ESI Pharmaceuticals). Vesicular stomatitis virus, Indiana serotype, was obtained from Dr. Carl Dieffenbach. It was plaque purified by using a modification of the procedure described by Wunner (1985) and stored at −80 °C. Titers were normally (1–2) × 10^8 pfu/mL as determined by plaque assay using a modification of the procedures described by Wunner (1985). VSV was serially diluted in ERM supplemented with 2% FCS and 0.06 mg/mL gentamycin. The dilutions of virus (1 mL) were each added to monolayers of confluent L929 cells on 6-well plates (35-mm diameter/well) purchased from Costar or Nuncleon Delta, SI, and incubated for 1 h at 37 °C to allow virus absorption. During this time, the wells were agitated every 15 min. The cells were washed with PBS and overlaid with 2% agar/2× media solution (1:1 v/v). After the plates were incubated for 2 days at 37 °C, the cells were stained with an 0.05% neutral red solution. The cells were incubated for 2 h at 37 °C, after which the stain was removed and the plaques were scored.

Radiolabeled VS virions were prepared by first infecting confluent mouse L929 cells in 75 cm^2 flasks with VSV at an moi of 5 and then incubating at 37 °C. At 4-h postinfection, the media were removed and replaced with leucine-free media containing dialyzed FCS. Infected cells were incubated with labeled media for 6 h at 37 °C. At this time, the media were removed and transferred to 15-mL centrifuge tubes. The cellular debris were removed by centrifugation at 2000*g* at 4 °C for 5 min, and the virions were pelleted by ultracentrifugation in an SW 50.1 rotor at 60000*g* for 2.5 h at 4 °C. Pellets were suspended in buffer containing 10 mM Tris-HCl (pH 7.4)/1% SDS, sonicated for 10 s, and stored at −80 °C. VSV proteins obtained from these pellets were used as markers for labeled protein analysis on polyacrylamide gels.

Preparation of VSV mRNA. Monolayers of confluent mouse L929 cells growing on 245 × 245 × 20 mm tissue cultures plates (Nuncleon Delta SI, Denmark) were infected with VSV at an moi of 5 in the presence of 5 μg/mL actinomycin D. The cells were washed 4-h postinfection with cold PBS which contained 137 mM sodium chloride, 2.7 mM potassium chloride, 8.1 mM dibasic sodium phosphate/monobasic potassium phosphate, 90 μM calcium chloride, and 50 μM magnesium chloride. The cells were then removed with a disposable cell scraper (Costar) and were pelleted at 2000*g* for 5 min at 4 °C. Cytoplasmic extracts were obtained by using the procedure described by Maniatis et al. (1982) except that the buffers were pH 7.4. Cytoplasmic RNA was obtained by extraction with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1 v/v). The distilled phenol was saturated with 0.1 M Tris-HCl, pH 9.0. The RNA was then ethanol precipitated as described by Maniatis et al. (1982) and stored in a 0.5 mg/mL solution at −80 °C.

In Vitro Translation of VSV mRNA in the Presence of Oligodeoxyribonucleoside Methylphosphonates. Cell-free translation reactions were carried out by using rabbit reticulocyte lysates obtained from Bethesda Research Laboratories, Inc. as previously described (Blake et al., 1985a). 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 aliquot of each amino acid except methionine, 88.4 mM potassium acetate, 4.2 μCi of [^{35}S]-methionine, 0–150 μM oligonucleoside methylphosphonate, 2 μg of VSV mRNA, and 8.3 mL of rabbit reticulocyte lysate in a total volume of 25 μL. The translation reactions were carried out at 30 °C for 60 min. Three-microliter aliquots of the reaction mixture were removed at 20, 40, and 60 min and precipitated in 10% TCA. Filtered TCA precipitates were counted in Betafluor (National Diagnostics).

After 60 min, a 3-μL aliquot of each translation reaction was added to 30 μL of discontinuous gel loading buffer which contained 0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, and 10% mercaptoethanol. The solution was boiled for 2.5 min, and 15-μL aliquots of each sample were then analyzed by discontinuous gel electrophoresis, using a modification of the

[1] Abbreviations: VSV, vesicular stomatitis virus; HSV, Herpes simplex virus; pfu, plaque-forming units; moi, multiplicity of infection; SDS, sodium dodecyl sulfate; FCS, fetal calf serum; PBS, phosphate-buffered saline; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonate; TCA, trichloroacetic acid; ERM, Eagle's reinforced media; d-Np*NNNNN*, an oligodeoxyribonucleoside methylphosphonate; the symbol p indicates a phosphodiester linkage; nucleoside units linked by methylphosphonate groups are in italics.

method described by Laemmli (1970). The acrylamide stock solution contained acrylamide/methylenebis(acrylamide) in a ratio of 29.6:0.4. The stacking and running gels contained 5% and 10% acrylamide, respectively. The stacking gel was run at 15 mA for 1.5 h and the running gel at 30 mA for 3.0 h. The gels were fixed in 50% methanol/10% acetic acid for 1 h, soaked in ENLIGHTENING (New England Nuclear, Inc.) for 15–30 min, and dried onto Kodak blotting paper. VSV proteins were located by fluorography at −80 °C overnight with Kodak XAR-5 film. Bands containing VSV proteins were excised and counted in 10 mL of Betafluor.

