BIOORGANIC CHEMISTRY 19, 283–299 (1991)

Uridine Analogs of 2′,5′-Oligoadenylates: On the Biological Role of the Middle Base of 2-5A Trimer[1]

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Received November 13, 1990

In order to further delineate the role of the second nucleotide residue of 2-5A in its interaction with the 2-5A-dependent endonuclease, RNase L, a series of uridine-substituted sequence-specific analogs were synthesized and evaluated for their ability to bind to and activate the nuclease. Substitution of only the 5′-terminal adenosine by uridine caused up to a 100-fold loss in binding and activation of RNase L. Replacement of the middle adenosine residue of 2-5A trimer by uridine also resulted in some loss of binding and activation ability. When the 2′-terminal adenosine was replaced by uridine, a dramatic decrease in activation ability was observed. The results reinforced earlier conclusions that elements of the adenine base of the 5′-nucleotide are involved in binding to the 2-5A-dependent endonuclease, whereas activation is dependent upon structural determinants in the adenine moiety of the third adenosine nucleotide residue of 2-5A. These results also implicated some as yet undefined structural or conformational feature associated with the second nucleotide unit of 2-5A that may be involved in binding to or activation of RNase L.

INTRODUCTION

Among the most notable of the diverse biological activities of interferon are the antiviral effects which are mediated through several mechanisms including the 2-5A[6] system (*1*, *2*). 2-5A represents a unique oligonucleotide series of the form

[1] A part of this work was presented at the Joint Meeting of the American Society of Biological Chemists and the American Chemical Society, June 8–12, 1986 Washington, D.C., and also at the 107th Congress of the Pharmaceutical Society of Japan, April, 1987, Kyoto, Japan.

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[6] Abbreviations used: 2-5A, ppp5′A2′p5′A2′p5′A; DMF, dimethylformamide; HPLC, high performance liquid chromatography; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid. In reference to the 2-5A trimer, ade_1, ade_2, and ade_3 refer to the adenines of the 5′-terminal nucleotide unit, the middle nucleotide, and the 2′-terminal nucleotide, respectively; similarly, rib_1, rib_2, and rib_3 refer to the similarly located ribose moieties. The 2-5A-dependent endonuclease is also referred to as RNase L. The derivative in which the 2′-terminal ribose unit has been oxidized, reacted with *n*-hexylamine,

0045-2068/91 $3.00

ppp5′A2′(p5′A)$_n$($n \geq 1$) generated by the interferon inducible enzyme 2′,5′-oligoadenylate synthetase [ATP: (2′-5′) oligoadenylate transferase, EC 2.7.7.19] in the presence of a double-stranded RNA activator (*3*). The oligonucleotide 2-5A binds to and activates a latent ribonuclease (RNase L) capable of degrading viral mRNA (reviewed in (*1, 2*)). The 2′,5′-linked oligonucleotide is degraded by a 2′,5′-phosphodiesterase activity (*4*). To delineate oligonucleotide structural motifs which play a fundamental role in defining binding and activation of RNase L, various alterations in 2-5A structure have been executed and examined for biological activity (reviewed in (*5*)). In studies reported heretofore (*6–8*), it appeared that, numbering from the 5′-terminus of 2-5A, the adenine bases of the first and third nucleotides were of particular importance in influencing oligonucleotide–RNase L interactions. For instance, the N1/N6 amino function of ade$_1$ was vital for *binding* to the endonuclease, whereas the N1/N6 functionality of ade$_3$ was a sine qua non for *activation* of RNase L. Similarly, the purine N7 of either ade$_1$ or ade$_3$ could be replaced by CH without much change in mouse RNase L binding ability; however, these same substitutions led to significant decreases in the capacity to activate mouse RNase L as judged by protein synthesis inhibition studies. Conversely, similar alterations to the N1/N6 or N7 moieties of ade$_2$ resulted in minor or nonexistent changes in oligomer binding or activating ability. Based on this limited data, the base domains of ade$_2$ would seem not to assume an influential function in the interplay of 2-5A with its endonuclease. This is certainly not the situation when the ribose domains of 2-5A are considered since the 3′-OH group of the rib$_2$ of 2-5A seems indispensable for activation of the mouse endonuclease (*9*).

