2′,5′-Oligoadenylates and Related 2′,5′-Oligonucleotide Analogues. 2. Effect on Cellular Proliferation, Protein Synthesis, and Endoribonuclease Activity†

Bronwyn G. Hughes* and Roland K. Robins

ABSTRACT: A number of the new enzymatically synthesized 2′,5′-oligonucleotide trimers, namely, those containing the nucleosides 8-azaadenosine, toyocamycin, sangivamycin, formycin, 8-bromoadenosine, tubercidin, and guanosine, were found to inhibit protein synthesis and cellular proliferation after uptake into intact L and HeLa cells. 2′,5′-Oligonucleotide trimers containing cytidine, inosine, uridine, and 1,N^6-ethenoadenosine had some effect while those containing 2-chloroadenosine, 3-ribosyladenine, ribavirin, and 2-β-D-ribofuranosylthiazole-4-carboxamide had no detectable effect on protein synthesis or cellular proliferation after uptake into L or HeLa cells. All of these 2′,5′-oligonucleotide analogues inhibited protein synthesis in the in vitro rabbit reticulocyte lysate system except for the trimer containing ribavirin. Such nucleoside substitutions have further defined the substrate-specificity requirements for the endoribonuclease and/or the inhibitors for the 2′,5′-phosphodiesterase. Most of the 2′,5′-analogues were degraded in L-cell extracts so the endogenous nucleases are not very specific. The 2′,5′-trimers containing tubercidin and 2-β-D-ribofuranosylthiazole-4-carboxamide were quite stable in comparison to the 2′,5′-A trimer. The inhibition of protein synthesis and cellular proliferation observed correlated well with the degradation of rRNA and polyadenylated mRNA observed after uptake of the 2′,5′-analogues into intact L cells. The degradation of the polyadenylated mRNA appeared to be a more sensitive test than inhibition of cellular protein synthesis for determining biological activities of the 2′,5′-oligonucleotide analogues.

A series of 5′-triphosphate 2′,5′-oligoadenylates, ppp-$(A_{2'p5'})_nA$ (2′,5′-A), are known to be involved in the mechanism of action of interferon [consult Lengyel (1982) for a recent review], and hence, 2′,5′-A's or related 2′,5′-oligonucleotide analogues are potential antiviral and antitumor agents. We have succeeded in enzymatically synthesizing a number of new 2′,5′-oligonucleotides with murine 2′,5′-oligoadenylate synthetase and certain nucleoside 5′-triphosphates [refer to the preceding paper (Hughes et al., 1983)] and have evaluated these 2′,5′-oligonucleotides for biological activity in the hope that the cellular role(s) for 2′,5′-A might be further elucidated and that more potent, stable 2′,5′-oligonucleotides might be found.

2′,5′-A may be a natural cell regulator controlling the rate of cell division since 2′,5′-oligoadenylate synthetase has been found in a wide variety of avian and mammalian cells and tissues and varies with the hormonal and nutritional status of the cell (Hovanessian et al., 1977; Ball, 1979; Minks et al., 1979; Kimchi et al., 1981). The ratio of the 2′,5′-oligoadenylate synthetase to 2′,5′-phosphodiesterase, which degrades 2′,5′-A, determines the level of 2′,5′-A and is lower in fast-growing monkey kidney cells (BSC-1) than in confluent cells (Kimchi et al., 1981). Also, the exogenous addition of 2′,5′-A blocks concanavalin A induced mitogenesis in lymphocytes (Kimchi et al., 1979b). The primary effect for the added 2′,5′-A seems to be the impairment of synthesis of various proteins, especially of the histones. Kimchi et al. (1981) have also reported that serum starvation decreased the level of 2′,5′-phosphodiesterase in BSC-1 cells whereas serum addition increased it.

2′,5′-A has been introduced into a variety of cell types, e.g., human, hamster, monkey, and mouse with a resultant cessation of growth and inhibition of protein synthesis (Hovanessian et al., 1979a,b). The inhibition of protein synthesis seems to be the consequence of the transient activation of a latent endoribonuclease by 2′,5′-A binding, which then leads to degradation of both rRNA[1] (Hovanessian et al., 1979a,b) and polyadenylated mRNA [poly(A) mRNA] (Clemens & Williams, 1978; Hovanessian et al., 1979a,b; Nilsen et al., 1981). rRNA was shown to be partially degraded in L cells after calcium phosphate facilitated uptake of 2′,5′-A (Hovanessian et al., 1979a,b) and also in interferon-treated SV40 virus infected monkey cells (Revel et al., 1979) or in interferon-treated encephalomyocarditis (EMC) virus infected L cells (Wreschner et al., 1981) in which the level of 2′,5′-A is presumably increased. Poly(A) mRNA was also found to be reduced, and evidence is presented for its degradation in 2′,5′-A-treated MRC5 cells (Hovanessian et al., 1979a,b). Nilsen et al. (1981) have shown that in HeLa cells treated with interferon and double-stranded RNA that the increase in the 2′,5′-A level correlates with the enhanced decay of poly(A) mRNA. In addition, Clemens & Williams (1978) have reported that one or two nicks per mRNA molecule resulted when 2′,5′-A was incubated with rabbit reticulocyte lysates.

2′,5′-A has been implicated in the antiviral mechanism of interferon since accumulation of viral RNA is inhibited in direct proportion with the increase in the 2′,5′-oligoadenylate synthetase activity (Baglioni et al., 1979). An interesting exception is the case of HeLa cells, which have a high basal level of 2′,5′-A in which interferon seems to prevent EMC virus mediated inhibition of the endoribonuclease so that the 2′,5′-A can be active (Silverman et al., 1982). 2′,5′-A has been introduced into cells by membrane permeability (Williams & Kerr, 1978), calcium phosphate coprecipitation (Hovanessian & Wood, 1980), and microinjection (Higashi & Sokawa,

† From the Department of Chemistry and Cancer Research Center, Brigham Young University, Provo, Utah 84602. *Received September 22, 1982.* This investigation was supported in part by Public Health Service Grant AI 15324 awarded by the National Institute of Allergy and Infectious Diseases, Department of Health and Human Services.

[1] Abbreviations: rRNA, ribosomal ribonucleic acid; poly(A) mRNA, polyadenylated mRNA; EMC virus, encephalomyocarditis virus; DEAE, diethylaminoethyl; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP40, nonidet P40; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate.

0006-2960/83/0422-2127$01.50/0

1982) with resultant antiviral activity against EMC virus in BHK-21 cells (Williams et al., 1979b) and vesicular stomatitis virus in L cells (Hovanessian & Wood, 1980; Higashi & Sokawa, 1982). In addition, the nonphosphorylated "core" (2′,5′-ApApA) has been used to protect tobacco plant tissues from infection by tobacco mosiac virus (Devash et al., 1982).

In the present study, the biological activities of a number of our new enzymatically synthesized 2′,5′-oligonucleotide analogues are reported. The analogues were studied (1) as inhibitors for cellular proliferation and protein synthesis in intact L and HeLa cells, (2) as inhibitors for in vitro protein synthesis in both animal (rabbit reticulocyte lysate) and plant (wheat germ extract) translation systems, (3) as endoribonuclease activators or 2′,5′-phosphodiesterase inhibitors as determined by ribosomal RNA (rRNA) and poly(A) mRNA cleavage, and (4) for relative stabilities to endogenous nucleolytic enzymes in comparison to the naturally occurring 2′,5′-A's. These 2′,5′-oligonucleotides have also been tested for antiviral activity and found to possess activity (to be published elsewhere) that can be correlated in general to that shown here for cellular protein synthesis inhibition, except that the viruses were inhibited by more of the 2′,5′-oligonucleotide analogues.