In Vitro Translation of Globin mRNA in the Presence of Oligodeoxyribonucleoside Methylphosphonates. The translation reactions were carried out with 0.3 μg of rabbit globin mRNA in the presence of 0–100 μM oligodeoxyribonucleoside methylphosphonates. The reaction mixtures were incubated for 60 min at 30 °C. Three-microliter aliquots were removed at this time and immediately added to 50 μL of continuous gel loading buffer. After being boiled, 10-μL aliquots were analyzed by continuous gel electrophoresis as previously described (Blake et al., 1985a). Bands comigrating with α- and β-globin were excised and counted in Betafluor.

Effects of Oligodeoxyribonucleoside Methylphosphonates on VSV Protein Synthesis in Cultured Cells. Monolayers of mouse L929 cells were grown in LAB-TEK 8-chamber (1 cm^2) microscope slides (Miles Scientific). Confluent cells [(1–1.2) × 10^5 cells/well] were preincubated at 37 °C with 0–150 μM oligodeoxyribonucleoside methylphosphonate in media containing 2% FCS for 16 h. The cells were then infected with VSV at an moi of 5. At 6-h postinfection, the media were removed, and the cells were incubated with methionine-free media containing oligonucleoside methylphosphonate and 2% dialyzed FCS for 15 min at 37 °C. The media were then removed, and cells were labeled for 30 min at 37 °C with methionine-free media containing [^{35}S]methionine (60 μCi/mL). The cells were swelled in ice-cold buffer containing 10 mM Tris-HCl (pH 7.4), 2.5 mM magnesium chloride, and 30 mM potassium chloride and then suspended in ice-cold buffer containing 0.75% NP40 for 5 min. The cells were then scraped with a pipet tip, and 50-μL aliquots of each lysate were transferred to Eppendorf tubes containing discontinuous gel loading buffer. The buffer solution was boiled for 2.5 min and stored at −80 °C. This procedure was also followed with mock-infected cells in the presence of 0–150 μM oligodeoxyribonucleoside methylphosphonate. Ten-microliter aliquots of each sample were loaded onto discontinuous gels and run exactly as described for cell-free translation reactions. The amounts of viral protein present in each sample were determined by using a modification of the difference analysis procedure described by McAllister and Wagner (1976). This procedure corrects for cellular protein that comigrates with viral protein. For each sample, all areas not containing viral protein were excised from the gel. The total cpm from infected cells, I, and the total cpm from uninfected cells, U, were determined, and the ratio $I/U = R$ was calculated. The amount, A, of each viral protein in a sample was then determined by using the formula $A = V - RM$, where V is the total ^{35}S activity present in a sample from VSV-infected cells at a gel fraction containing a particular viral protein and M is the ^{35}S activity in the corresponding gel fraction from uninfected cells.

Assays for VSV Production. Monolayers of mouse L929 cells were preincubated with oligodeoxyribonucleoside methylphosphonates as described above. The cells were then infected with VSV at an moi of 5. The media were removed at either 6- or 24-h postinfection and stored at −80 °C. The amount of virus released into the media from each sample was determined by a standard plaque assay, as previously described.

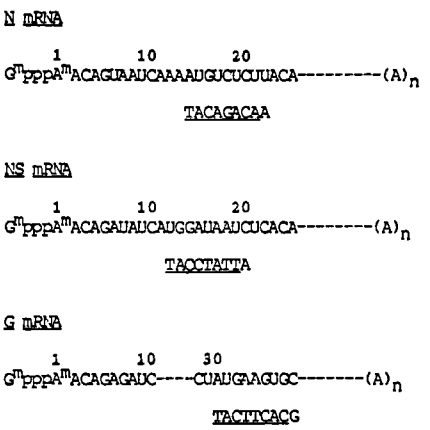

FIGURE 1: Partial nucleotide sequence of the initiation codon regions of vesicular stomatitis virus (VSV) N, NS, and G protein mRNAs. The sequence of the complementary oligonucleoside methylphosphonate is shown below each mRNA sequence.