In order to understand more precisely the role of the ade$_2$ of 2-5A, we sought to replace the adenine ring system completely. Specifically, we wondered whether or not the entire adenine ring of the 2-5A molecule might be replaced without deleterious consequences. We chose to substitute the ade$_2$ with the base uracil since uracil bears none of the common protein–nucleic acid recognition markers that adenine possesses, and because it represents a replacement which is relatively straightforward in a synthetic sense.

EXPERIMENTAL

Materials

The sources of the various chemicals, biochemicals, and enzymes used herein have been described previously (*7, 9–11*). The linkage isomers were prepared by the method of Sawai and Ohno (*12, 13*) using a recent modification (*7, 11*). Uridine 5′-monophosphate (sodium salt) was desalted on Dowex 50W-X8(H^+) to yield the free acid which was used to make uridine 5-phosphoromorpholidate(MopU) or

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and reduced with cyanoborohydride bears, in place of a terminal adenosine, a 9-(3′-aza-4′-hexyl-1′,2′,3′,4′-tetradeoxyhexopyranos-1′-yl)-adenine moiety. This oligonucleotide is abbreviated as p5′A-2′p5′U2′p5′A2′p5′(ahp)Ade, where ahp stands for azahexapyranose.

uridine 5'-phosphoroimidazolidate(ImpU). The preparation of other nucleotide morpholidates and imidazolidates has been described in detail elsewhere (*7, 11*).

METHODS

Preparation of pA2'p5'U2'p5'A via the Dimer pU2'p5'A

$Pb(NO_3)_2$ (0.25 M, 1 ml) was added to a mixture of uridine 5'-phosphoromorpholidate (210 mg, 0.5 mmol, sodium salt), and adenosine 5'-phosphoroimidazolidate (210 mg, 0.5 mmol, sodium salt) dissolved in 1-methylimidazolium nitrate buffer (10 ml, 0.2 M, pH 7.5) at 4°C. After 4 days of stirring at 4°C, Chelex 100 (NH_4^+ form, 5 ml) was added to the turbid reaction mixture which then gradually became clear. The Chelex was removed by filtration, and the filtrate was diluted with water to 100 ml and applied to a DEAE-Sephadex A-25 column (1.6 × 20 cm) preequilibrated with water. Elution was with a linear gradient of 0.0 M (500 ml) to 0.3 M (500 ml) triethylammonium bicarbonate (pH 7.5). 192 fractions were collected. Fractions 72–86 were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The product, the 5'-phosphoromorpholidate of U2'p5'A, was obtained as the triethylammonium salt (392 OD_{260}). This dinucleotide phosphoromorpholidate was dissolved in 10% acetic acid solution (5 ml), and the mixture was incubated at 37°C for 2 h. The solvent was removed under reduced pressure and the residue was dissolved in 0.1 M ammonium acetate buffer (pH 5.75) (5 ml). The reaction mixture then was incubated at 37°C for 2 h with P1 nuclease (50 μg), and the resulting solution was heated at 100°C for 5 min. The solution was diluted with water to 15 ml and applied to a DEAE-Sephadex A-25 column (1.0 × 15 cm) preequilibrated with water. Elution was with a linear gradient of 0.0 M (250 ml) to 0.5 M (250 ml) triethylammonium bicarbonate (pH 7.6). Ninety-six fractions were collected. Fractions 43–59 were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, pU2'p5'A, was isolated as the triethylammonium salt (266 OD_{258}).

The dinucleotide pU2'p5'A (500 OD_{258}, 0.022 mmol), triethylammonium salt, was dissolved in dry DMF (2 ml) and pulverized triphenylphosphine (14 mg, 0.055 mmol), imidazole (7.5 mg, 0.11 mmol), and 2,2'-dipyridyldisulfide (12 mg, 0.055 mmol) were added to this solution. The resulting yellow-colored reaction mixture was stirred at room temperature for 2 h. The whole mixture was poured into 0.1 M sodium iodide solution in acetone (30 ml) with stirring. The white precipitate was collected by centrifugation and washed several times with fresh acetone until the yellow color was gone. After drying at room temperature for 1 h under vacuum, the sodium salt of the 5'-phosphoroimidazolidate of U2'p5'A was used directly in the next step of the synthesis without further purification.

