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Effect of interferon and 2′,5′-oligoadenylates on rotavirus RNA synthesis

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Abstract

Based on the antiviral effect of interferon on rotavirus replication the inhibitory effect of 2′,5′-oligoadenylates on MRNA and double-stranded RNA synthesis was studied using an in vitro assay. The chemically synthesized oligonucleotides were used to determine several characteristics of the inhibitory effect, such as chain length, presence of phosphate residues at the 5′-end, and the 2′,5′-phosphodiester bond itself. In vitro transcription was inhibited by oligos with 5 or more adenine residues at a final concentration of 100 μM or greater. This result makes rotavirus transcriptase different from other viruses in which the inhibitory effects are associated with dinucleotides and trinucleotides. The inhibitory effect was increased when the oligo contained a phosphate residue at the 5′-end; in this case, inhibition was also seen at lower oligo concentrations as well as at shorter oligo chain length. The study of the kinetics of inhibition showed that the inhibition by p(A2′p5′)$_3$3A was competitive with a K_i value of 256 μM. The effect of the oligonucleotides on the in vitro viral RNA replication showed that the 2′,5′-oligoadenylates were not able to significantly inhibit the in vitro rotavirus RNA synthesis. The lack of inhibition in the in vitro assay was very peculiar since RNA transcription and replication involves the viral RNA polymerase, VP1.

Keywords: Interferon; 2′,5′-Oligoadenylate; Rotavirus

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1. Introduction

In interferon-treated cells, the synthesis of several new proteins has been reported, including the 2′,5′-oligoadenylate synthetase that catalyzes the synthesis of 2′,5′-oligoadenylates of different chain lengths (Hovanessian and Kerr, 1979; Yang et al., 1981; Joklik, 1990). The 2′,5′-oligoadenylates have been associated with the activation of ribonuclease L, which has been shown to be directly involved in the degradation of newly synthesized viral mRNA, by interference with the early stages of viral infection (Slattery et al., 1979; Nilsen et al., 1981; Joklik, 1990). Also, the presence of double-stranded RNA in the cytoplasm of viral infected cells has been suggested to be a mechanism for the activation of the oligoadenylate synthetase (Minks et al., 1979; Slattery et al., 1979; Yang et al., 1981). The involvement of the oligoadenylate synthetase and ribonuclease L in the specific degradation of viral mRNA was determined primarily in picornavirus- and reovirus-infected cells (Nilsen et al., 1982; Kumar et al., 1988). In cell free systems wherein replicative intermediates of both viruses were incubated with a interferon-treated HeLa cell extract, viral mRNA was degraded thereby showing the relation between the synthesis of oligoadenylates and the antiviral response (Nilsen and Baglioni, 1979; Baglioni et al., 1984).

2′,5′-Oligoadenylates have been implicated in the suppression of viral transcription and replication by their ability to inhibit the activity of enzymes involved in viral morphogenesis (Henderson et al., 1982; Liu and Owens, 1987; Subramanian et al., 1990; Schroder et al., 1992). The VSV RNA polymerase and topoisomerases are inhibited by different mechanisms since the dependence upon the chain length of the oligo seems to differ for each enzyme (Subramanian et al., 1990; Castora et al., 1991).

In the present paper, we describe the effect of the 2′,5′-oligonucleotides, taking advantage of an in vitro transcription and replication assay for rotavirus RNA synthesis that obviates problems derived from cell uptake and intracellular degradation of the 2′,5′-oligoadenylates.

2. Materials and methods

2.1. Virus and cells

SA-11 virus stocks were a gift from Dr. John Patton. The SA-11 rotavirus strains were grown by infection of MA-104 cells, using m.o.i. of 1–2 as described elsewhere (Sandino et al., 1988). The virus was purified from the cell lysate as previously described (Sandino et al., 1988).