Effects of Oligodeoxyribonucleoside Methylphosphonates on Cell Growth. The effects of oligodeoxyribonucleoside methylphosphonates on cell growth were determined by comparing the plating efficiencies of treated and nontreated cells. Confluent monolayers of mouse L cells growing in an 8-chamber microscope slide were treated with media containing 2% FCS and 0 or 150 μM d-Gp*CACTTCAT*. The cells were incubated for 40 h at 37 °C at which time they were trypsinized and diluted to a concentration of 100–300 cells/mL. The cell number was determined by using a Coulter counter (Coulter Electronics). Three milliliters of each dilution was seeded onto 6-well plates (35-mm diameter/well) (Costar). After 9 days, the media were removed, the cells were fixed with methanol and stained with Giemsa, and the number of colonies were counted. The plating efficiency is the ratio of colonies formed to the number of cells plated multiplied by 100.

RESULTS

Complementarity between Oligodeoxyribonucleoside Methylphosphonates and VSV or Rabbit Globin mRNA. Four oligodeoxyribonucleoside methylphosphonate sequences were synthesized on an insoluble polystyrene support and were characterized as previously described (Miller et al., 1986; Murakami et al., 1985). These oligomers have their 5′-terminal nucleoside linked via a phosphodiester bond which simplifies their purification and characterization. As shown in Figure 1, the oligomers d-Ap*ACAGACAT* (I), d-Ap*TTATCCAT* (II), and d-Gp*CACTTCAT* (III) are complementary to sequences including the initiation codon of N, NS, and G VSV mRNAs, respectively. An additional oligomer, d-Tp*TTTTTTTT* (IV) was also prepared which is complementary to the poly(rA) tail of mRNA.

The complementarity between oligomers I, II, and III and VSV or globin mRNA was determined by using a computer program which matches the oligodeoxyribonucleoside methylphosphonate sequence with complementary nucleotide sequences of VSV (Rose & Gallione, 1981; Gallione et al., 1981; Schubert et al., 1984) and globin mRNAs (Pavlakis et al., 1980). The program was used to search for mRNA sequences which are complementary to five or more contiguous bases of the methylphosphonate oligomers.

The results of this search are shown in Table I. Oligomers I–III are each completely complementary only to the initiation

Table I: Complementarity between Oligodeoxyribonucleoside Methylphosphonates and VSV mRNA or Rabbit Globin mRNA

oligonucleoside methylphosphonate	no. of contiguous oligomer bases	no. of complementary sites[a]								
		VSV mRNA						globin mRNA		
		L	G	NS	N	M	total	α	β	total
d-Ap*ACAGACAT* (I)	9	0	0	0	1	0	1	0	0	0
	8	0	0	0	0	0	0	0	0	0
	7	2	0	0	0	0	0	0	0	0
	6	4	4	0	1	0	9	0	0	0
				1[b]			1[b]			
	5	16	1	2	3	1	23	1	3	4
				1[b]		1[b]	2[b]	1[b]		1[b]
d-Ap*TTATCCAT* (II)	9	0	0	1	0	0	1	0	0	0
	8	1	0	0	0	0	1	0	0	0
	7	2	0	0	1	0	3	0	0	0
	6	8	1	0	2	0	11	0	0	0
	5	33	10	5	6	3	57	0	1	1
				1[b]	1[b]		2[b]			
d-Gp*CACTTCAT* (III)	9	0	1	0	0	0	1	0	0	0
	8	1	0	0	0	0	1	0	0	0
	7	1	1	0	0	0	2	0	0	0
	6	9	1	2	0	1	13	0	1[b]	1[b]
	5	40	7	8	6	6	67	0	1	1
		2[b]	1[b]	2[b]	1[b]	1[c]	7[b]			

[a] These values represent the number of nonoverlapping sites on the mRNA which are complementary to five or more contiguous bases of the oligomer. [b] Site is located in the 3′-noncoding region of the mRNA. [c] Site is located in the 5′-noncoding region of the mRNA.

codon regions of their respective target VSV mRNA. However, partial complementarity involving five to eight contiguous bases of each methylphosphonate oligomer was found with each of the VSV mRNAs. As expected, the largest number of sites involved only five contiguous bases of the oligomer, while with the exception of L mRNA, very few sites in VSV mRNA involving six, seven, or eight contiguous bases were found. Most of the complementary sites were found on mRNA of L protein. This was expected since the L mRNA is much longer than the other VSV mRNAs.

No regions of rabbit globin α or β mRNA were found to be completely complementary to three methylphosphonate oligomers. Partial complementarity involving five contiguous oligomer bases was found between oligomer I and α- and β-globin mRNAs. In contrast, oligomers II and III were partially complementary only to β-globin mRNA.