Materials and Methods

Cell Culture. Mouse L cells (American Type Culture Collection CCL 1 clone 929) and HeLa cells (American Type Culture Collection CCL 2) were grown in Eagle's minimal essential medium with Earle's salts in 10% bovine serum. For cellular-proliferation experiments, cells were plated in four-well plastic plates (Vangard International Inc.) at 4.0×10^4 cells per well, and various 2′,5′-A's or related 2′,5′-analogues were added to nonconfluent cells after 24 h. For determination of the effect of the various 2′,5′-A's or 2′,5′-analogues on in vivo protein synthesis, L cells were plated at 2×10^5 cells per well and HeLa cells at 1×10^5 cells per well in Linbro 24-well plates (Flow Laboratories, Inc.), and cells were used at confluency after 24 h of growth. For determination of the effect of various 2′,5′-A's and related analogues on cytoplasmic RNAs in intact cells, 100-mm plates were seeded with L cells at 5.1×10^6 cells per plate, and [^{3}H]uridine was added to confluent cells after 20 h of growth, followed by treatment with the 2′,5′-A's or related analogues.

Enzymatic Synthesis, Isolation, and Characterization of 2′,5′-A and Related 2′,5′-Analogues. The 2′,5′-oligonucleotides were synthesized with mouse L-cell 2′,5′-oligoadenylate synthetase free in solution according to a modification (Hughes et al., 1983) of the procedure of Derynck et al. (1980). The 2′,5′-oligonucleotides were separated by DEAE-cellulose column chromatography and characterized as to chain length and number of 5′-phosphates by the HPLC system described in the preceding paper (Hughes et al., 1983). In order to calculate the molarity concentrations of the 2′,5′-A and related analogue solutions at pH 7.0, it was necessary to use the following molar extinction coefficients at the indicated wavelength of maximum absorbance, where the nucleoside indicates that found in the trimer: adenosine (35.714 at A = 259 nm according to P-L Biochemicals, Inc.), 8-azaadenosine (21.568 at A = 279 nm), 2-chloroadenosine (29.228 at A = 265 nm), sangivamycin (38.757 at $A \approx 278$ nm), toyocamycin (31.540 at A = 275 nm), formycin (17.393 at A = 303 nm), 3-ribosyladenine (28.061 at A = 275 nm), ribavirin (2.695 at A = 220 nm), tubercidin (28.061 at A = 278 nm), 2-β-D-ribofuranosylthiazole-4-carboxamide (23.790 at A = 236 nm), inosine (31.540 at A = 248 nm), 1,N^6-ethenoadenosine (14.541 at A = 265 nm), guanosine (31.772 at A = 252 nm), 8-bromoadenosine (35.020 at A = 264.8 nm), uridine (25.911 at A = 262 nm), and cytidine (22.293 at A = 271 nm). These extinction coefficients were calculated on the assumption of the same hyperchromicity value calculated for the 2′,5′-pppApApA of 1.2936 except that the trimers containing ribavirin and 2-β-D-ribofuranosylthiazole-4-carboxamide were assumed not to demonstrate hyperchromicity and those containing uridine and cytidine were assumed to be the same as those for the corresponding 3′,5′-trimers (Toal, 1970). Extinction coefficients at A = 259 nm for the 2′,5′-A dimer and tetramer of 26.5 and 40 were used, respectively, and were calculated with data for the analogous 3′,5′-oligoadenylates from our laboratory.

Effect of Various 2′,5′-A's and Related 2′,5′-Analogues on Cellular Proliferation and Protein Synthesis in Intact Cells. The medium was decanted from either the four-well plates for the studies on cellular proliferation or the 24-well plates for the studies on protein synthesis, and either 0.5 mL of Hepes-buffered saline, pH 7.05 [prepared according to Hovanessian & Wood (1980) except that the Na_2HPO_4 concentration was 0.47 mM] was added to the controls, or 0.5 mL of 2′,5′-A's or related analogues in Hepes-buffered saline, pH 7.05, was added. $CaCl_2$ was then added to 71 mM to all wells (including the controls), and the procedure for uptake of the 2′,5′-A into the cells by the calcium phosphate coprecipitation technique of Hovanessian & Wood (1980) was followed. The 90-min incubation at 37 °C was carried out in a CO_2 incubator. For cellular proliferation experiments, the Hepes-buffered saline was replaced with regular minimal essential medium (1 mL) after the 90-min incubation, and cells were trypsinized and counted after 2 further days of growth. Cells from each well were counted at least 6 times in a hemacytometer, and each reported value for the cell-count determinations represents data from generally eight wells. For protein-synthesis determinations, the medium was decanted after 90 min and replaced with medium containing methionine at 10% the normal level, and 4 μCi of [^{35}S]methionine (New England Nuclear) was added per well. The cells were incubated for 1 h at 37 °C in a CO_2 incubator and then washed and solubilized according to Hovanessian & Wood (1980). The radioactivity of the entire sample was measured in 10 mL of Aquasol or Scintiverse II. Data for 2′,5′-A's and the related analogues were the average of several determinations made from generally a total of 15 wells per 2′,5′-oligonucleotide.

Stability of 2′,5′-A and Related 2′,5′-Analogues in a Cell-Free System from L Cells. 2′,5′-A and analogues were adjusted to pH 7.5, lyophilized, and resuspended in buffer B of Kimchi et al. (1979a). Aliquots (2 μL) of the 2′,5′-A or related analogues (1.32 mM in the stability reaction mixture) in buffer B were incubated for the indicated times at 37 °C with NP40 cell extracts (6 μL, 125 μg) prepared according to Hughes et al. (1983) that had been concentrated before the assay in a Millipore immersible CX-30 ultrafiltration unit. Reactions were terminated by freezing (−20 °C) and analyzed for relative degradation by the HPLC reversed-phase Ultrasphere C_{18} ODS system described in the preceding paper (Hughes et al., 1983). Samples without any 2′,5′-A or analogue (2 μL of buffer B + 6 μL of concentrated cell extracts) were also analyzed at all of the wavelengths used for the 2′,5′-A and related analogue HPLC analyses, and absorbancies were found to be at the background level so that corrections for protein absorption were not required.

Effect of Various 2′,5′-A's and Related 2′,5′-Analogues on Rabbit Reticulocyte Lysate or Wheat Germ Extract in Vitro Translation Systems. The various 2′,5′-A's or analogues were

Table I: Inhibition of L- and HeLa-Cell Proliferation and in Vivo Protein Synthesis by Various 2′,5′-A's

		L cells		HeLa cells	
treatment	concn[a] (M)	% inhibition of cellular proliferation	% inhibition of protein synthesis	% inhibition of cellular proliferation	% inhibition of protein synthesis
control[b]		0	0	0	0
pppApA[c]	5×10^{-6}		0		48
	5×10^{-7}	0	0	0	0
ApApA[d]	1×10^{-4}	0	0		0
	5×10^{-7}	0	0		
pApApA[d]	5×10^{-7}	0	0	0	0
pppApApA[e]	5×10^{-7}	83	80	75	88
	1×10^{-7}	56	59	65	79
	5×10^{-8}	33	29	52	74
	1×10^{-8}	0	0	38	52
	5×10^{-9}	0	0	35	16
pppApApApA[f]	5×10^{-7}	85	83	80	89

