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Radiation Inactivation Analysis of Influenza Virus Reveals Different Target Sizes for Fusion, Leakage, and Neuraminidase Activities

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ABSTRACT: The size of the functional units responsible for several activities carried out by the influenza virus envelope glycoproteins was determined by radiation inactivation analysis. Neuraminidase activity, which resides in the glycoprotein NA, was inactivated exponentially with an increasing radiation dose, yielding a target size of 94 ± 5 kilodaltons (kDa), in reasonable agreement with that of the disulfide-bonded dimer (120 kDa). All the other activities studied are properties of the HA glycoprotein and were normalized to the known molecular weight of the neuraminidase dimer. Virus-induced fusion activity was measured by two phospholipid dilution assays: (i) relief of energy transfer between *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (N-NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-dioleoyl-L-α-phosphatidylethanolamine (N-Rh-PE) in target liposomes and (ii) relief of self-quenching of N-Rh-PE in target liposomes. Radiation inactivation of fusion activity proceeded exponentially with radiation dose, yielding normalized target sizes of 68 ± 6 kDa by assay i and 70 ± 4 kDa by assay ii. These values are close to the molecular weight of a single disulfide-bonded (HA_1 + HA_2) unit (75 kDa), the "monomer" of the HA trimer. A single monomer is thus inactivated by each radiation event, and each monomer (or some part of it) constitutes a minimal functional unit capable of mediating fusion. Virus-induced leakage of calcein from target liposomes and virus-induced leakage of hemoglobin from erythrocytes (hemolysis) both showed more complex inactivation behavior: a pronounced shoulder was present in both inactivation curves, followed by a steep drop in activity at higher radiation levels. These curves showed that virus-induced leakage is mediated by a larger and more complex functional unit than is required for the prerequisite fusion process, for which the minimum functional unit is a single monomer.

Influenza is an enveloped virus that carries two transmembrane glycoproteins called HA and NA in its lipid membrane (Nayak, 1977). The predominant one, HA, is a trimer consisting of three identical subunits of ca. 75 kDa held together by noncovalent interactions. Each subunit consists of two disulfide-linked polypeptide chains, HA_1 and HA_2, of ca. 50 and 25 kDa, respectively. The X-ray crystal structure of the extracellular portion of HA, comprising over 90% of the molecule, has been determined to 3-Å resolution (Wilson et al., 1981). The second influenza glycoprotein, NA, is a tetramer of subunits of ca. 60 kDa. The X-ray crystal structure of its external portion has been determined to 2.9-Å resolution (Varghese et al., 1983). About 90% of total influenza glycoprotein is HA, and 10% is NA (Nayak, 1977).

HA is the sole influenza protein to catalyze fusion reactions, which the virus undergoes readily at pHs below 6.0 (White et al., 1983). Fusion of the viral envelope with a cellular membrane at low pH is an essential step in the viral infectious cycle and is a necessary prerequisite of virus-induced membrane leakage reactions (White et al., 1983; Lenard & Miller, 1983). HA also functions to bind influenza virions to target cells, through a sialic acid binding site on each monomer (Wilson et al., 1981; White et al., 1983). Binding is independent of the fusion function, since specific proteolytic activity of the HA precursor, HA_0, is absolutely required for fusion (and hence for leakage), but not for hemagglutination (White et al., 1983). NA possesses a readily measurable neuraminidase activity, which may be important in multicycle infections (Nayak, 1977).

An important unanswered question is how many HA molecules constitute a minimum functional unit for fusion. This paper reports results from a radiation inactivation study carried out to answer this question. Radiation inactivation has been successfully used to determine the functional target size and, from this, the molecular weight of a variety of both soluble and membrane-bound enzymes in a frozen, hydrated state (Kempner & Schlegel, 1979; Jung, 1984). In this paper we report different target sizes for the inactivation of two HA-catalyzed functions: a surprisingly small target size is calculated for fusion, while leakage assays reveal a much larger and more complex functional unit. A convenient internal reference for these determinations was provided by measuring the target size for the neuraminidase activity of NA.

Materials and Methods

Influenza Virus. Strain APR/8/34 was grown in 10- to 11-day-old embryonated eggs and purified on 5–40% potassium tartrate gradients.