Lead nitrate solution (0.25 M, 100 μl) was added to a solution of adenosine 5'-phosphoromorpholidate (47.6 mg, 0.11 mmol, sodium salt) and all of the 5'-phosphoroimidazolidate of U2'p5'A prepared above in 1-methylimidazolium nitrate

buffer (0.2 M, 2 ml, pH 7.5) at 4°C. After 5 days of stirring at 4°C, Chelex 100 (NH_4 form, 2 ml) was added to the turbid reaction mixture which gradually became clear. The Chelex was removed by filtration, and the filtrate was added to 10% acetic acid solution (20 ml). The mixture was incubated at 37°C for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in 0.1 M ammonium acetate buffer (pH 5.75) (5 ml). The reaction mixture was incubated at 37°C for 2 h in the presence of P1 nuclease (100 μg) followed by heating at 100°C for 5 min. The solution was diluted with water to 15 ml and applied to a DEAE-Sephadex A-25 column (1.6 × 20 cm) preequilibrated with water. Elution was with a linear gradient of 0.0 M (400 ml) to 0.5 M (400 ml) triethylammonium bicarbonate (pH 7.6). A total of 161 fractions was collected. Fractions 108–122 were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, pA2′p5′U2′p5′A, was isolated as the triethylammonium salt (88 OD, 12.5%).

Preparation of pU2′p5′A2′p5′A

The dinucleotide pA2′p5′A (3000 OD, triethylammonium salt, 0.115 mmol) was dissolved in dry DMF (2 ml) and pulverized triphenylphosphine (80 mg, 0.763 mmol), imidazole (40 mg, 2.2 mmol), and 2,2′-dipyridyldisulfide (70 mg, 0.772 mmol) were added to this solution. The resulting yellow-colored reaction mixture was stirred at room temperature for 2 h and then the whole mixture was poured with stirring into a 0.1 M sodium iodide solution in acetone (100 ml). The white precipitate was collected by centrifugation and washed several times with fresh acetone until the yellow color was gone. After drying at room temperature for 1 h under vacuum, the sodium salt of the 5′-phosphoroimidazolidate of A2′p5′A was used directly in the next step of the synthesis.

Lead nitrate solution (0.25 M, 100 μl) was added to a solution of uridine 5′-phosphoromorpholidate (21 mg, 0.05 mmol, sodium salt) and the imidazolidate of pA2′p5′A in 1-methylimidazolium nitrate buffer (0.2 M, 2 ml, pH 7.5) at 4°C. After 5 days of stirring at 4°C, Chelex 100 (NH_4^+ form, 5 ml) was added to the reaction mixture. The Chelex was removed by filtration, and the filtrate was added to 10% acetic acid solution (20 ml). The mixture was incubated at 37°C for 2 h. The solvent was removed under reduced pressure and the residue was dissolved in 0.1 M ammonium acetate buffer (pH 5.75, 5 ml). The reaction mixture was incubated at 37°C for 2 h in the presence of P1 nuclease (200 μg), followed by heating at 100°C for 5 min. The solution was diluted with water to 15 ml and applied to a DEAE-Sephadex A-25 column (1.6 × 20 cm) preequilibrated with water. Elution was with a linear gradient of 0.0 M (400 ml) to 0.5 M (400 ml) triethylammonium bicarbonate (pH 7.6). A total of 163 fractions was collected. Fractions 112–128 were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, pU2′p5′A2′p5′A, was isolated as the triethylammonium salt (207 OD_{259}, 13.6%).