[a] Concentrations were calculated from the combined percentages for the 5′-di- and 5′-triphosphorylated forms and the extinction coefficients given under Materials and Methods. [b] The controls contained 71 mM $CaCl_2$, which showed a decrease of 12% over controls without $CaCl_2$ for HeLa cells but had no effect in L cells. L-cell controls had 800 000 cpm, and HeLa-cell controls had 180 000 cpm. [c] 88% pppApA and 8.8% ppApA as shown by HPLC characterization. [d] 98% ApApA or pApApA as shown by HPLC characterization. [e] 85% pppApApA and 9.2% ppApApA as shown by HPLC characterization. (Refer to the legend for Figure 3 in the preceding paper for the complete composition.) [f] 78% pppApApApA and 6.6% ppApApApA as shown by HPLC characterization. (Refer to the legend for Figure 3 in the preceding paper for the complete composition.)

concentrated by lyophilization when necessary with subsequent resuspension in water. The effect of the 2′,5′-A's or analogues on cell-free protein synthesis in both the rabbit reticulocyte lysate and wheat germ extract systems was determined, using commercially available kits (Bethesda Research Laboratories, Inc.) programmed with globin mRNA and with [^{3}H]leucine (Amersham) as the labeled amino acid. These standard in vitro translation reactions were carried out under the conditions of Pelham & Jackson (1976) for the rabbit reticulocyte lysate system and Roberts & Paterson (1973) for the wheat germ extract system. Globin mRNA at 0.1 and 0.2 μg/mL (30 μL/assay) was used for the rabbit reticulocyte lysate and wheat germ extract systems, respectively. Reaction times were for 60 min at 30 °C for the rabbit reticulocyte lysate system and at 25 °C for the wheat germ extract system.

Isolation of Cytoplasmic RNA. [^{3}H]RNA (35 000 cpm/μg) was isolated from L cells that had been treated at confluency with 240 μCi of [^{3}H]uridine (ICN) per 100-mm plate for 20 h, followed by treatment with Hepes-buffered saline for controls or 2′,5′-A's or related 2′,5′-analogues according to the calcium phosphate coprecipitation technique described above for studies on inhibition of protein synthesis except that incubations were terminated and RNA was isolated after the 90-min incubation period. (The additional 1-h incubation used for [^{35}S]methionine labeling was eliminated.) Two 100-mm plates per control of 2′,5′-A or analogue were chilled on ice for 4 min and washed 3 times with 5 mL of cold phosphate-buffered saline (PBS), and the cells were scraped off with a rubber policeman in 2 mL of PBS/plate. Cells were collected by centrifugation, swollen 10 min at 0 °C in hypotonic buffer (1 mM Tris-HCl, pH 7.4, 1 mM NaCl, and 0.5 mM $MgCl_2$), and disrupted by 25 strokes of a Dounce homogenizer with a tight-fitting pestle. One-tenth volume of 100 mM Tris-HCl, pH 7.4, 1 M NaCl, and 10 mM EDTA was added, and the nuclei were pelleted at 1100*g*. The RNA was then extracted 2 times with equal volumes of phenol and chloroform and precipitated according to Robertson & Varmus (1979). RNA pellets were resuspended in TE buffer (10 mM Tris-HCl, pH 7.4, and 10 mM EDTA) after centrifugation.

Electrophoretic Analysis of Cytoplasmic RNA for Degradation on Agarose Gels. Denatured cytoplasmic RNA (30 μg/sample) isolated from either controls or 2′,5′-A-treated or related analogue treated L cells was electrophoresed in horizontal 1.8% agarose slab gels containing 10 mM methylmercuric hydroxide (Alfa Division, Ventron Chemical, Danvers, MA) in circulated borate buffer (3 mL/min) as previously described by Bailey & Davidson (1976). After electrophoresis at 90 V for 7 h, the gels were stained with 20 μg/mL ethidium bromide containing 0.1 M β-mercaptoethanol and the RNA bands in the wet gels visualized on a short-wavelength UV light box (Ultra-Violet Products, Inc.). For fluorography, gels were equilibrated in 8.75% 2,5-diphenyloxazole (PPO) in acetone (3 h), rinsed repeatedly in water, dried, and exposed to Kodak X-Omat AR film in Du Pont CRC cassettes containing two Quanta III screens.

Oligo(deoxythymidylic acid)–Cellulose Chromatographic Analysis for Degradation of Poly(A) mRNA. Cytoplasmic RNA isolated from either controls or 2′,5′-A-treated or related analogue treated L cells was heat denatured in TE buffer at 90 °C for 2 min, quick cooled on ice, and brought up to 0.5 M NaCl and 0.2% $NaDodSO_4$. This solution was then passed over a 7-mg oligo(deoxythymidylic acid) [oligo(dT)]–cellulose (Sigma) column equilibrated in binding buffer according to Robertson & Varmus (1979). The unbound material was removed by extensive washing with binding buffer and poly(A) mRNA subsequently released by elution with 10 mM Tris-HCl, pH 7.5. Radioactivity of the fractions was determined in 10 mL of Aquasol. The ratio of the poly(A) mRNA to the total RNA was then calculated, and the percent degradation of poly(A) mRNA was expressed as the percentage of labeled poly(A) mRNA recovered from 2′,5′-A- or analogue-treated cells compared to that recovered from control (Hepes-buffered saline treated) cells. Generally, three additions of each RNA sample was made to the columns, and the average of the three results was reported. The value for the control poly(A) mRNA (2.38% of the total RNA) was the average result for eight determinations. Nearly 100% of the input RNA was recovered in these determinations.

Results

Inhibition of L- and HeLa-Cell Proliferation and in Vivo Protein Synthesis by Various 2′,5′-A's and Related 2′,5′-Analogues. The effect of various 2′,5′-A's on both cellular proliferation determined 2 days after the addition of the 2′,5′-A's and protein synthesis determined 3 h 15 min after the addition of the 2′,5′-A's is shown in Table I. The 5′-

Table II: Inhibition of L- and HeLa-Cell Proliferation and in Vivo Protein Synthesis by 2′,5′-A and Related 2′,5′-Analogues

treatment (ppNpNpN and pppNpNpN where N is)[a]	L cells		HeLa cells	
	% inhibition of cellular proliferation	% inhibition of protein synthesis	% inhibition of cellular proliferation	% inhibition of protein synthesis
control	0	0	0	0
adenosine[b]	83	80	75	88
8-azaadenosine	85	84	75	88
toyocamycin	95	29	55	79
sangivamycin	70	50	75	46
formycin	55	28	98	28
8-bromoadenosine	42	66	54	39
tubercidin	28	56	42	32
guanosine	29	33	40	50
cytidine	0	45	0	33
inosine	0	0	0	12
uridine	0	0	0	6
1,N^6-ethenoadenosine	0	0	8	0
2-chloroadenosine	0[c]	0[c]	0[c]	0[c]
3-ribosyladenine	0	0	0	0
ribavirin	0	0[d]	0	0
2-β-D-ribofuranosylthiazole-4-carboxamide	0	0[d]	0	0

[a] The concentration for ppNpNpN and pppNpNpN was 5×10^{-7} M and was calculated as in Table I. The percentage of pppNpNpN (assuming ppNpNpN + pppNpNpN was 100%) was as follows for each N: 2-chloroadenosine, 96%; adenosine, 8-azaadenosine, toyocamycin, formycin, and guanosine, 90 ± 4%; 8-bromoadenosine, tubercidin, inosine, and 1,N^6-ethenoadenosine, 67 ± 7%; cytidine, 49%; sangivamycin, uridine, 3-ribosyladenine, ribavirin, and 2-β-D-ribofuranosylthiazole-4-carboxamide, 22 ± 10%. The controls had 800 000 cpm for L cells and 180 000 cpm for HeLa cells. [b] Similar results were obtained for the P-L Biochemicals, Inc., or enzymatically prepared 2′,5′-A trimers. [c] 0% inhibition also resulted at a concentration of 5×10^{-6} M. [d] 0% inhibition also resulted at a concentration of 5×10^{-6} M.