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Chemicals. Cardiolipin, the sodium salt from bovine heart, and calcein were purchased from the Sigma Chemical Co. *N*-(Lissamine rhodamine B sulfonyl)dioleoylphosphatidylethanolamine (N-Rh-PE)[1] was obtained from Avanti Polar Lipids, Inc. *N*-(7-Nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine (N-NBD-PE) was from Molecular probes, Inc., and brain gangliosides (lyophilized Folch extract) were from Supelco. All other chemicals were of the highest purity available.

Radiation Inactivation. The virus suspension was dialyzed into 0.1 M NaCl, 0.01 M Tris, and 0.001 M EDTA, pH 8.0, and the viral protein concentration adjusted to ca. 1 mg/mL. The suspension was plated into open aluminum trays to a depth of 0.5–0.6 mm and frozen at −80 °C. The frozen samples were irradiated in a closed chamber under flowing liquid nitrogen vapor with a Van de Graaff generator, using a 1.5-MeV electron beam at a dose of 2 Mrd/min. The temperature was maintained at −45 to −50 °C during irradiation. Radiation doses were determined at sample irradiation temperature using Blue Cellophane film (Du Pont MSC-300) calibrated against a Fricke dosimeter as described previously (Jung et al., 1980).

Target Size Analysis. In those cases where radiation inactivation followed the exponential relationship predicted by a "single-hit–single-target" model (Kempner & Schlegel, 1979; Jung, 1984), the empirical relationship of Kepner and Macey (1968)

$$M_r = 6.4 \times 10^5 / D_{37}$$

was used, where D_{37} is the dose in megarads resulting in 37% residual activity and M_r is the molecular mass of the target in daltons. Data were fitted by unweighted least-squares regression analysis. The best fit line was not arbitrarily constrained to pass through the 100% activity–zero dosage intercept.

When assays were performed for either calcein leakage or hemolysis, a pronounced shoulder was seen in the inactivation curves, indicating a general "multiple-target–multiple-hit" mechanism of radiation inactivation (Oliver & Shepstone, 1964). The generalized equation describing this inactivation can be simplified on the assumption of a "multiple-target–single-hit" or a "single-target–multiple-hit" model (Oliver & Shepstone, 1964). The best fit of the data to these models was obtained by a Simplex algorithm (Nedler & Mead, 1965).

Preparation of Liposomes. Cardiolipin in ethanol (4.8 mg/mL) and ethanolic solutions of the fluorescent phospholipid probes to be used were mixed in the desired proportions and injected into 25 volumes of buffer (140 mM NaCl, 10 mM sodium citrate, 5 mM HEPES, 0.1 mM EDTA, pH 5.0) and then adjusted when necessary to pH 7.0 (Batzri & Korn, 1973). The resulting small unilamellar vesicles were used the same day.

Fusion Assay Using Resonance Energy Transfer. The procedure was originally described by Struck et al. (1981) and applied to influenza virus fusion by Stegmann et al. (1985). Portions of 1.0 mol % each of N-NBD-PE and N-Rh-PE were incorporated into small unilamellar cardiolipin vesicles prepared as described above. In most studies the liposomes and viruses were mixed, incubated at 37 °C for 15 min, and cooled to room temperature, and the end point fluorescence (total increase in donor emission) was measured. Fusion activity was expressed as a fraction of the donor emission measured after addition of 0.5% (v/v) Triton X-100 to the mixture. The kinetics of fusion were followed continuously by first determining the fluorescence base-line levels of the target liposomes alone and then adding the virus suspension directly to the cuvette in a small volume. Excitation and emission wavelengths were 465 and 530 nm, respectively.

Fusion Assay Using Relief of Self-Quenching of N-Rh-PE. In this assay 10 mol % of N-Rh-PE was incorporated into the cardiolipin vesicles. At this concentration, the probe was 90% quenched. During fusion, membrane dilution results in a linear increase in fluorescence due to unquenching. Excitation and emission wavelengths were 570 and 588 nm, respectively.

Calcein Leakage from Liposomes. The procedure used was that described by Stegmann et al. (1981). Liposomes for this assay were prepared as described above, with the following exceptions: (i) buffer contained 100 mM calcein; (ii) sample was frozen at −80 °C for 1 h after ethanol injection of cardiolipin to produce large unilamellar vesicles (Pick, 1981); (iii) vesicles were freed of nonencapsulated calcein by gel filtration on a Sephadex G-50 column. Excitation and emission wavelengths were 490 and 530 nm, respectively.

Hemolysis. Hemolysis of human red blood cells was measured as previously described (Lenard & Miller, 1981), using a citrate–phosphate buffer system (Gomori, 1955) at pH 5.0.