Preparation of pA2'p5'A2'p5'U

The dinucleotide pA2'p5'A (4000 OD, triethylammonium salt, 0.154 mmol) was dissolved in dry DMF (5 ml). Pulverized triphenylphosphine (200 mg, 0.763 mmol), morpholine (240 μl, 2.76 mmol), and 2,2'-dipyridyldisulfide (170 mg, 0.772 mmol) were added to this solution, and the resulting yellow-colored reaction mixture was stirred at room temperature for 2 h. The whole mixture was poured with stirring into 0.1 M sodium iodide solution in acetone (100 ml). The white precipitate was collected by centrifugation and washed several times with fresh acetone until the yellow color was gone. After drying at room temperature for 1 h under vacuum, the sodium salt of the 5'-phosphoromorpholidate of A2'p5'A was used directly in the next step of synthesis.

Lead nitrate solution (0.25 M, 100 μl) was added to a solution of ImpU (63 mg, 0.15 mmol, sodium salt) and all of the morpholidate (0.2 M, 5 ml, pH 7.5) of p5'A2'p5'A prepared above in 1-methylimidazolium nitrate buffer (0.25 M, 250 μl) at 4°C. After 5 days of stirring at 4°C, Chelex 100 (NH_4^+ form, 5 ml) is added to the turbid reaction mixture. The Chelex was removed by filtration, and the filtrate was added to 10% acetic acid solution (20 ml). The mixture was incubated at 37°C for 2 h. The solvent was removed under reduced pressure, and the residue was dissolved in 0.1 M ammonium acetate buffer (pH 5.75, 5 ml). The reaction mixture was incubated at 37°C for 2 h in the presence of P1 nuclease (50 μg), followed by heating at 100°C for 5 min. The solution was diluted with water to 15 ml and applied to a DEAE-Sephadex A-25 column (1.6 × 20 cm) preequilibrated with water. Elution was with a linear gradient of 0.0 M (400 ml) to 0.5 M (400 ml) triethylammonium bicarbonate (pH 7.6). A total of 166 fractions was collected. Fractions 111–130 were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, pA2'p5'A2'p2'U, was isolated as the triethylammonium salt (1028 OD_{259}, 21.9%).

Preparation of p5'A2'p5'U2'p5'A2'p5'A

The trinucleotide p5'U2'p5'A2'p5'A (960 OD_{258}, triethylammonium salt, 0.03 mmol) was dissolved in dry DMF (5 ml). Pulverized triphenylphosphine (39.3 mg, 0.15 mmol), imidazole (20 mg, 0.3 mmol), and 2,2-dipyridyldisulfide (33 mg, 0.15 mmol) were added to this solution, and the resulting yellow-colored reaction mixture was stirred at room temperature for 1 h. The mixture was poured into a 0.1 M sodium iodide solution in acetone (50 ml) with stirring. The white precipitate was collected by centrifugation and washed several times with fresh acetone until the yellow color disappeared. After drying the resulting precipitate at room temperature for 1 h under vacuum, the sodium salt of the 5'-phosphoroimidazolidate of U2'p5'A2'p5'A was obtained.

Lead nitrate solution (0.25 M, 270 μl) was added to a solution of adenosine 5'-phosphoromorpholidate (62.4 mg, 0.15 mmol, sodium salt) and the imidazolidate of p5'U2'p5'A2'p5'A prepared above in 1-methylimidazolium nitrate buffer (0.2 M, 1.8 ml, pH 7.5) at 4°C. After 4 days of stirring at 4°C, Chelex 100 (NH_4 form, 5

ml) was added to the turbid reaction mixture which became clear. The Chelex was removed by filtration, and the filtrate was added to a 10% acetic acid solution (20 ml). The mixture was incubated at 37°C for 2 h. The solvent was removed under reduced pressure and the residue was dissolved in 0.1 M ammonium acetate buffer (pH 5.75, 10 ml). The reaction mixture was incubated at 37°C for 2 h in the presence of P1 nuclease (250 μg) and then heated at 100°C for 5 min. The solution was diluted with water to 50 ml and applied to a DEAE-Sephadex A-25 column (1.6 × 20 cm) preequilibrated with water. Elution was with a linear gradient of 0.0 M (500 ml) to 0.5 M (500 ml) triethylammonium bicarbonate (pH 7.6). A total of 196 fractions were collected. Fractions 172–195 were pooled and concentrated *in vacuo*, and water was added to and evaporated from the resulting residue to remove residual triethylammonium bicarbonate. The desired product, p5'A-2'p5'U2'p5'A2'p5'A, was isolated as the triethylammonium salt (339 OD_{258}, 26%). Fractions 136–158 gave the trinucleotide p5'U2'p5'A2'p5'A (537 OD_{358}, 56%).