Effects of Oligodeoxyribonucleoside Methylphosphonates on Cell-Free Translation of VSV mRNA and Globin mRNA. VSV mRNA was translated in a rabbit reticulocyte lysate system at 30 °C in the absence and presence of oligomers I, II, and III. In the absence of oligomer, 0.04–0.06 μCi or 40–60 fmol of [^{35}S]methionine was incorporated into VSV protein after 60-min incubation. The translation products, which included NS, N, and M proteins, were analyzed by discontinuous gel electrophoresis. As expected, only low levels of G and L proteins were detected when gels were fluorographed for 5 days at −80 °C (data not shown). A fluorogram of VSV proteins synthesized in the absence and presence of oligomer (I) is shown in Figure 2. The amounts of each VSV protein synthesized were determined by counting regions of the dried gel corresponding to the proteins. Between 500 and 3000 cpm were observed in each protein band.

The effects of oligodeoxyribonucleoside methylphosphonates I, II, and III on NS, N, and M cell-free protein synthesis are shown in Table II. Oligomer I specifically inhibited N protein synthesis in the concentration range of 50–100 μM. At 150 μM oligomer I, N protein synthesis was inhibited by 77% whereas NS and M protein syntheses were also inhibited to the extent of 38% and 43%, respectively. Oligomer II inhibited the synthesis of NS, N, and M proteins at 100 and 150 μM in a concentration-dependent manner. However, the effect of oligomer II was less selective than that of oligomer I. At

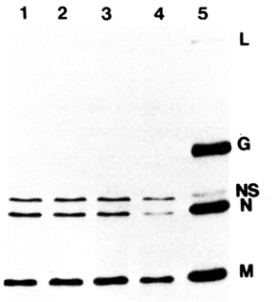

FIGURE 2: Analysis by gel electrophoresis of [^{35}S]methionine-labeled VSV proteins synthesized in rabbit reticulocyte lysate after incubating at 30 °C for 60 min in the presence of the following concentrations of d-Ap*ACAGACAT* (I): lane 1, 0 μM; lane 2, 50 μM; lane 3, 100 μM; lane 4, 150 μM. Lane 5 contains [^{3}H]leucine-labeled VS virion proteins.

Table II: Effects of Oligodeoxyribonucleoside Methylphosphonates on VSV and Globin Protein Synthesis in a Rabbit Reticulocyte Lysate at 30 °C[a]

oligonucleoside methyl-phosphonate	concn (μM)	% change[b]				
		NS	N	M	α-globin	β-globin
d-Ap*ACAGACAT*	50	+7	−26	+20	−6	−9
(I)	100	−7	−36	+12	−15	−9
	150	−38	−77	−43		
d-Ap*TTATCCAT*	50	+5	−4	+7	+18	+14
(II)	100	−15	−25	+2	+63	+50
	150	−24	−35	−18		
d-Gp*CACTTCAT*	50	−4	−15	0	+26	+20
(III)	100	+2	−16	+6	+40	+55

[a] Average of two or three experiments with a range of ±6% (VSV) or ±4% (globin). [b] Minus sign indicates inhibition of translation, while plus sign indicates stimulation of translation.

100 μM, oligomer II inhibited both N protein synthesis and to a lesser extent NS protein synthesis, whereas at 150 μM the synthesis of all three proteins was inhibited. Oligomer III which is complementary to G mRNA had little effect on the synthesis of either NS, N, or M proteins.

Similar experiments were carried out to determine the effects of oligomers I–III on translation of rabbit globin mRNA. The results of these experiments are shown in Table II. Oligomer I, which is partially complementary to one coding

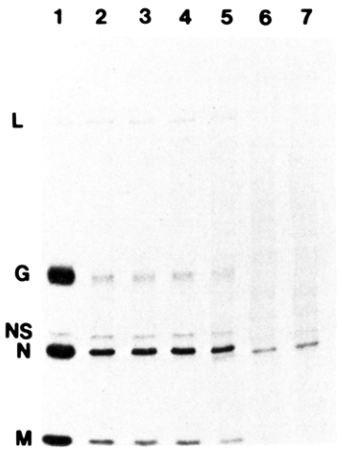

FIGURE 3: Analysis by gel electrophoresis of [^{35}S]methionine-labeled proteins obtained from VSV-infected (lanes 2–5) and mock-infected cells (lanes 6 and 7) 6-h postinfection in the presence of the following concentrations of d-Ap*ACAGACAT* (I): lanes 2 and 6, 0 μM; lane 3, 50 μM; lane 4, 100 μM; lanes 5 and 7, 150 μM. Lane 1 contains [^{3}H]leucine-labeled VS virion protein markers.

region of α-globin mRNA and three coding regions of β-globin mRNA, is a weak inhibitor of globin mRNA translation. Oligomers II and III are partially complementary to one and two regions of β-globin mRNA, respectively. Surprisingly, both of these compounds stimulate globin protein synthesis in a concentration-dependent manner.