2.2. Preparation of viral particles

Single-shelled particles were obtained by EDTA treatment from purified double-shelled virus particles, as previously described (Cohen et al., 1979; Sandino et al., 1986). Intracellular viral particles with double-stranded RNA synthesis activity were obtained from confluent monolayers of MA-104 cells infected with virus stock at a m.o.i. of 5, at

6 h postinfection. From the infected cell lysates, the replicative subviral particles were then purified as previously described (Patton, 1986). Subviral particles were resuspended in a final volume of 10 μl of HGD buffer (10 mM *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES)-HCl, pH 7.6/10% glycerol/2 mM DTE) per starting tissue culture flask. Particles were used in reactions immediately or stored at −70°C until needed.

2.3. Interferon effect on rotavirus-infected MA-104 cells

MA-104 cells were seeded on 5-cm^2 culture tubes and incubated for 24 h at 37°C in MEM containing 5% fetal bovine serum. Confluent monolayers were washed twice with MEM and pretreated with MEM without serum containing 0, 1000, 2000, 5000 or 10,000 units/ml of IFN-α_{2a} (recombinant human leukocyte interferon-α_{2a}, Roferon A, was provided by Dr. Claudio Blank, Laboratorio Roche, Chile) and incubated for 24 h at 37°C. The cultures were then washed twice with MEM and infected with 10 PFU/cell or 1 PFU/cell of trypsin-activated SA-11 rotavirus (10 μg/ml trypsin, 30-min incubation at 37°C). Following 1-h adsorption at 37°C, the inoculum was removed and replaced with phosphate-free MEM containing 20 μCi/ml [^{32}P]orthophosphoric acid and the same IFN-α_{2a} concentrations as the monolayers had in the preincubation period. The tubes infected with 10 m.o.i. of trypsin-activated SA-11 rotavirus were incubated for 9 h at 37°C, whereas for those tubes infected with 1 m.o.i., the period of incubation was 24 h. At the end of the incubation, the medium was removed and the cells were resuspended in hypotonic buffer (3 mM Tris-HCl, pH 8.1; 0.5 mM $MgCl_2$; 3 mM NaCl), extracted with phenol-chloroform and ethanol precipitated. After overnight incubation at −20°C with ethanol, the viral RNA was obtained by centrifugation at 12,000 *g* for 30 min at 4°C. Rotavirus double-stranded RNA genome segments were analyzed by 7% polyacrylamide gel electrophoresis and autoradiography. Control mock-infected cells treated for 48 h with the same IFN- α_{2a} doses as in the above-described experiment, did not present any cytopathic effect.

2.4. Synthesis of 2′,5′-oligoadenylates

The 5′-monophosphorylated 2′,5′-oligoadenylates used in this study were prepared by the lead ion-catalyzed oligomerization of adenosine 5′-phosphoroimidazolidate as previously described (Torrence et al., 1984). 5′-Triphosphates were made by reaction of the corresponding phosphoroimidazolidate with tri-*n*-butylammonium pyrophosphate (Imai and Torrence, 1985). The 2′,5′-oligoadenylates (lacking a 5′-phosphate moiety) were obtained from commercial sources (Calbiochem, Sigma) and repurified as described previously (Torrence et al., 1984).

2.5. RNA polymerase assay

mRNA synthesis

Purified single-shelled rotavirus particles were assayed for RNA polymerase activity by incubation in a 25-μl reaction mixture containing 120 mM Tris-HCl (pH 8.5), 1 mM

$MgCl_2$, 40 mM NaCl, 0.1 mM *S*-adenosylmethionine, 0.2 mM each ATP, CTP and GTP and 0.24 mM of [^{3}H]UTP (SA: 40 cpm/pmol). The mixture was incubated for 30 min at 45°C. To analyze the transcriptional product, the acid-soluble radioactivity was determined.

Double-stranded RNA synthesis

In the assay to determine the replicase activity, conditions identical to those described above for mRNA synthesis were used, except that [α-^{32}P]UTP (SA: 3000 cpm/pmol) was used instead of tritium-labeled UTP and the *S*-adenosylmethionine was eliminated from the mixture. The reaction was then incubated at 45°C for 60 min. The replicase activity was determined by analysis of the reaction products after phenol extraction and ethanol precipitation by RNA gel electrophoresis in 8% polyacrylamide gels where the presence of the 11 double-stranded RNA segments was determined as described elsewhere (Patton and Gallegos, 1988). Viral particles assayed to measure RNA replication activity were purified from infected cells as described above.