Preparation of 2'-Terminally Modified p5'A2'p5'U2'p5'A2'p5'A[p5'*A2'p5'U2'p5'A2'p5'(ahp)ade*]

The preceding compound was prepared by addition of sodium periodate (0.1 M, 129 μl) to ice cold p5'A2'p5'U2'p5'A2'p5'A (3.5 μmol) in H_2O (1 ml). The reaction was kept on ice for 50 min until completion of the reaction as determined by HPLC. The reaction was quenched with ethylene glycol (1 M, 25 μl) and stirred for 30 min. Hexylamine (0.05 M, 620 μl) was added and the pH of the solution immediately adjusted to pH 8.6 with acetic acid (0.1%). The reaction was stirred on ice for 20 min and sodium cyanoborohydride (0.5 M, 150 μl) was added and again the pH was adjusted with acetic acid (0.1%) to pH 6.5. The reaction mixture was purified by HPLC using a Zorbax ODS (21.2 mm × 25 cm) column and a linear gradient of 0–100% buffer B (methanol/H_2O, 1 : 1) in buffer A (50 m*M* ammonium phosphate, pH 7.0) in 30 min with a 9 ml/min flow rate. The appropriate fractions were applied to a DEAE-Sephadex A-25 column (1 cm × 25 cm) and eluted with a linear gradient of 0.1 M (250 ml) to 0.6 M (250 ml) triethylammonium bicarbonate (pH 7.6). The final product was isolated after repeated addition and evaporation of water, in a yield of 40%.

Preparation of Triphosphates of Uridine Analogs

All monophosphates were converted to triphosphates using the method described earlier (*7, 11*). For example, p5'A2'p5'U2'p5'A (50 OD_{260}, 1.56 μmol, triethylammonium salt) was converted to the imidazole derivative, Imp5'A-2'p5'U2'p5'A, by reaction with triphenylphosphine (4 mg, 15 μmol), imidazole (2 mg, 30 μmol), and 2,2'-dipyridyldisulfide (3.8 mg, 16 μmol). It was isolated as the sodium salt, which was dissolved in tri-*n*-butylammonium pyrophosphate (300 μl) in DMF (0.5 M). The reaction mixture was kept at room temperature for 20 h. After dilution with H_2O (15 ml) the solution was applied to DEAE-Sephadex A-25 (1 cm × 20 cm) and eluted with triethylammonium bicarbonate (pH 7.6). The final triphosphate products were isolated after addition and evaporation of water to remove volatile buffer from the column chromotography. Yields were as follows:

ppp5'U2'p5'A2'p5'A, 59%; ppp5'A2'p5'U2'p5'A, 42%; ppp5'A2'p5'A2'p5'U, 56%; ppp5'A2'p5'U2'p5'A2'p5'A, 76%: ppp5'A2'p5'U2'p5'A2'p5'Aahp, 55%.