Inhibition of VSV Protein Synthesis by Oligodeoxyribonucleoside Methylphosphonates in Infected Mouse L Cells. Confluent mouse L929 cells were preincubated with methylphosphonate oligomer for 16 h and then infected with VSV in the presence of the oligomer. At 6-h postinfection, the incorporation of [^{35}S]methionine was measured by TCA precipitation. In the absence of oligomer, 0.014–0.023 and 0.025–0.038 μCi of [^{35}S]methionine was incorporated into proteins present in VSV-infected and mock-infected cells, respectively. The total amount of incorporation of [^{35}S]-methionine increased with increasing amounts of oligomers I–III in VSV-infected cells (data not shown).

The effects of oligomers I–IV on the synthesis of VSV proteins were analyzed by gel electrophoresis. A fluorogram of VSV proteins synthesized in VSV-infected cells in the presence of varying concentrations of oligomer I is shown in Figure 3. Increasing concentrations of oligomer I resulted in a decrease in synthesis of all five viral proteins and a concomitant increase in the synthesis of cellular proteins. Similar trends were observed for VSV-infected cells treated with oligomers II and III. The amounts of each protein in a sample were determined by a difference analysis procedure described under Experimental Procedures. Between 500 and 5000 cpm were detected in each band containing VSV protein. As shown in Table III, oligomers I–III inhibit the synthesis of all five VSV proteins in a concentration-dependent manner. Oligomer III appears to have the greatest effect on VSV protein synthesis in mouse L cells. The effect of oligomer I at 150 μM on VSV protein synthesis was greater than that of oligomer II. Oligomer IV had only a slight effect on VSV protein synthesis even at 150 μM.

The effects of a 150 μM aliquot of each oligodeoxyribonucleoside methylphosphonate on protein synthesis in mock-infected cells were also determined by gel electrophoresis as shown in Figure 3 for oligomer I. Similar results were obtained when mock-infected cells were treated with oligomers II–IV. In addition, the total TCA-precipitable counts in mock-infected cells were determined in the presence and absence of oligomers.

Table III: Effects of Oligodeoxyribonucleoside Methylphosphonates on VSV Protein Synthesis in Mouse L Cells[a]

oligonucleoside methylphosphonate	concn (μM)	% inhibition[b] L	G	NS	N	M
d-Ap*ACAGACAT* (I)	50	4	+3	7	12	13
	100	12	2	12	9	27
	150	69	73	57	67	63
d-Ap*TTATCCAT* (II)	50	20	4	7	4	9
	100	38	31	26	29	38
	150	59	42	43	47	46
d-Gp*CACTTCAT* (III)	50	35	25	45	15	10
	100	94	88	90	80	63
	150	96	94	97	99	92
d-Tp*TTTTTTTT* (IV)	50	14	10	9	20	5
	100	15	13	17	22	11
	150	16	30	8	25	15

[a] Average of two or three experiments with a range of ±5%. [b] Plus sign indicates stimulation of protein synthesis.

Table IV: Effects of Oligodeoxyribonucleoside Methylphosphonates on VSV Production[a]

oligonucleoside methylphosphonate	concn (μM)	log reduction (pfu/mL)[b] at hours postinfection 6	24
d-Ap*ACAGACAT* (I)	50	0.07	0.07
	100	0.40	0.07
	150	1.08	0.62
d-Ap*TTATCCAT* (II)	50	0.08	0.10
	100	0.36	0.20
	150	0.52	0.39
d-Gp*CACTTCAT* (III)	50	0.40	+0.02
	100	1.18	0.79
	150	1.52	1.02
d-Tp*TTTTTTTT* (IV)	50	0.10	0.07
	100	0.12	0.09
	150	0.15	0.07

[a] Average of two or three experiments with a range of ±0.03 log reduction (pfu/mL). [b] Plus sign indicates stimulation of VSV production.

The results of these studies showed that none of the methylphosphonate oligomers affected protein synthesis in mock-infected cells (data not shown).