triphosphorylated dimer (pppApA) had no effect in L cells even at 5×10^{-6} M, but it did inhibit protein synthesis by 48% in HeLa cells at this concentration. It is likely that the dimer is converted to the trimer in HeLa cells, which then demonstrates the inhibitory activity (Minks et al., 1980). The nonphosphorylated core trimer (ApApA) did not inhibit cell growth or protein synthesis even at 1×10^{-4} M in either L or HeLa cells. The 5′-monophosphorylated trimer (pApApA) was also inactive in both cell lines. The 5′-triphosphorylated form of the trimer (pppApApA) showed similar inhibition in L cells for both inhibition of cellular proliferation and inhibition of protein synthesis. Inhibitory activity resulted even at 5×10^{-8} M. The HeLa cells were shown to be even more sensitive to the 5′-triphosphylated trimer since inhibition resulted at concentrations as low as 5×10^{-9} M. Similar inhibition was shown for the 5′-triphosphorylated trimer and tetramer forms at the same concentrations (5×10^{-7} M) when they were compared in either L or HeLa cells. The dimer, trimer, and tetramer 2′,5′-A's were predominantly the 5′-triphosphorylated forms (see footnotes *c–f* to Table I for percent compositions). The 5′-tri- and 5′-diphosphorylated forms were both used in calculating concentrations since both appear to be active (Baglioni et al., 1981). There was little of the 5′-monophosphorylated forms present as shown by the HPLC analyses reported in the preceding paper (Hughes et al., 1983), and since it is presumed to be inactive (pApApA was not active as shown in Table I), it was not added in for concentration calculations.

Quite a number of the 2′,5′-analogues were effective in inhibiting cellular proliferation and protein synthesis as shown in Table II. The trimer containing the nucleoside 8-azaadenosine was particularly effective, and inhibition of cellular proliferation and protein synthesis equaled that exhibited by the naturally occurring 2′,5′-A trimer in both L and HeLa cells. The 2′,5′-A trimer that had been synthesized with the enzyme 2′,5′-oligoadenylate synthetase in our laboratory gave similar inhibition of cellular proliferation and protein synthesis to that obtained from P-L Biochemicals, Inc., that had been prepared chemically. The trimers containing toyocamycin, sangivamycin, or formycin were very effective in inhibiting cellular proliferation although their effect on protein synthesis was less marked than that shown by the trimer containing adenosine. The trimers containing 8-bromoadenosine or tubercidin were about 50% as effective in inhibiting cell growth or protein synthesis as the natural 2′,5′-A trimer. The guanosine-containing trimer showed quite good inhibition in both L and HeLa cells. This is of particular interest since we (Hughes et al., 1983) have shown that the enzyme, 2′,5′-oligoadenylate synthetase, will utilize guanosine 5′-triphosphate as a substrate, and hence, the 2′,5′-oligoguanylate trimer may occur naturally. The trimer with cytidine as the nucleoside showed good inhibition of protein synthesis although the effect seemed to be rather short-lived since no effect was shown on cellular proliferation. The trimer containing cytidine may also be naturally occurring since cytidine 5′-triphosphate was a recognizable substrate for the so-called 2′,5′-oligoadenylate synthetase (Hughes et al., 1983). The inosine- and uridine-containing analogues did show some inhibition of protein synthesis in HeLa cells, which were also the more sensitive to the 2′,5′-A trimer (Table I). The 1,N^6-ethenoadenosine-containing analogue was rather inactive except that it did inhibit cellular proliferation to a rather limited extent in HeLa cells. The 2′,5′-oligonucleotide analogues of 2-chloroadenosine, 3-ribosyladenine, ribavirin, and 2-β-D-ribofuranosylthiazole-4-carboxamide were not active in L or HeLa cells at 5×10^{-7} M or in some cases at 5×10^{-6} M as indicated in Table II.

The concentrations for these trimers were calculated by using the known amounts for the 5′-di- and 5′-triphosphorylated forms. The trimer of sangivamycin showed good inhibition, and this was mostly the 5′-diphosphorylated form (78%—refer to footnote *a* in Table II for percent compositions), helping to substantiate the work of Baglioni et al. (1981), who reported similar endoribonuclease activation for the 5′-di- and 5′-triphosphorylated forms of the 2′,5′-A trimer. In the case of the sangivamycin trimer, it is also possible that the inhibition results due to elevated 2′,5′-A levels if the sangivamycin trimer is acting as an inhibitor for the 2′,5′-phosphodiesterase.

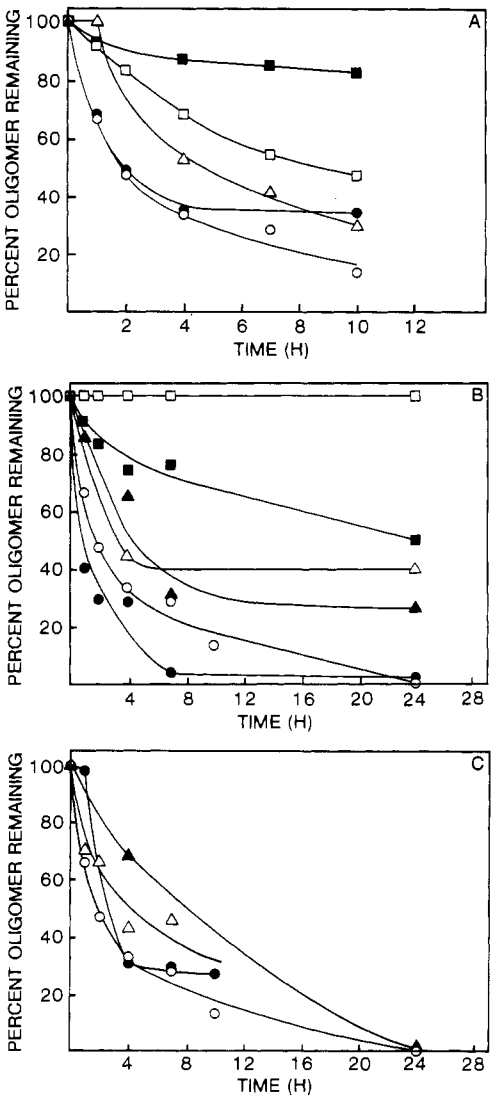

FIGURE 1: Stability of 5′-di- and 5′-triphosphorylated 2′,5′-oligoadenylate trimer (ppApApA and pppApApA) and related 2′,5′-oligonucleotide trimer (ppNpNpN and pppNpNpN) and tetramer (ppNpNpNpN and pppNpNpNpN) analogues in a cell-free system from L cells as determined by HPLC analyses. (A) ppApApA and pppApApA (○); ppNpNpN and pppNpNpN where N is sangivamycin (●), toyocamycin (△), and tubercidin (■); ppNpNpNpN and pppNpNpNpN where N is tubercidin (□). (B) ppApApA and pppApApA (○); ppNpNpN and pppNpNpN where N is 8-azaadenosine (●), 2-chloroadenosine (▲); cytidine (△), and 2-β-D-ribofuranosylthiazole-4-carboxamide (□); ppNpNpNpN and pppNpNpNpN where N is 2-β-D-ribofuranosylthiazole-4-carboxamide (■). (C) ppApApA and pppApApA (○); ppNpNpN and pppNpNpN where N is 8-bromoadenosine (●), guanosine (△), or ribavirin (▲). Relative percent compositions for the trimers were the same as in Tables I and II. The degradation shown for the tubercidin-containing tetramer represents results where the tetramer (7.7%) was present along with the dimer (9.5%) and trimer (78%) forms in the reactions. The degradation shown for the 2-β-D-ribofuranosylthiazole-4-carboxamide-containing tetramer represents results where the tetramer (32%) was present along with the trimer (16%), pentamer (18%), and hexamer (5%) forms in the reactions.