Chemical and Enzymatic Degradation

5'-Dephosphorylation of synthetic oligonucleotides was effected via alkaline phosphatase exposure using 0.4 OD_{258} of oligomer substrate and 0.06 unit of enzyme in Tris–acetate (0.2 M, pH 8.8), $MgCl_2$ (0.001 M), and a total volume of 100 μl. Incubation of this mixture was at 37°C for 2 h. Snake venom phosphodiesterase degradation of synthetic oligonucleotides was performed with 0.4 OD_{258} of substrate and 0.02 unit of enzyme in Tris–acetate (0.01 M, pH 8.8), $MgCl_2$ (0.001 M), in a total volume of 100 μl, with incubation at 37°C for 1 h. Periodate oxidation and base elimination of trinucleotides was done using 0.2–0.5 OD_{258} of substrate treated with sodium periodate (0.1 M, 100 μl) at 25°C in darkness for 30 min. Lysine (1.0 M, pH 9.0, 100 μl) was added, and the mixture was incubated at 56°C for 1 h. Then to the reaction mixture, 50 μl of Tris–acetate (1 M, pH 6.5) was added together with 0.1 unit of bacterial alkaline phosphatase. The mixture was incubated at 37°C for 18 h. The digested product was then compared to an authentic sample of the appropriate dinucleotide by use of HPLC. In all cases, putative products were identified by comparison with authentic materials. Ratios of nucleotide and/or nucleoside products were determined via integration of the chromatogram and correction for differences in ε values. Table 2 provides the results of such experiments as well as HPLC systems employed.

Biological Assays

Materials. [^{3}H]Leucine (sp act 147 Ci/mmol) and [5'-^{32}P]cytidine'-3',5'-bisphosphate (sp act 3000 Ci/mmol) were obtained from New England Nuclear. T4 ligase was from Pharmacia and poly(U) from either P-L Biochemicals or Boehringer-Mannheim. Other sources have been described previously (*6, 7, 9, 10*).

The radiobinding probe (pp)p5'A2'p5'A2'p5'A2'p5'A3'[^{32}P]p5'C3'p was prepared by addition of [5'-^{32}P]cytidine-3',5'-bisphosphate to either p5'A2'p5'A-2'p5'A2'p5'A or ppp5'A2'p5'A2'p5'A2'p5'A with T4 ligase using a previously described procedure (*14*). The reacted probe was purified using HPLC. Poly(U)-[^{32}P)p5'C3'p and core cellulose was prepared using the methods described by Silverman (*15*).

Techniques. Biochemical assays employed to measure the activity of uridine isomers were repeated at least three times and the reported values are averages. The assays included:

(i) Determination of the ability of the oligonucleotide triphosphates to inhibit protein synthesis in mouse L cell-free translational system programmed with encephalomyocarditis virus RNA (*16*). Increasing quantities of analog were added to the cell-free system and the minimum concentration of oligomer necessary to achieve half-maximal inhibition of translation was measured.

(ii) A second method involved a radiobinding assay, using mouse liver or L_k cell extracts and ppp5'A2'p5'A2'p5'A2'p5'A3'[^{32}P]p5'C3'p as a radioactive probe which, in a nitrocellulose binding assay, could be displaced by oligonucleotides

that bind to the 2-5A site on the endonuclease. In this case the concentration of oligomer needed to displace 50% of the radiolabeled probe from binding to the endonuclease–nitrocellulose complex was used as a measure of affinity to the 2-5A-dependent endonuclease (*14*).

(iii) A third assay, like the protein synthesis assay, was a functional assay for RNase L degrading activity. This method was developed by Silverman (*15*), and involved first the prior partial purification of the RNase L by absorption to 2-5A core (A2'p5'A2'p5'A2'p5'A) cellulose and addition of 2-5A or an analog to activate the endonuclease. The ability of the activated RNase L to degrade poly (U)[^{32}P]p5'C3'p (vide supra) was then determined by measuring the acid-insoluble radioactivity.