3. Results

The effect of interferon on rotavirus infection was determined by pretreatment of MA-104 cells with different amounts of recombinant human IFN-α_{2a}, before infection of cell monolayers. The interferon effect on rotavirus multiplication was assayed by determining radioactivity associated with the double-stranded viral RNA by gel electrophoresis after labeling the virus RNA with [^{32}P]orthophosphoric acid. As seen in Fig. 1, MA-104 cells were effectively protected, in a concentration-dependent manner, against SA-11 rotavirus infection by IFN, based on the decrease of the viral double-stranded RNA synthesis detected by PAGE. When the cells were infected at an m.o.i. of 10, the effect of interferon was observed at a concentration of 2000 IU/ml and total inhibition at 10,000 IU/ml of IFN. Using this high virus-to-cell m.o.i. the IFN effect was observed as early as 9 h postinfection. At lower m.o.i. (1 PFU/cell) with multiple rounds of infection (24 h.p.i.), the effect of IFN was also evident. This shows that the IFN probably inhibits early steps of rotavirus morphogenesis.

Based on the effect that IFN has on the synthesis of 2′,5′-oligoadenylates, the effect of several 2′,5′-oligoadenylates on in vitro rotavirus transcription was determined using purified single-shelled SA-11 rotavirus particles. The assay involved the addition of different amounts of 2′,5′-oligoadenylates to the reaction mixture, and after 30 min of incubation at 45°C, the amount of RNA synthesized was determined as acid-insoluble radioactivity. The effect on the reaction of the different 2′,5′-oligoadenylates was expressed as the percentage of the RNA polymerase activity compared with a control with no additions. In Fig. 2, the effect of chain length is shown. Non-5′-phosphorylated oligomer containing 4 residues was able to inhibit MRNA synthesis of rotavirus by about 30%, but one with 5 adenosines was more than 50% inhibitory at a concentrations lower than 0.2 mM. Oligos with less than 4 adenosine residues did not inhibit, even at higher concentrations (Fig. 2A). When 2′,5′-oligoadenylates with the 5′-terminal monophosphates were used, an increase of the inhibitory effect was obtained. As seen in