Stability of 2′,5′-A and Related 2′,5′-Analogues in a Cell-Free System from L Cells. A comparison of the stabilities of the 2′,5′-analogues to that of the natural 2′,5′-A should be useful in elucidating possible mechanisms for these 2′,5′-oligonucleotides and also in defining the activity of endogenous nucleolytic enzymes. The 2′,5′-A trimer was broken down rapidly in cell-free extracts from L cells as shown by the data in Figure 1 (with 47% remaining at 2 h, open circles). HPLC analyses showed that degradation was quite complete since resultant products were mostly nucleoside and smaller products even after a 1-h incubation period (data not shown). Figure 1A shows that only about 16% of the 2′,5′-A trimer remained after 10 h (open circles). The trimer of sangivamycin (closed circles) was degraded similarly to the adenosine-containing trimer up to 4 h with 34% remaining at 10 h. The toyocamycin-containing trimer (open triangles) was somewhat more stable while the tubercidin trimer (closed squares) was quite stable in comparison to the 2′,5′-A trimer, as shown by the fact that 83% remained at 10 h. Interestingly, the tubercidin tetramer (open squares) was degraded more rapidly (with 47% remaining at 10 h) than the tubercidin trimer form.

The 8-azaadenosine-containing trimer was degraded somewhat more rapidly than the natural 2′,5′-A trimer (Figure 1B, closed and open circles, respectively). The 2-chloroadenosine- (closed triangles) and cytidine- (open triangles) containing trimers were more stable than the adenosine-containing trimer, but these oligomers were slowly degraded with 30 and 40% remaining at 10 h, respectively. The trimer of 2-β-D-ribofuranosylthiazole-4-carboxamide (open squares) was very stable and was still 100% intact after 24 h. The 2′,5′-A trimer was completely degraded by this time. It is interesting, however, that the 2-β-D-ribofuranosylthiazole-4-carboxamide tetramer (closed squares) was degraded, with 50% remaining at 24 h.

Data in Figure 1C show that the trimer of 8-bromoadenosine (closed circles) was degraded in a similar fashion to that for adenosine (open circles). Trimers containing guanosine (open triangles) and ribavarin (closed triangles) were somewhat more stable, but they too were degraded with time: 32% of the 2′,5′-oligoguanylate trimer remained at 10 h compared to about 16% for the 2′,5′-oligoadenylate trimer.

Inhibition of Protein Synthesis in a Rabbit Reticulocyte Cell-Free System by Various 2′,5′-A's and Related 2′,5′-Analogues. The various 2′,5′-A's and related 2′,5′-analogues were studied as inhibitors of the in vitro rabbit reticulocyte lysate translation system. The trimer core (ApApA) and 5′-monophosphorylated form (pApApA) did not inhibit protein synthesis as shown in Table III in agreement with data from other laboratories (Williams & Kerr, 1978; Martin et al., 1979). The tetramer was found to inhibit protein synthesis to a somewhat greater extent than the trimer at the same concentrations (calculated from extinction coefficients given under Materials and Methods and not on the basis of the AMP equivalents sometimes reported). It should be noted that both our enzymatically synthesized and the chemically synthesized (from P-L Biochemicals, Inc.) 2′,5′-A trimer 5′-triphosphate showed similar inhibition (Table III, footnote *c*) at lower concentrations than reported by Williams et al. (1979a). This probably reflects differences in the methods used to prepare the rabbit reticulocyte lysates. Most of the analogues inhibit protein synthesis very much like the 5′-triphosphorylated 2′,5′-A trimer in this in vitro system. The 8-bromoadenosine-containing trimer inhibits protein synthesis to a somewhat lesser extent while the ribavirin-containing trimer was not an inhibitor. The 2′,5′-trimers containing the naturally occurring nucleosides (guanosine, cytidine, inosine, and uridine) were all found to be equally effective to the adenosine-containing trimer in inhibiting protein synthesis.

Inhibition of Protein Synthesis in a Wheat Germ Cell-Free System by Various 2′,5′-A's and Related 2′,5′-Analogues. 2′,5′-A's and the related 2′,5′-analogues were tested for protein synthesis inhibitory activity in the plant in vitro translation system from wheat germ. The 5′-monophosphorylated form of the trimer (pApApA) was found to be inactive (Table IV)

Table III: Inhibition of Protein Synthesis in a Rabbit Reticulocyte Cell-Free System by Various 2′,5′-A's and Related 2′,5′-Analogues

treatment	% inhibition at a concn[a] (nM) of 12.5	25	50	100	125	250	380
ApApA[b]						0	0
pApApA[b]					0	0	0
pppApApA[b,c]	36	40	42	62	69	78	80
pppApApApA[b]	40	56	67	82		90	92
ppNpNpN and pppNpNpN where N is[d]							
8-azaadenosine	16				42	77	74
toyocamycin						80	95
sangivamycin						89	91
formycin				79		88	91
8-bromoadenosine				35		53	67
tubercidin						96	97
guanosine				50		89	94
cytidine		86				88	88
inosine				86		94	
uridine			40	49			
2-chloroadenosine					52	75	
3-ribosyladenine				94		96	
ribavirin						0	0
2-β-D-ribofuranosylthiazole-4-carboxamide						73	81

[a] Concentrations were calculated as in Table I. Incorporation in the absence of inhibitors was 45 011 cpm. [b] Percent compositions were the same as in Table I. [c] The same inhibition resulted for our enzymatically prepared or the P-L Biochemicals, Inc., chemically prepared 2′,5′-A trimer 5′-triphosphate. [d] Percent compositions were the same as in Table II.

Table IV: Inhibition of Protein Synthesis in a Wheat Germ Cell-Free System by Various 2′,5′-A's and Related 2′,5′-Analogues

treatment	% inhibition at a concn[a] (nM) of 250	380
pApApA[b]	0	0
pppApApA[b]	76	88
ppNpNpN and pppNpNpN where N is[c]		
toyocamycin	77	90
sangivamycin	72	70
8-bromoadenosine	93	93
tubercidin	95	96

[a] Concentrations were calculated as in Table I. Incorporation in the absence of the inhibitors was 61 200 cpm. [b] Percent compositions were the same as in Table I. [c] Percent compositions were the same as in Table II.

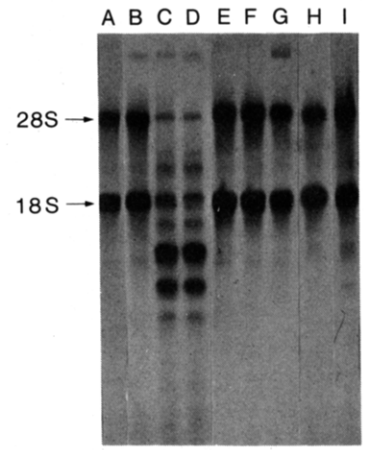

FIGURE 2: Degradation of rRNA in intact L cells treated with various 2′,5′-A's or related 2′,5′-oligonucleotide analogues. Cytoplasmic RNA was subjected to electrophoresis in 1.8% agarose gels containing methylmercuric hydroxide as described under Materials and Methods, and an autoradiograph of the dried gel is shown. (A) Control; (B) 2′,5′-A dimer (ppApA and pppApA); ppNpNpN and pppNpNpN where N is (C) adenosine, (D) 8-azaadenosine, (E) toyocamycin, (F) 8-bromoadenosine, (G) sangivamycin, (H) tubercidin, and (I) guanosine.

while the 5-triphosphorylated form (pppApApA) was found to inhibit protein synthesis at a level comparable to that for the animal system using rabbit reticulocyte lysates (Table III). The four 2′,5′-oligonucleotide analogues studied all showed very similar inhibitory activity to that found for the 2′,5′-A trimer, pppApApA. In this case, the 8-bromadenosine-containing trimer gave similar inhibition to the other analogues.