To carry out this assay, CB buffer was prepared from 868 μl of buffer A (100 mM Hepes, pH 7.5, 50 mM $Mg(OAc)_2$, 70 mM β-mercaptoethanol, 900 mM KCl), 5.0 μl β-mercaptoethanol, 936 μl ATP (10 mM, pH 7.0), 75 μl leupeptin (10 mg/ml) and 1885 μl of sterile H_2O. Sufficient core cellulose (*15*) suspension was used to give 25 μM core in the final assay mixture. Core cellulose was washed twice with 10 vol of 1/2 × CB buffer (CB buffer: H_2O, 1 : 1). L-cell extract (100 μg/protein/assay) was added to the core cellulose together with an equal volume of ice-cold CB buffer. After incubation at 0°C for 1 h, the core cellulose was washed three times with 1/2 × CB buffer. The cellulose was resuspended in buffer and distributed among the requisite number of assay tubes such that each tube contained the core cellulose with 20 μl of 1/2 × CB buffer. Water or analog dilution (5 μl) containing the poly(U)[^{32}P]p5'C3'p probe was added. This mixture was incubated for 2 h at 30°C and then 1 ml of ice cold 5% trichloroacetic acid with 0.2% sodium pyrophosphate was added together with 100 μl of a saturated solution of yeast RNA as a carrier. Radioactivity was determined by collecting the precipitate on GF/C glass fiber discs (No. 30, 25-mm diameter, Whatman) and washing three times with ice cold 5% trichloroacetic acid containing 0.2% pyrophosphate and then with ice cold ethanol. After drying, radioactivity was determined with a liquid scintillation counter. The minimum concentration of oligomer necessary to achieve half-maximal degradation of poly(U)[^{32}P]p5'C3'p was measured.

Methodology of Determination of the Stability of Oligonucleotide Analogs

The stability of 5'-monophosphate analogs (5×10^{-5} M) was determined under the conditions of proteins synthesis inhibition described above except that H_2O was substituted for [^{3}H]leucine and encephalomyocarditis mRNA (*17*). Aliquots were removed at various times during incubation and processed by heating to 100°C for 5 min followed by centrifugation for 5 min in a Fisher Micro-Centrifuge Model 235B. The resultant supernatant was used for analysis. The amount of 5'-monophosphate analog remaining and the presence of degradation products was determined by HPLC using two Beckman System Model 110A pumps and 421 controller. The detector was an ISCO Model UA-5 absorbance/fluorescence monitor. The integration of the chromatograph was determined with an Altex Model C-RIA integrator. The column employed was μBondapak C_{18}(Waters). The concentration of the analogs was determined by measurement of the integrated peaks

in conjunction with an internal standard. The measurement of the stability of analogs under radiobinding conditions was identical to that described above except that H_2O was substituted for the radiolabeled probe. The stability of triphosphate analogs was determined under the core cellulose assay conditions except that H_2O replaced poly(U)[^{32}P]p5′C3′p.

RESULTS

Chemistry

Synthesis of the requisite uridine analogs of 2-5A was accomplished according to an adaptation (*11*) of the lead ion-catalyzed polymerization procedure (*12, 13*). This method permits, within certain limits, the preparation of sequence-specific, 2′,5′-oligonucleotides. The crux of this approach (*11*) is the use of the 5′-phosphoromorpholidate moiety as a protecting group for the nucleoside destined to become the 5′-terminus. Under normal conditions of the lead ion-catalyzed polymerization, the morpholidate is unreactive, but it can be removed later by mild acid treatment to regenerate the 5′-monophosphate, or it can be converted to a 5′-triphosphate moiety as found in 2-5A itself. With this procedure, four 2′,5′-linked oligonucleotides were generated; namely p5′A2′p5′A2′p5′U, p5′A2′p5′U2′p5′A, p5′U2′p5′A2′p5′A, and p5′A2′p5′U2′p5′A2′p5′A. The assigned structures were confirmed by degradation methods as well as NMR (Tables 1 and 2). For instance, when treated with a mixture of snake venom phosphodiesterase and bacterial alkaline phosphatase, the oligonucleotide products gave the nucleosides adenosine and uridine in the expected stoichiometric ratios. In addition, the sequence of periodate oxidation, base elimination, and bacterial alkaline phosphatase treatment removed the 2′-terminal nucleotide and yielded a simple 5′-dephosphorylated dinucleotide which could be identified by comparison with authentic material. Using the enzyme digestion data and the chemical degradation technique, the sequence of an oligonucleotide was assigned. Moreover, proton NMR was used to establish the requisite type and number of adenine and/or uracil protons and the number of anomeric protons (Table 1).