Effects of Oligonucleoside Methylphosphonates on VSV Production. Confluent mouse L929 cells were infected with VSV after preincubating with oligomer for 16 h. The amount of virus released into the cell culture media at 6- and 24-h postinfection was determined by plaque assay. The results are shown in Table IV. The effects of oligomers I–III on VSV production are concentration dependent and appear to parallel the effects of these oligomers on VSV protein synthesis in cultured cells. For example, oligomer III inhibits VSV production to the greatest extent while oligomer IV has very little effect on VSV production. Oligomers I–III have a greater effect on VSV production at 6-h postinfection than at 24-h postinfection.

Effects of d-GpCACTTCAT on Cell Growth. Monolayers of mouse L929 cells were treated with 150 μM d-Gp*CACTTCAT* for 40 h at 37 °C. Treated and untreated cells were counted. No difference in cell counts was detected in treated and untreated cells. The plating efficiencies, which are sensitive indicators of cell growth, were also determined. Untreated cells and cells treated with oligomer III had plating efficiencies of 13–21% and 12–20%, respectively, when 100–300 cells/mL were plated. The plating efficiencies increased as the cell number increased. These results show that 150 μM oligomer III did not have any effect on the colony

formation by mouse L929 cells.

Discussion

Studies on the cell-free translation of globin mRNA in rabbit reticulocyte lysates showed that oligodeoxyribonucleotides and oligodeoxyribonucleoside methylphosphonates complementary to the initiation codon region of the mRNA inhibited translation (Blake et al., 1985a,b). These oligomers, which presumably inhibit the initiation step of translation, are more effective in inhibiting protein synthesis than oligomers complementary to the coding region of globin mRNA. The oligodeoxyribonucleoside methylphosphonates used in the present study are designed to bind to the initiation codon regions of VSV N, NS, or G mRNA. These methylphosphonate oligomers, which contain nine nucleoside units, are completely complementary only to their target mRNAs (Table I) and are expected to selectively inhibit translation of their respective mRNAs.

The effects of the methylphosphonate oligomers on translation of VSV mRNA and globin mRNA were studied in a cell-free rabbit reticulocyte system. The VSV mRNA was prepared from VSV-infected mouse L cells which had been treated with actinomycin D to prevent host cell mRNA transcription. In agreement with previous studies in cell-free systems (Rose & Lodish, 1976; Ball & White, 1976; Preston & Szilagyi, 1977), we found that only N, NS, and M proteins were synthesized in significant amounts in the lysate (Figure 2). The lack of L protein synthesis may be due to the low amount of L mRNA in the VSV mRNA preparation. The amount of L mRNA is estimated to be 20-fold less than N mRNA (Villareal et al., 1976). Since G protein is membrane bound during its biogenesis, only a small amount of its unglycosylated precursor is synthesized in the lysate system. Synthesis of this protein in cell-free systems occurs only when microsomal membranes are added (Toneguzzo & Ghosh, 1977).

The synthesis of N protein was inhibited in a specific manner by d-Ap*ACAGACAT* (I), which is complementary to N mRNA (Table II). This oligomer had little or no inhibitory effect on globin mRNA translation. The observed inhibition of NS and M protein synthesis in the presence of 150 μM oligomer I may be due to formation of partial duplexes between oligomer I and NS and mRNAs. As shown in Table I, oligomer I could potentially form duplexes containing five contiguous base pairs with the coding regions of NS and M mRNA. Such partial duplex formation is more likely to occur at high oligomer concentration. Previous results have shown that a tetramer, d-*CCAT*, at 200 μM can inhibit globin mRNA translation in a reticulocyte cell-free system (Blake et al., 1985b). Thus, partial duplexes formed between oligomer I and NS and M mRNAs may be sufficiently stable at high oligomer concentrations to inhibit translation of these messages.

Oligomer II, d-Ap*TTATCCAT*, showed less specific inhibition of VSV mRNA translation than oligomer I. This oligomer inhibits both NS and N protein synthesis in the concentration range 100–150 μM. Although oligomer II is completely complementary only to the initiation codon region of NS mRNA, it could form a partial duplex containing seven contiguous base pairs with the coding region of N mRNA. The observation that oligomer II inhibits N protein synthesis to a somewhat greater extent than NS protein synthesis could reflect the relative binding strengths of the oligomer for the binding sites on the N and NS mRNAs. As previously demonstrated in our studies with globin mRNA (Blake et al., 1985b; Murakami et al., 1985), methylphosphonate oligomer binding is influenced by the secondary structure of the mRNA. Thus, it is possible that the partial oligomer binding site for oligomer II on N mRNA is more exposed than is the complete binding site of the NS mRNA. The differences in accessibility of the oligomer binding sites could account for the similar extent of inhibition of both N and NS protein synthesis by oligomer II.