Degradation of rRNA in Intact L Cells Treated with Various 2′,5′-A's or Related 2′,5′-Analogues. Figures 2 and 3 show the results of the various 2′,5′-A or analogue treatments of L cells from which cytoplasmic RNA was subsequently isolated and analyzed under denaturing conditions on agarose gels. Little difference was noted for rRNA-cleavage band patterns from 2′,5′-A trimer (ppApApA + pppApApA) treatments under protein synthesis conditions as outlined under Materials and Methods or for the 1-h shorter time period used for these rRNA-cleavage studies, so results depicted here are for 1-h shorter incubations than those used in the protein-synthesis inhibition studies. Figure 2 lane A shows the locations and the intact nature of the 18S and 28S rRNA bands for controls in which L cells were treated with Hepes-buffered saline, followed by the calcium phosphate uptake technique. Lane B reveals that there was only a slight effect for treatment with the dimer, while lane D shows that the 2′,5′-trimer analogue containing 8-azaadenosine resulted in a very similar band pattern for rRNA breakdown products to that for the 2′,5′-A trimer (lane C). Lanes E–H show that the 2′,5′-trimer analogues containing toyocamycin, 8-bromoadenosine, sangivamycin, and tubercidin all result in some rRNA degradation but that it was much less than that shown for the 2′,5′-A trimer treatment (lane C). The 2′,5′-oligoguanylate-trimer treatment resulted in marked rRNA degradation (lane I), but it was still less than that shown for the 2′,5′-oligoadenylate trimer (lane C).

Figure 3 lane A shows that rRNA isolated from L cells treated with the 2′,5′-trimer analogue containing 3-ribosyladenine resulted in only a slight effect if any on the rRNA while the 2′,5′-trimer analogue of formycin (lane B), 2′,5′-A tetramer (lane C), and 2′,5′-A trimer (lane D) all resulted in marked degradation with similar cleavage band patterns. The 2′,5′-oligonucleotide trimer containing cytidine (lane E) also resulted in notable degradation. The 2-chloroadenosine-trimer

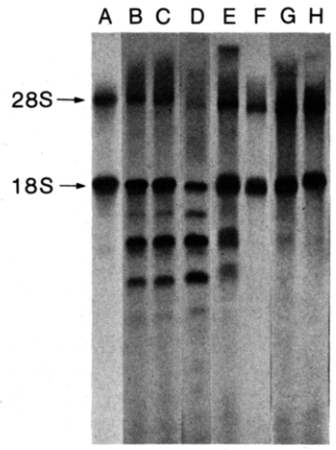

FIGURE 3: Degradation of rRNA in intact L cells treated with various 2′,5′-A's or related 2′,5′-oligonucleotide analogues. Cytoplasmic RNA was subjected to electrophoresis in 1.8% agarose gels containing methylmercuric hydroxide as described under Materials and Methods, and an autoradiograph of the dried gel is shown. ppNpNpN and pppNpNpN where N is (A) 3-ribosyladenine, (B) formycin, (E) cytidine, (F) 2-chloroadenosine, (G) inosine, and (H) uridine; (C) 2′,5′-A tetramer (ppApApApA and pppApApApA); (D) 2′,5′-A trimer (ppApApA and pppApApA).

(lane F) and uridine-trimer (lane H) analogues did not show an effect on rRNA while the inosine-trimer analogue (lane G) had a slight effect. The 2′,5′-trimer analogues containing 1,N^6-ethenoadenosine, ribavirin, or 2-β-D-ribofuranosylthiazole-4-carboxamide did not show any effect on rRNA since bands for these looked like those shown for the controls (data not shown). The 5′-monophosphorylated adenosine containing trimer (pApApA) also was without demonstrable effect (data not shown).

Degradation of Poly(A) mRNA in Intact L Cells Treated with Various 2′,5′-A's or Related 2′,5′-Analogues. The effect of the various 2′,5′-A and related 2′,5′-analogue L-cell treatments on the degradation of poly(A) mRNA was determined by comparing the relative decrease in oligo(dT)–cellulose-bound poly(A) mRNA for the 2′,5′-A or analogue treatments to controls. The core 2′,5′-A trimer (ApApA) and 5′-monophosphorylated 2′,5′-A trimer (pApApA) did not lead to breakdown of poly(A) mRNA (Table V), while the dimer had a slight effect (15% degradation relative to the controls). The 2′,5′-A trimer (pppApApA) and tetramer (pppApApApA) cell treatments resulted in similar poly(A) mRNA degradation, 69 and 66%, respectively, as did all of the 2′,5′-oligonucleotide analogue trimers except those containing 2-chloroadenosine and 2-β-D-ribofuranosylthiazole-4-carboxamide, which did not have any effect on poly(A) mRNA cleavage.

Table V: Degradation of Poly(A) mRNA in Intact L Cells Treated with Various 2′,5′-A's or Related 2′,5′-Analogues

treatment[a]	% degradation[b]
control	0
pppApA[c]	15
ApApA[c]	0
pApApA[c]	0
pppApApA[c]	69
pppApApApA[c]	66
ppNpNpN and pppNpNpN where N is[d]	
8-azaadenosine	54
toyocamycin	78
sangivamycin	81
formycin	63
8-bromoadenosine	85
tubercidin	83
guanosine	85
cytidine	54
inosine	78
uridine	56
1,N^6-ethenoadenosine	69
2-chloroadenosine	0
3-ribosyladenine	54
ribavirin	59
2-β-D-ribofuranosylthiazole-4-carboxamide	0

[a] The concentration for the various 2′,5′-A's and related 2′,5′-oligonucleotide analogues was 5×10^{-7} M and was calculated as in Table I. [b] Percent degradation was calculated relative to the control. Cytoplasmic RNA samples (5.48 μg) had 215 000 cpm. [c] Percent compositions were the same as in Table I. [d] Percent compositions were the same as in Table II.