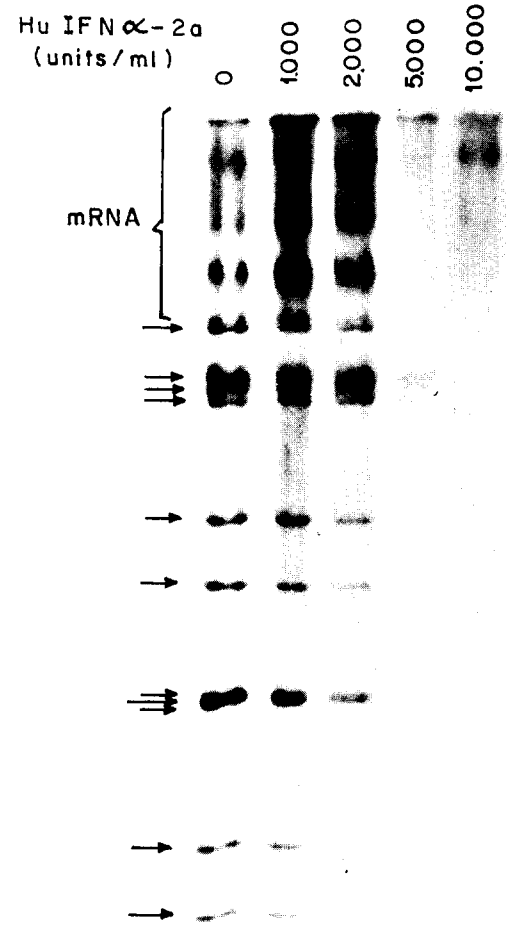
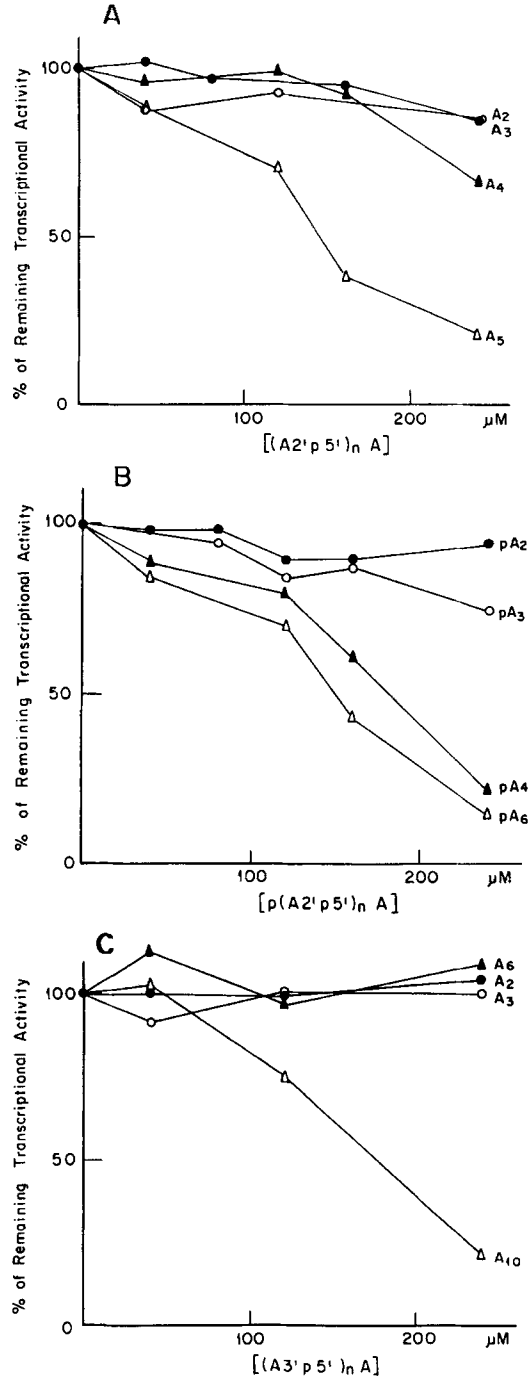


Fig. 1. Inhibition of SA-11 multiplication by interferon. The effect of interferon on rotavirus infection was determined by pretreatment of MA-104 cells for 24 h with 0–10,000 units per ml of recombinant human interferon-α_{2a} Control and IFN-treated cells were infected with rotavirus SA-11 at a m.o.i. of 10, at 9 hours postinfection, the cells were harvested, and the RNA purified and analyzed by PAGE and autoradiography as described in Materials and methods. In the figure, the IFN doses used, as well as the 11 dsRNA segments of the rotavirus genome, are indicated.

Fig. 2B, an 80% inhibition was obtained with the 2′,5′-tetraadenylate 5′-monophosphate as compared with the non-phosphorylated oligo, which at a similar concentration yielded only 35% inhibition. In order to determine the specificity of the inhibitory effect exerted by the 2′,5′-bond in the oligonucleotides, the effect of 3′,5′-oligoadenylates was studied. As seen in Fig. 2C, only oligonucleotides with a chain length over 7 were able to inhibit, and oligos containing less than 6 adenine residues were not inhibitory. In these experiments, an inhibitory effect could be detected at concentrations over 200 μM for a 3′,5′-oligoadenylate of a chain length of 10 residues.