Oligomer III, which is complementary to G protein mRNA, did not affect NS or M protein synthesis and had only a small inhibitory effect on N protein synthesis. This inhibition, however, appeared to be independent of oligomer concentration. Since G mRNA is not translated to a detectable extent, it was not possible to determine the effect of oligomer III on G protein synthesis. Both oligomers II and III, in contrast to oligomer I, stimulated globin protein synthesis in a concentration-dependent manner. The reason for this stimulatory effect is not clear at this time. However, stimulation of protein synthesis has been observed by others when translations were carried out in the presence of oligodeoxyribonucleotides (Zamecnik & Stephenson, 1978; Blake et al., 1985a).

In summary, the results from the cell-free experiments indicate that the oligomers only inhibit the translation of VSV mRNAs. The effects of the oligomers on the translation of globin mRNA were either neutral or stimulatory. The specificity of inhibition of VSV mRNA translation is dependent upon the sequence of the oligomer. For example, at 100 μM, oligomer I targeted toward N mRNA specifically inhibited N protein synthesis. However, oligomer II, which is targeted toward NS mRNA, appeared to inhibit both NS and N protein synthesis. Oligomer III, targeted toward G mRNA, had little effect on the translation of NS, N, or M mRNA. This complex situation of specificity can be interpreted from the analysis of sequence complementarity shown in Table I. This analysis and the experimental data support the conclusion that the inhibition of translation of VSV mRNAs by these oligomers is due to duplex formation between the oligomers and the viral mRNAs.

Oligomers I–III each effectively inhibited VSV protein synthesis in a concentration-dependent manner in VSV-infected mouse L cells (Figure 3 and Table III). To allow maximum uptake of the oligomers by the L cells, the cells were preincubated with the oligomers 16 h prior to infection. The viral proteins were labeled 6-h postinfection, a time when most cellular protein synthesis has ceased. Each oligomer inhibited the synthesis of all five viral proteins in infected cells. In addition, in the presence of oligomer, the level of cellular protein synthesis increased as the level of viral protein synthesis decreased (Figure 3). This result and the observation that the oligomers had no detectable effects on cellular protein synthesis in uninfected cells, even at concentrations of 150 μM, suggest that the oligomers are able to exert a specific inhibitory effect on viral protein synthesis.

The oligomers appeared to have the greatest inhibitory effect at concentrations of 100 μM or above, although oligomer III did give significant inhibition at a concentration of 50 μM. The different amounts of inhibition produced by the different oligomers at the same concentration suggest that the inhibition is dependent upon the sequence of the methylphosphonate oligomer and not on some general inhibitory property of the methylphosphonate oligomers. Thus, the data are consistent with the interpretation that inhibition is due to interaction between the oligomer and the VSV mRNA. The data on the effect of d-Tp*TTTTTTTT* (IV) on VSV protein synthesis support this interpretation. Previous results have shown that oligothymidine methylphosphonates form duplexes of low

stability with poly(rA) (Miller et al., 1979). Therefore, this oligomer would not be expected to interact with oligo(A) regions of viral or cellular RNAs. As shown in Table III, oligomer IV has only a small inhibitory effect on VSV protein synthesis over the concentration range 50–150 μM. In addition, oligomer IV did not have any detectable effect on protein synthesis in mock-infected mouse L cells. These results demonstrate that inhibition of viral protein synthesis is not due to the mere presence of a methylphosphonate oligomer or its metabolic products.

Although oligomers I–III gave selective inhibition of VSV mRNA translation in vitro, each oligomer inhibited VSV protein synthesis of all five VSV proteins in VSV-infected mouse L cells. This result may reflect the role of the viral proteins in virus function. It is known that the target mRNAs for oligomers I and II both code for proteins involved in virus replication or transcription. For example, N protein is bound to viral RNA and is required for replication of VSV RNA (Patton et al., 1984). Although N protein is not required for the primary transcription step, initial inhibition of N protein synthesis by oligomer I could result in an overall decrease in the synthesis of plus- and minus-strand viral RNA. Minus-strand RNA serves as a template for subsequent rounds of mRNA synthesis or secondary transcription. Thus, initial inhibition of N protein synthesis by oligomer I could lead to a reduction in the levels of all five virus mRNAs and proteins. On the basis of the in vitro translation results (Table II), oligomer II would be expected to inhibit both N and NS protein synthesis. Since NS protein is part of viral transcriptase (Emerson & Yu, 1975), reduction in levels of this protein could directly lead to reduced levels of VSV mRNAs and thus inhibition of all five viral proteins. This type of complex involvement is probably not an explanation for the observed inhibition of VSV protein synthesis by oligomer III which is targeted toward G protein mRNA. This glycoprotein is responsible for binding virus to susceptible host cells, induces uptake of the virus by the cell (Bishop et al., 1975), and facilitates budding during virus maturation (Lodish & Porter, 1980). This protein is not known to play any direct role in virus RNA or protein synthesis.