Neuraminidase Activity. Hydrolysis of the synthetic substrate neuramin-lactose was determined fluorometrically by measuring the NADH produced by coupled reactions of the lactose reaction product with β-galactosidase and galactose dehydrogenase. A lactose/galactose test kit (Boehringer Mannheim) was used as described by Cabezas et al. (1983).

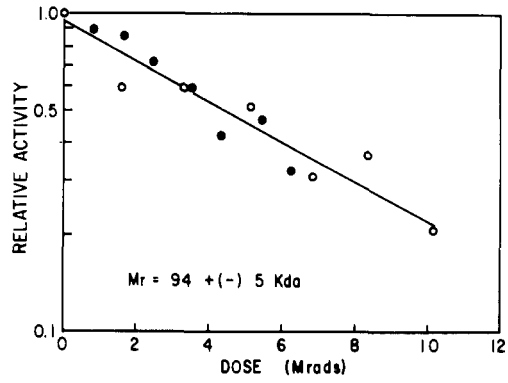

FIGURE 1: Radiation-induced loss of neuraminidase activity from influenza virions. Results of two experiments are shown.

RESULTS

The inactivation of the neuraminidase activity associated with the viral glycoprotein NA was determined as an initial test of the suitability of intact influenza virions for radiation inactivation studies and as an internal molecular weight standard. Activity decayed exponentially with radiation dose (Figure 1), yielding a target molecular weight of 94 ± 5 kDa, a value fairly close to the molecular weight of a dimer of NA. This finding implies that the four subunits of a native NA molecule are not strictly equivalent in their interactions with each other. It has been suggested that dimers may be formed by disulfide bonding within the tetramer (Varghese et al., 1983) and disulfide bonds are efficient transmitters of destructive radiation energy (Haigler et al., 1985). This finding therefore places an upper limit of two monomers on the size of the minimum functional unit required for neuraminidase activity. It does not rule out the possibility that one monomer may be sufficient for activity, since an entire disulfide-bonded dimer is likely destroyed by each single radiation hit.

[1] Abbreviations: N-Rh-PE, *N*-(lissamine rhodamine B sulfonyl)-dioleoyl-L-α-phosphatidylethanolamine; N-NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)dipalmitoyl-L-α-phosphatidylethanolamine.

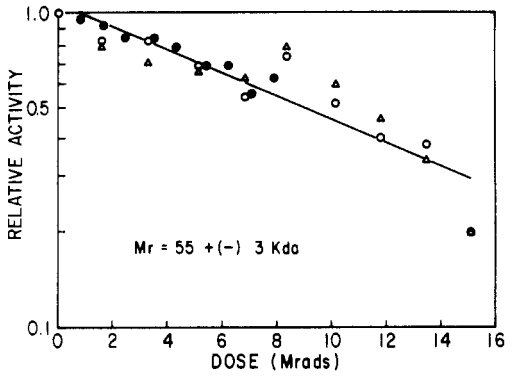

FIGURE 2: Radiation-induced loss of fusion activity from influenza virions, as measured by relief of resonance energy transfer between NBD-PE and N-Rh-PE in target liposomes: (●) experiment 1; (○, △) experiment 2; (▲) 10 mol % brain gangliosides present in target liposomes.

Table I: Calculated Target Sizes for Influenza Virus Envelope-Related Activities

assay	targets per functional unit[a]	target $M_r \times 10^{-3}$
neuraminidase	1[b]	94 ± 5[c] (120[d])
fusion: resonance energy transfer	1	55 ± 3 (70)
fusion: relief of self-quenching	1	53 ± 5 (68)
leakage of calcein	7–8[e]	~300[e] (388)
	3–4[f,g]	~350[f] (447)
hemolysis	6–7[e]	~335[e] (428)
	3–4[f,g]	~383[f] (489)

[a] Assuming single-hit inactivation. [b] Monomer or dimer could be functional unit. [c] Mean ± standard deviation. [d] Basis for normalized values (in parentheses). [e] Assuming single-hit–multiple-target model. [f] Assuming multiple-hit–single-target model. [g] Hits required to inactivate a single target.