A
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50
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% of Remaining Transcriptional Activity
A2
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B
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50
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% of Remaining Transcriptional Activity
pA2
pA3
pA4
pA6
100
200
μM
[p(A2'p5')n A]
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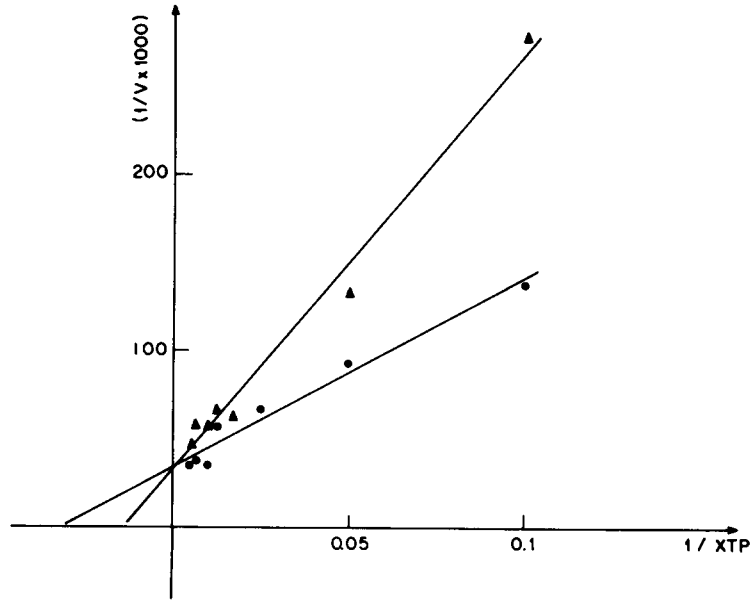

Fig. 3. Kinetics of inhibition of rotavirus mRNA synthesis by 5′-monophosphate 2′,5′-tetraadenylate. Double-reciprocal plot of the reaction rate in the presence of different concentrations of XTP (ATP, CTP, GTP), and with p(A2′p5′)$_3$A. The initial velocities were expressed as cpm of [^{3}H]UMP incorporated into acid-insoluble material. p(A2′p5′)$_3$A concentrations were 0 (●) and 144 μM (▲).

The kinetic parameters of the reaction were studied for the 5′-monophosphate 2′,5′-tetraadenylate. The characteristics of the inhibition were determined using a concentration of the oligo of 144 μM that inhibited over 50% the RNA polymerase activity. In Fig. 3, the Lineweaver–Burk plot indicated that the oligo exerted a competitive mechanism of inhibition with a K_i of 256 μM.

The study of the inhibitory effect exerted by the 2′,5′-oligoadenylates on the viral RNA polymerase, was extended to the ability to inhibit the viral enzyme when catalyzing the synthesis of the double-stranded viral RNA. The only difference between the viral activity involved in mRNA and dsRNA synthesis, is that for the transcription activity, RNA polymerase (VP1) is active in a single-shelled particle that contains the 11

Fig. 2. Inhibition mRNA synthesis catalyzed by single-shelled SA-11 virus particles by 2′,5′-oligoadenylates. Purified single-shelled SA-11 particles were assayed for RNA polymerase activity for 30 min at 45°C. The RNA polymerase activity was determined as incorporation of [^{3}H]UMP into acid-insoluble material in the presence of the indicated amounts of the different 2′,5′-oligoadenylates. The results are expressed as percentage of a control reaction carried out without addition of any oligoadenylate (100% corresponds to 257 pmol of [^{3}H]UMP incorporated into acid-insoluble material). (A) The effect of the addition of oligoadenylates with a non-phosphorylated 5′-end, from 2–5 adenine residues in length. (B) The effect of 2′,5′-oligoadenylates with the 5′-monophosphate end. (C) The effect of the addition of the 3′,5′-oligoadenylates.