Although oligomers I–III are specifically targeted to N, NS, and G proteins, respectively, examination of the data in Table I reveals that seven or eight contiguous bases of each oligomer are complementary to the coding regions of L protein mRNA. This rather extensive complementary could result in the formation of stable, partial duplexes between each of these oligomers and L mRNA with subsequent inhibition of L protein synthesis. As described previously, it was not possible to test the effects of these oligomers on L mRNA translation in the cell-free system. However, since L protein constitutes a part of the viral transcriptase (Emerson & Yu, 1975), inhibition of its synthesis would be expected to lead to overall inhibition of virus mRNA and protein synthesis.

Oligomers I–III could directly inhibit virus replication by interacting with complementary sequences on the full-length plus-strand transcript initially produced after virus infection. Such interactions would reduce the number of minus-strand RNAs which serve as templates for mRNA synthesis during secondary transcription.

The effects of oligomers I–IV on VSV production were examined at two points during the infectious cycle, 6- and 24-h postinfection. Inhibition is greater at 6-h postinfection, a period of rapid virus production, than at 24-h postinfection, a period when the infectious cycle is complete and virus production has plateaued (Grosch & Erickson, 1975). These methylphosphonate oligomers exert sequence-specific inhibitory effects on VSV production at both time points (Table IV). For example, at 6-h postinfection and at 150 μM, oligomer III targeted toward G mRNA is most effective (1.52 log reduction), oligomer I targeted toward N mRNA is next (1.08 log reduction), and oligomer II targeted toward NS mRNA is less (0.52 log reduction), while oligomer IV [oligo(T)] is hardly effective (0.13 log reduction). These results paralleled the results obtained from the studies on the effects of the oligomers on VSV protein synthesis in infected cells. The strict correspondence of these two effects clearly supports the conclusion that the reduction of virus yield by these oligomers is due to their specific inhibition of the VSV protein synthesis inside the infected cells. It is noteworthy that these oligomers had no effects on protein synthesis in uninfected mouse L cells and, correspondingly, the growth of L cells at 150 μM. It is likely that some duplex formation of cellular RNA with these methylphosphonate oligomers may take place but these duplexes may be small in percentage as compared to the total cellular RNA comprising many species. Thus, inactivation of a small percentage of cellular RNA of many species may have little effect on cellular protein synthesis and cell growth. This reasoning also favors the interpretation that general inhibitory effects of a given oligomer on viral protein synthesis are less likely related to formation of short duplexes of five to six contiguous bases with the viral mRNAs, but are more likely due to the multiple and close involvement of all the viral genes and gene products during the infection process.

The above results establish a causal relationship between hybridization arrest of VSV mRNA translation by oligodeoxyribonucleoside methylphosphonates in a cell-free system and their inhibitory effects on VSV protein synthesis and virus production in VSV-infected mouse L cells. These results further demonstrate that virus vs. host cell function can be selectively controlled by sequence-specific methylphosphonate oligomers. However, it is obvious that certain obstacles must be overcome before this technique can be used to study and/or control either viral or cellular gene expression at the mRNA level in an unambiguous manner.

The present studies and previous studies on globin mRNA translation (Blake et al., 1985a) show that rather high concentrations of oligomers in the chain-length range of octamer to dodecamer are requied in order to obtain significant levels of translation inhibition in cell-free systems. This is due to the equilibrium nature of the duplex formation between the oligomers and the target mRNAs. In considering the nature of the physical binding process, two important issues are self-evident: (1) Thermodynamic considerations indicate that at low binding levels, the association process predominates and oligomer binding is efficient, while at high binding levels the opposite occurs. Thus, physical binding above the 50% level is much less efficient and approaches zero as the binding approaches 100%. At high concentrations of oligomer, the tendency for partial duplex formation with other short sequences increases. Thus, if high concentrations of oligomer are required to significantly inhibit translation of the target mRNA, the inhibition of translation of other mRNAs is likely to occur. (2) Due to the on–off nature of the physical binding process, inhibition of viral mRNA function by the bound oligomer is only temporary. Processes for degrading mRNAs in the cell also exist. Therefore, these degradative processes could permanently destroy mRNA whose function has been temporarily inhibited by the bound oligomer.

These two considerations clearly indicate that the efficiency and specificity of inhibition of target mRNA function could

be increased significantly if duplex formation also leads to permanent and immediate destruction of the target mRNA. In addition, it would be desirable to have a direct demonstration of the specificity of duplex formation between the oligomer and the target RNA. Our laboratory is currently working toward these goals.

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