Discussion

A number of the new enzymatically synthesized 2′,5′-oligonucleotides, namely, the trimers containing the nucleosides 8-azaadenosine, toyocamycin, sangivamycin, formycin, 8-bromoadenosine, tubercidin, and guanosine, were found to inhibit protein synthesis and cellular proliferation after uptake into intact L and HeLa cells. The trimer with cytidine as the nucleoside inhibited protein synthesis but not cellular proliferation, while the 2′,5′-oligonucleotides with inosine and uridine inhibited protein synthesis to a rather limited extent in HeLa cells. The trimers with 2-chloroadenosine, 3-ribosyladenine, ribavirin, and 2-β-D-ribofuranosylthiazole-4-carboxamide were inactive in these in vivo systems. The trimers containing toyocamycin, sangivamycin, and formycin were found to inhibit cellular proliferation to a greater extent than protein synthesis, which would appear to be a measure of their cellular toxicity. Since these 2′,5′-oligonucleotide trimers were degraded when incubated with cell extracts, it seems likely that breakdown products, e.g., the nucleoside, nucleotide, or the resultant products of kinase reactions such as the nucleoside 5′-triphosphates, might be exhibiting secondary inhibitory effects in addition to the inhibition by the corresponding 2′,5′-oligonucleotides. Toyocamycin, administered as the nucleoside, has been shown to inhibit rRNA processing (Auger-Buendia et al., 1978) as well as synthesis of total cellular RNA (Brdar & Reich, 1976). In addition, sangivamycin has been reported to inhibit RNA synthesis in several tumor and normal cell lines (Glazer & Hartman, 1981; Glazer et al., 1981; Ritch et al., 1981) while formycin has been reported to inhibit base methylation in nuclear RNA (Stern & Glazer, 1980), to inhibit tRNA synthesis (Majima et al., 1977), and to decrease both adenosine 5′-triphosphate and adenosine 3′,5′-monophosphate levels (Zimmerman et al., 1979). The trimer of 1,N^6-ethenoadenosine inhibited cellular proliferation but not protein synthesis in HeLa cells, which also seems to be indicative of the formation of a toxic degradation product. The 2′,5′-oligocytidylate trimer inhibited protein synthesis but not cellular proliferation in both L and HeLa cells. Stability studies carried out with cell-free extracts indicated that the 2′,5′-oligocytidylate trimer was at least as stable as the 2′,5′-A trimer so there is no obvious explanation for this.

It is apparent from the results of the incorporation of the 2′,5′-oligonucleotide analogues into L and HeLa cells that a 2-chloro or a 3-ribose substituent on the base inactivates the 2′,5′-oligonucleotide trimer and that a 6-amino group seems to be somewhat preferred since trimers with the inosine and 1,N^6-ethenoadenosine nucleotides were not very active although an exception was found with the guanosine trimer, which was quite active. The 2′,5′-oligonucleotides containing ribavirin or 2-β-D-ribofuranosylthiazole-4-carboxamide were inactive so such base substitutions were not tolerated. Biological activity was retained, however, for replacement of the

N-9 nitrogen with a carbon atom, replacement of the 8-carbon with a nitrogen atom, replacement of the N-7 nitrogen with a carbon atom, addition of an 8-bromo or 2-amino substituent, or addition of an amido or cyano group at C-5 (in sangivamycin or toyocamycin) in the base of the 2′,5′-oligonucleotide. Either these nucleoside substitutions in the 2′,5′-trimers seem to define the substrate-specificity requirements for the endoribonuclease or certain of the 2′,5′-analogues may be acting as inhibitors for the 2′,5′-phosphodiesterase so that endogenous 2′,5′-A levels increase, which may then lead to the effects observed. No attempt was made to differentiate between these two possibilities in the present study.

The 2′,5′-oligonucleotide analogues were studied as inhibitors for both plant and animal in vitro protein translation systems. Less specificity for the 2′,5′-analogues was observed in the in vitro systems than for the uptake into intact L and HeLa cells since all of the 2′,5′-trimer analogues had inhibitory activity except the ribavirin trimer in the rabbit reticulocyte system. Perhaps, the trimers arrive at their targets more readily in the in vitro cell-free systems. Silverman et al. (1981) have shown that another analogue, ppp($A_{2'p}$)$_3$ApCp, was an inhibitor in the rabbit reticulocyte system but that it was much less active if at all in extracts from L or Ehrlich ascites tumor cells.

Similar inhibition resulted for the 2′,5′-analogues in either the plant or the animal systems so either the endoribonuclease and/or the 2′,5′-phosphodiesterase appears to be similar in plants or animals. This suggests the possible usage of these new 2′,5′-oligonucleotides as potential antiviral or antitumor agents for both plants and animals.

Most of the 2′,5′-oligonucleotides were degraded in L-cell extracts so the nucleolytic enzymes do not seem to be very specific. The sangivamycin-, 8-azaadenosine-, and 8-bromoadenosine-containing trimers were degraded very similarly to the 2′,5′-A trimer. The toyocamycin, 2-chloroadenosine, cytidine, guanosine, and ribavirin trimers were found to be somewhat more stable while the tubercidin and 2-β-D-ribofuranosylthiazole-4-carboxamide trimers were quite stable. The tetramers of tubercidin and 2-β-D-ribofuranosylthiazole-4-carboxamide were degraded more rapidly than the corresponding trimers, probably due to the contribution of the additional phosphate making these more recognizable substrates.

The stabilities of our 2′,5′-oligonucleotide analogues could probably be further enhanced by preparing the corresponding terminal 3′-*O*-monomethyl derivatives (Baglioni et al., 1981) or the 3′-deoxy analogues (Doetsch et al., 1981) or by adding the 3′,5′-linked pCp terminal group (Silverman et al., 1981) since other investigations have reported that such modifications greatly increased stabilities for the 2′,5′-A's with retention of many of the biological activities.

The incorporation of the 5′-triphosphorylated 2′,5′-A trimer or tetramer and certain of our 2′,5′-oligonucleotides into intact L cells led to the degradation of rRNA similar to that observed for 2′,5′-A by Wreschner et al. (1981) in cell-free extracts or for 2′,5′-A taken up into intact cells by Hovanessian et al. (1979a). The cleavage of the rRNA shown after the uptake of some of the 2′,5′-analogues correlated quite well with the inhibition of protein synthesis observed. The 2′,5′-A dimer, 5′-monophosphorylated 2′,5′-A trimer, and 2′,5′-A trimers containing inosine, uridine, 1,N^6-ethenoadenosine, 2-chloroadenosine, 3-ribosyladenine, ribavirin, and 2-β-D-ribofuranosylthiazole-4-carboxamide had either no effect or only a slight effect on either protein synthesis or rRNA breakdown. Both inhibition of protein synthesis and rRNA cleavage were observed for the 2′,5′-A trimer, 2′,5′-A tetramer, and 2′,5′-trimers containing 8-azaadenosine, formycin, guanosine, or cytidine. Some of the 2′,5′-analogues, namely, toyocamycin, 8-bromoadenosine, sangivamycin, and tubercidin, did inhibit protein synthesis, but only a slight effect on rRNA was detected. These four 2′,5′-oligonucleotides did lead to marked poly(A) mRNA degradation though, so preferential breakdown of poly(A) mRNA over rRNA can be achieved with some of these 2′,5′-oligonucleotide analogues, and the poly(A) mRNA degradation seems to be responsible for the inhibition of protein synthesis observed in these cases.

Poly(A) mRNA was also found to be degraded in intact L cells after uptake of the 5′-triphosphorylated 2′,5′-A trimer or tetramer or most of the 2′,5′-oligonucleotide analogues. All the 2′,5′-oligonucleotide analogues led to poly(A) mRNA degradation except the 2-chloroadenosine and 2-β-D-ribofuranosylthiazole-4-carboxamide trimers. The 2′,5′-A dimer did cause some breakdown of poly(A) mRNA even though no effect on protein synthesis had been observed. This indicates that poly(A) mRNA cleavage may be a more sensitive measure of activity for these 2′,5′-analogues. Several other 2′,5′-analogues, i.e., those containing inosine, uridine, 1,N^6-ethenoadenosine, 3-ribosyladenine, and ribavirin, led to degradation of poly(A) mRNA without demonstrable effect on cellular protein synthesis in L cells. It is worth noting that three of these, i.e., those containing inosine, 1,N^6-ethenoadenosine, and 3-ribosyladenine, also showed some antiviral activity against vesicular stomatitis virus in L cells, which also seems to be a more sensitive test for biological activity (unpublished data). These 2′,5′-oligonucleotide analogues should be investigated further as antiviral and antitumor agents since these initial studies have shown that they possess some interesting biological activities.

Acknowledgment

The skillful technical assistance of M. Joleen Glassett is gratefully acknowledged.