Fusion of influenza virions with cardiolipin liposomes was measured in two ways: by relief of resonance energy transfer between N-NBD-PE and N-Rh-PE incorporated into target liposomes and by relief of self-quenching of N-Rh-PE in target liposomes. A target of 55 ± 3 kDa was found by relief of resonance energy transfer (Figure 2), while a target of 53 ± 5 kDa was obtained by relief of self-quenching (not shown). These values became 70 ± 4 and 68 ± 6 kDa, respectively, after normalization to the known molecular weight of the neuraminidase dimer (Table I). These values are in reasonable agreement with the 75-kDa molecular weight of the minimum structural unit of HA, the disulfide-bonded (HA_1 + HA_2) subunit, or "monomer", of the native trimeric HA molecule. The monomer, or some part of it, thus constitutes the minimum functional unit required for virus-mediated fusion.

This finding implies that a single subunit of the trimeric HA structure is inactivated by a single radiation event, without damage to the other two subunits of the native molecule. Similar behavior was observed by Kempner and Miller in a study of the noncovalently stabilized hexameric enzyme glutamate dehydrogenase (Kempner & Miller, 1983). In that study the target size for enzyme inactivation corresponded to the hexamer, while extrinsic fluorescence and the presence of the monomeric polypeptide chain on gels were both lost, with a target size corresponding to a monomer. We have been unable to measure the target size for loss of HA protein directly by its disappearance from gels, as done by Kempner and Miller (1983), because both HA_1 and HA_2 band broadly, stain poorly, and are often incompletely resolved from internal viral proteins. The finding of a target size corresponding to a minimal structural unit by functional assay, however, makes a determination by disappearance from gels unnecessary.

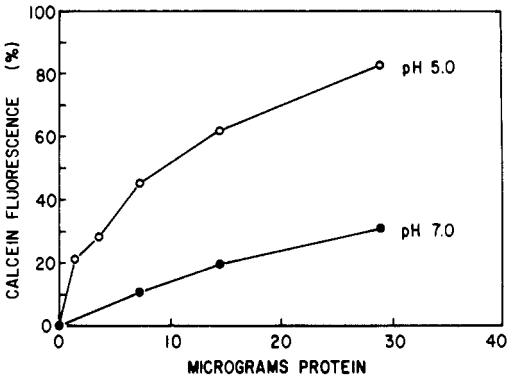

FIGURE 3: Influenza virus induced leakage of calcein from target liposomes at pH 5.0 and 7.0, as a function of the amount of added influenza viral protein.

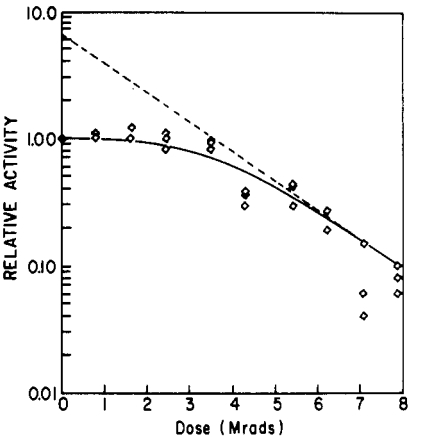

FIGURE 4: Radiation-induced loss of influenza virus mediated calcein leakage from liposomes at pH 5.0.

The data in Figure 2 were obtained by measuring end points of fusion, after 15-min incubation at 37 °C. This result is not affected if initial rate is measured instead, since this remained constant relative to end point at 0.83 ± 0.25%/s at 22 °C, regardless of radiation dose or final activity level. The end point was generally used for reasons of convenience and accuracy.

The presence of 10 mol % brain gangliosides to serve as viral receptors had no effect on the apparent target size (Figure 2). The use of large cardiolipin vesicles, obtained by freezing and thawing the standard preparation [which resulted in a doubling of OD_{340} of the liposome preparation due to light scattering (Pick, 1981)] slowed the rate of fusion significantly but did not affect the end point (data not shown).

The virus-induced leakage of calcein from cardiolipin liposomes was studied in order to compare the target size for this activity with that observed for fusion. A nonlinear dependence of leakage on added influenza protein was found at pH 5.0 (Figure 3); this was used as a standard curve to determine the extent of radiation-induced inactivation. We could not repeat the observation of Stegmann et al. (1985) of extensive virus-induced leakage at pH 7.0 (Figure 3).