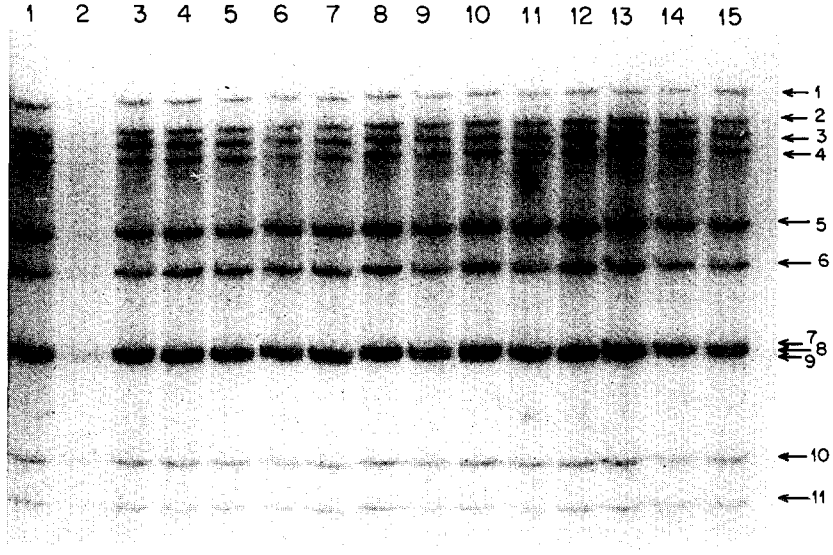

Fig. 4. Effect of the addition of 2′,5′-oligoadenylates on the double-stranded RNA synthesis catalyzed by partially purified SA-11 subviral particles. Partially purified SA-11 subviral particles were assayed for double-stranded RNA synthesis by incubation in a 25 μl reaction mixture containing 150 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 80 mM NaCl, 2 mM each ATP, CTP and GTP and 0.02 mM [α-^{32}P]UTP (SA: 3000 cpm/pmol). The reaction was then incubated at 45°C for 60 min and the reaction products analyzed by gel electrophoresis in 8% polyacrylamide gels and further autoradiography. Lane 1 corresponds to a reaction carried without the addition of any oligoadenylate. Lane 2 is a control where 200 μM of 9(β-D-arabinofuranosyl)adenine (Ara-A) was added. In the rest of the autoradiogram, the oligoadenylates were added according to the following order: 2′,5′-diadenylate (lane 3); 2′,5′-diadenylate-5′-monophosphate (lane 4); 2′,5′-triadenylate (lane 5); 2′,5′-triadenylate-5′-monophosphate (lane 6); 2′,5′-triadenylate-5′-triphosphate (lane 7); 2′,5′-tetraadenylate (lane 8); 2′,5′-tetraadenylate-5′-monophosphate (lane 9); 2′,5′-tetraadenylate-5′-triphosphate (lane 10); 2′,5′-pentaadenylate (lane 11); 2′,5′-pentaadenylate-5′-monophosphate (lane 12); 3′,5′-triadenylate (lane 13); 3′,5′-hexaadenylate (lane 14) and 3′,5′-decaadenylate (lane 15). The arrows indicate the migration of each of the 11 double-stranded RNA segments determined by marker obtained by direct extraction from purified virus particles and stained with silver nitrate.

RNA genes and polypeptides: VP1, VP2, VP3 and VP6 and the particle with replicase activity is made of VP1, VP3 and non-structural NS34, NS35, and NS54 polypeptides and the mRNA. The assay conditions for the replicase activity were identical to those used to determine in vitro viral mRNA synthesis, but the activity was measured by the specific detection of the 11 double-stranded RNA segments when the reaction products were analyzed by RNA gel electrophoresis. As shown in Fig. 4, the subviral particles were able to synthesize the 11 double-stranded RNA segments (lane 1), and the 2′,5′-oligoadenylates at concentrations of 0.6 mM, were unable to inhibit viral RNA replication independent of the chain length or the number of phosphate residues at the 5′-end (lanes 3–12). In this case, 9(β-D-arabinofuranosyl)adenine-5′-triphosphate (Ara-ATP), a powerful inhibitor of rotavirus in vitro replication, was used as a control (lane 2).

4. Discussion

The antiviral effects of the 2′,5′-oligoadenylates include the inhibition of the synthesis of viral RNA and the degradation of viral MRNA as a direct consequence of the activation of a specific endonuclease. The presence in the infected cells of an increased concentration of 2′,5′-oligoadenylates is a direct consequence of the presence of interferon, synthesized in response to viral infection (Nilsen et al., 1981; Slattery et al., 1979; Joklik, 1990). Among the mechanisms involved for the antiviral response to interferon, the direct inhibitory effect of 2′,5′-oligoadenylates on viral enzymes has also been reported. The results of the studies on the inhibition of rotavirus replication by interferon, using different m.o.i. showed that inhibition was dependent on the IFN concentration suggesting that inhibition may involve a direct effect on RNA synthesis such as described above.