Registry No. pppApA·ppApA, 84811-30-3; ApApA, 70062-83-8; pApApA, 61172-40-5; pppApApA·ppApApA, 84811-31-4; pppApApApA·ppApApApA, 84823-62-1; pppNpNpN·ppNpNpN (N = 8-azaadenosine), 84811-34-7; pppNpNpN·ppNpNpN (N = toyocamycin), 84811-37-0; pppNpNpN·ppNpNpN (N = sangivamycin), 84811-40-5; pppNpNpN·ppNpNpN (N = formycin), 84823-99-4; pppNpNpN·ppNpNpN (N = 8-bromoadenosine), 84824-02-2; pppNpNpN·ppNpNpN (N = tubercidin), 84824-05-5; pppNpNpN·ppNpNpN (N = guanosine), 84824-08-8; pppNpNpN·ppNpNpN (N = cytidine), 84811-43-8; pppNpNpN·ppNpNpN (N = inosine), 84811-46-1; pppNpNpN·ppNpNpN (N = uridine), 84811-49-4; pppNpNpN·ppNpNpN (N = 1,N^6-ethenoadenosine), 84823-65-4; pppNpNpN·ppNpNpN (N = 2-chloroadenosine), 84811-52-9; pppNpNpN·ppNpNpN (N = 3-ribosyladenine), 84811-55-2; pppNpNpN·ppNpNpN (N = ribavirin), 84811-58-5; pppNpNpN·ppNpNpN (N = 2-β-D-ribofuranosylthiazole-4-carboxamide), 84811-61-0; pppNpNpNpN·ppNpNpNpN (N = tubercidin), 84811-64-3; pppNpNpNpN·ppNpNpNpN (N = 2-β-D-ribofuranosylthiazole-4-carboxamide), 84811-67-6; endoribonuclease, 59794-03-5.

References

Auger-Buendia, M. A., Hamelin, R., & Tavitian, A. (1978) *Biochim. Biophys. Acta 521*, 241–250.

Baglioni, C., Maroney, P. A., & West, D. K. (1979) *Biochemistry 18*, 1765–1770.

Baglioni, C., D'Alessandro, S. B., Nilsen, T. W., den Hartog, J. A. J., Crea, R., & van Boom, J. H. (1981) *J. Biol. Chem. 256*, 3253–3257.

Bailey, J. M., & Davidson, N. (1976) *Anal. Biochem. 70*, 75–85.

Ball, L. A. (1979) *Virology 94*, 282–294.
Brdar, B., & Reich, E. (1976) *Period. Biol. 78*, 51–61.
Clemens, M. J., & Williams, B. R. G. (1978) *Cell (Cambridge, Mass.) 13*, 565–572.
Derynck, R., Remaut, E., Saman, E., Stanssens, P., De Clercq, E., Content, J., & Fiers, W. C. (1980) *Nature (London) 287*, 193–197.
Devash, Y., Biggs, S., & Sela, I. (1982) *Science (Washington, D.C.) 216*, 1415–1416.
Doetsch, P., Wu, J. M., Sawada, Y., & Suhadolnik, R. J. (1981) *Nature (London) 291*, 355–358.
Glazer, R. I., & Hartman, K. D. (1981) *Mol. Pharmacol. 20*, 657–661.
Glazer, R. I., Hartman, K. D., & Cohen, O. J. (1981) *Biochem. Pharmacol. 30*, 2697–2702.
Higashi, Y., & Sokawa, Y. (1982) *J. Biochem. (Tokyo) 91*, 2021–2028.
Hovanessian, A. G., & Wood, J. N. (1980) *Virology 101*, 81–90.
Hovanessian, A. G., Brown, R. E., & Kerr, I. M. (1977) *Nature (London) 268*, 537–540.
Hovanessian, A. G., Wood, J., Meurs, E., & Montagnier, L. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3261–3265.
Hovanessian, A. G., Wood, J. N., Meurs, E., & Montagnier, L. (1979b) in *Regulation of Macromolecular Synthesis by Low Molecular Weight Mediators* (Koch, G., & Richter, D., Eds.) pp 319–327, Academic Press, New York.
Hughes, B. G., Srivastava, P. C., Muse, D. D., & Robins, R. K. (1983) *Biochemistry* (preceding paper in this issue).
Kimchi, A., Shulman, L., Schmidt, A., Chernajovsky, Y., Fradin, A., & Revel, M. (1979a) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3208–3219.
Kimchi, A., Shure, H., & Revel, M. (1979b) *Nature (London) 282*, 849–851.
Kimchi, A., Shure, H., & Revel, M. (1981) *Eur. J. Biochem. 114*, 5–10.
Lengyel, P. (1982) *Annu. Rev. Biochem. 51*, 251–282.
Majima, R., Tsutsumi, K. I., Suda, H., & Shimura, K. (1977) *J. Biochem. (Tokyo) 82*, 1161–1166.
Martin, E. M., Birdsall, N. J. M., Brown, R. E., & Kerr, I. M. (1979) *Eur. J. Biochem. 95*, 295–307.
Minks, M. A., Benvin, S., Maroney, P. A., & Baglioni, C. (1979) *J. Biol. Chem. 254*, 5058–5064.
Minks, M. A., Benvin, S., & Baglioni, C. (1980) *J. Biol. Chem. 255*, 5031–5035.
Nilsen, T. W., Maroney, P. A., & Baglioni, C. (1981) *J. Biol. Chem. 256*, 7806–7811.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247–256.
Revel, M., Kimchi, A., Schmidt, A., Shulman, L., Chernajovsky, Y., Rapoport, S., & Lapidot, Y. (1979) in *Regulation of Macromolecular Synthesis by Low Molecular Weight Mediators* (Koch, G., & Richter, D., Eds.) pp 284–302, Academic Press, New York.
Ritch, P. S., Glazer, R. I., Cunningham, R. E., & Shackney, S. E. (1981) *Cancer Res. 41*, 1784–1788.
Roberts, B. E., & Paterson, B. M. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2330–2334.
Robertson, D. L., & Varmus, H. E. (1979) *J. Virol. 30*, 576–589.
Silverman, R. H., Wreschner, D. H., Gilbert, C. S., & Kerr, I. M. (1981) *Eur. J. Biochem. 115*, 79–85.
Silverman, R. H., Cayley, P. J., Knight, M., Gilbert, C. S., & Kerr, I. M. (1982) *Eur. J. Biochem. 124*, 131–138.
Stern, H. J., & Glazer, R. I. (1980) *Biochem. Pharmacol. 29*, 1459–1464.
Toal, J. N. (1970) in *CRC Handbook of Biochemistry and Selected Data from Molecular Biology* (Sober, H. A., Ed.) 2nd ed., p G-217, The Chemical Rubber Co., Cleveland, OH.
Williams, B. R. G., & Kerr, I. M. (1978) *Nature (London) 276*, 88–90.
Williams, B. R. G., Kerr, I. M., Gilbert, C. S., White, C. N., & Ball, L. A. (1978) *Eur. J. Biochem. 92*, 455–462.
Williams, B. R. G., Golgher, R. R., Brown, R. E., Gilbert, C. S., & Kerr, I. M. (1979a) *Nature (London) 282*, 582–586.
Williams, B. R. G., Golgher, R. R., & Kerr, I. M. (1979b) *FEBS Lett. 105*, 47–52.
Wreschner, D. H., McCauley, J. W., Skehel, J. J., & Kerr, I. M. (1981) *Nature (London) 289*, 414–417.
Zimmerman, T. P., Wolberg, G., & Duncan, G. S. (1979) *J. Biol. Chem. 253*, 8792–8797.