The radiation inactivation curve for calcein leakage from liposomes was characterized by a pronounced shoulder followed by a steep drop (Figure 4). A very similar inactivation curve was obtained for hemolysis activity, which also measures virus-induced leakage (Figure 5). Leakage from liposomes and hemolysis thus display very similar inactivation behavior, suggesting that these activities are mediated by similar functional units. Figures 4 and 5 are both in marked contrast,

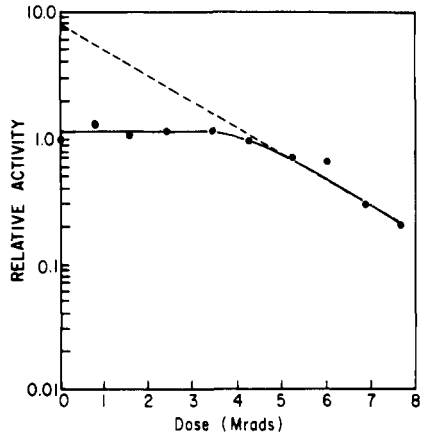

FIGURE 5: Radiation-induced loss of hemolysis activity from influenza virions.

however, with the shallower exponential inactivation of fusion activity (Figure 2), suggesting that leakage and fusion are mediated by quite different functional units.

The shape of the curves in Figures 4 and 5 may be analyzed in terms of a generalized multiple-hit–multiple-target model, in which the functional unit is assumed to consist of m targets of identical size, each of which requires n radiation-induced events for inactivation (Kempner & Schlegel, 1979). Many different values of m and n can generally be found that provide a reasonable fit to the experimental data, so that further structural information cannot generally be obtained from the inactivation curves alone. Two special cases of the general equation have been developed, however: the multiple-target–single-hit model ($n = 1$); the single-target–multiple-hit model ($m = 1$). Values calculated from the data in Figures 4 and 5 for these two special cases are listed in Table I. Of these, the single-target–multiple-hit model provides the more plausible interpretation, with a single target of ca. 400 kDa requiring 3–4 hits for inactivation. Assuming this model, each hit inactivates a portion of the target not too different in size from that of the HA monomer, which is known from Figure 2 to be the unit that is inactivated by a single radiation event.

Discussion

Two surprising and unexpected findings are reported in this paper. The first is that the smallest functional unit capable of mediating fusion—defined by the mixing of lipids between viruses and target liposomes—is also the smallest structural unit, a single monomer of the trimeric HA "spike". It has commonly been supposed that an ensemble of HA molecules is required for fusion, although published evidence supporting this point of view in a defined liposome system seems to be entirely lacking. The finding that the smallest structural unit is also the minimum functional unit serves to simplify and focus the problem of the mechanism of fusion; possible complexities arising from interactions between the individual structural units to form a functional unit need not be considered.

The HA trimer undergoes profound conformational changes when its environment is changed from neutral to acidic pH (Skehel et al., 1982; Doms et al., 1985), and it has been suggested that interactions between the monomers of each trimer may be weakened or even abolished altogether at low pH (Doms et al., 1985). The present findings are consistent with these observations in that any interactions between monomers that may remain at low pH are irrelevant to the fusion function.

The second surprising finding is that the functional unit that mediates membrane leakage is quite different from that which mediates membrane fusion. The size of the functional unit mediating calcein leakage from liposomes is closely similar to that mediating hemolysis. Although the size and complexity of the functional unit calculated from the data depend on the inactivation model assumed, it is clear that the functional unit for leakage is substantially larger and more complex than that for fusion. Leakage may well be medited by nonspecific aggregates of monomers existing under suitable conditions at the low pH required for fusion.

Previous work has shown that fusion by enveloped viruses can be dissociated from leakage. Carefully prepared viruses of various kinds are fusogenic and infectious, but are not hemolytic (Homma et al., 1976; Shimizu et al., 1976; Vaananen & Kaariainen, 1979). Such viral preparations can invariably be rendered leaky by various insults such as freezing and thawing, brief sonication, aging, or high-speed centrifugation (Homma et al., 1976; Shimizu et al., 1976; Vaananen & Kaariainen, 1979; Young et al., 1983). It has consequently been concluded that leakage is a nonspecific result of damage to the viral envelope: the incorporation of such a damaged, leaky membrane into the target membrane by fusion is thought to introduce the holes through which the internal contents of the target can pass. This model predicts that no well-defined target distinct from the fusion target would be found to be associated with leakage; on the contrary, radiation damage should promote leakage rather than inactivating it. The finding that fusion and leakage are inactivated with quite different radiation dependence suggests that leakage is an active process, mediated by an ensemble of viral proteins. How this ensemble is formed or activated by freeze-thawing, sonication, or other effective procedures remains unknown.

Registry No. Neuraminidase, 9001-67-6.

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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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