Here, the effect of 2′,5′-oligoadenylates on rotavirus mRNA and double-stranded RNA synthesis was studied using an in vitro assay. The chemically synthesized oligonucleotides permitted determination of several characteristics of the inhibitory effect on the in vitro mRNA synthesis, such as chain length, presence of phosphate residues at the 5′-end of the oligo, and the importance of the 2′,5′-bond itself. Under the assay conditions, used inhibitory effect was observed with the oligos containing 4 or more adenylate residues at a final concentration greater than 100 μM. This result was different from those previously reported for other viruses, where the greater inhibitory effects were obtained with shorter 2′,5′-oligonucleotides (Henderson et al., 1982; Liu and Owens, 1987; Subramanian et al., 1990); in those cases the dinucleotides and trinucleotides were highly inhibitory. In the present case, an increased inhibitory effect with the smaller oligo was obtained only when a phosphate residue was present at the 5′-end; in addition, inhibition was seen at lower oligo concentrations. Based on the above results and on the nature of the kinetics of inhibition seen in Fig. 3, where it is shown that the inhibition exerted by the 2′,5′-tetraadenylate-5′-monophosphate is competitive, it could be argued that the 2′,5′-oligoadenylates may affect initiation of the RNA chains. The value of K_i of 256 μM is similar to that described by others (Castora et al., 1991). The inhibitory effect of the oligo depended upon the nature of the phosphodiester bond, since inhibition with 3′,5′-oligoadenylates was observed, but only at higher concentration and with oligos of more of 6 residues in length. Thus, the results argue in favor of a specific effect of the 2′,5′-oligoadenylates and this effect depends upon the chain length.

The study of the effect of the oligonucleotides on the in vitro viral RNA replication showed that the 2′,5′-oligoadenylates are not able to inhibit in the in vitro rotavirus RNA replication assay. The lack of inhibition involves the synthesis of all 11 RNA segments. The lack of inhibition in the in vitro assay is very peculiar, since the RNA polymerase activity involved in both transcription and replication is based on the same viral protein, VP1. The difference in the inhibitory effect of the 2′,5′-oligoadenylates could be related to differences existing between the viral particles involved in transcription and those involved in replication. An additional explanation could be related to the fact that mRNA synthesis catalyzed by the single-shell particle involves both initiation and elongation of the RNA chain. In contrast, in vitro replication seems to involve more

elongation than initiation of new RNA chain. Since most of the RNA synthesizing activity during the in vitro replication assay, is related to elongation of preinitiated chains, the 2′,5′-oligo could then exert a more limited inhibitory effect. In the case of transcription, the viral particle is the single-shelled particle made of central core that contains the viral genome and VP1 (the polymerase), VP2 (RNA binding protein) and VP3 (the guanylyltransferase) and the inner capsid made of the major viral protein, VP6 (Sandino et al., 1986, 1988). On the other hand, the replicase particle is not very well defined in terms of structure, but is made by the 11 mRNA and VP1, VP2 and VP3 plus three non-structural polypeptides NS34, NS35 and NS54 (Helmberger-Jones and Patton, 1986; Sandino et al., 1988; Mansell and Patton, 1990). Thus, it is possible that the difference in the template of the reaction and in the interaction of VP1 with different polypeptides in both particles could be responsible for the different sensitivity to 2′,5′-oligoadenylates.

Interferon exerts an antiviral effect at several levels of viral morphogenesis. In the particular case of rotavirus, the synthesis of 2′,5′-oligoadenylates could be responsible of the inhibition of viral mRNA synthesis, thereby inhibiting viral protein synthesis. However, the antiviral effect of the oligo does not involve the inhibition of viral RNA replication in order to block viral morphogenesis. These considerations do not exclude the possibility that the interferon could inhibit viral replication through any other of the mechanisms described as part of the antiviral response.

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