The 5′-monophosphates were converted to the corresponding 5′-triphosphates in the usual manner through reaction of the phosphoroimidazolidate with pyrophosphate (*11*). The structures of such oligonucleotide 5′-triphosphates were corroborated by digestion with bacterial alkaline phosphatase. This procedure gave the relatively hydrophobic (by HPLC) "core" oligomers possessing only internucleotide phosphates. Such core molecules generated from 5′-triphosphates were identical with core molecules prepared by bacterial alkaline phosphatase treatment of the corresponding oligonucleotide 5′-monophosphate (Table 2).

To block the 2′-terminus of p5′A2′p5′U2′p5′A2′p5′A, the oligonucleotide was subjected to periodate oxidation, followed by cyclic hemialdal formation with *n*-hexylamine and finally generation of a morpholine ring by sodium cyanoborohydride (*18*). This procedure produced an oligonucleotide which was "tailed" (*18*) with the 2′-(9-adenyl)-6-hydroxymethyl-4-hexylmorpholine; namely, p5′A2′p5′U-

TABLE 1

Retention Time on HPLC and Characteristic Proton NMR[a] Signals of Oligonucleotides

	Analog anomeric time(min)	Retention C2–H, C8–H	Adenine C5–H, C6–H	Uracil protons
p5'U2'p5'A2'p5'A	9.64[b]	8.06(s)	7.44(d, 8.0Hz)	5.95(d, 3.5Hz)
		8.02(s)	5.25(d, 8.0Hz)	5.68(d, 2.6Hz)
		7.87(s)		5.62(d, 5.7Hz)
		7.80(s)		
p5'A2'p5'U2'p5'A	10.95[b]	8.18(s, 2H)	6.99(d, 8.0Hz)	6.03(d, 4.6Hz)
		8.02(s)	5.06(d, 8.0Hz)	5.85(d, 5.6Hz)
		7.94(s)		5.52(d, 3.2Hz)
p5'A2'p5'A2'p5'U	8.03[b]	8.00(s)	7.15(d, 8.0Hz)	5.95(d, 2.9Hz)
		7.98(s)	5.26(d, 8.0Hz)	5.81(d, 5.5Hz)
		7.92(s)		5.42(d, 2.6Hz)
		7.77(s)		
p5'U2'p5'A	8.91[b]	8.25(s)	7.51(d, 7.6Hz)	5.88(d, 4.5Hz)
		8.08(s)	5.24(d, 7.6Hz)	5.86(d, 4.5Hz)
p5'A2'p5'U2'p5'A2'p5'A	12.46[b]	8.18(s)	6.97(d, 8.0Hz)	6.00(d, 5.4Hz)
		7.99(s)	5.08(d, 8.0Hz)	5.92(d, 3.2Hz)
		7.97(d)		5.66(d, 2.2Hz)
		7.93(s)		5.29(s)
		7.85(s)		
		7.73(s)		
p5'A2'p5'U2'p5'A2'p5'(ahp)ade	8.25[c]	8.17(s)	7.03(d, 8.0Hz)	6.02(d, 4.8Hz)
		8.08(s)	5.11(d, 8.0Hz)	5.98(d, 3.9Hz)
		7.94(s)		5.70(d, 8.0Hz)
		7.92(s)		5.45(d, 2.4Hz)
		7.90(s)		
		7.84(s)		

[a] Chemical shifts were determined in D_2O with acetone (δ = 2.05 ppm) as an internal standard.

[b] Chromatograph performed on Ultrasphere ODS(Altex) reverse-phase column using a gradient of 0 to 50% buffer B (methanol: water, 1 : 1) over 25 min in buffer A (50 Mm ammonium phosphate, pH 7.5) at 1 ml/min.

[c] Chromatograph performed on μBondapak C_{18}(Waters) reverse-phase column using 70% buffer B in buffer A.

[d] Flow rate 1 ml/min.

2'p5'A2'p5'(ahp)ade. The corresponding 5'-triphosphate was obtained through the phosphoroimidazolidate procedure. Snake venom/alkaline phosphatase degradation data as well as proton NMR spectral data of the "tailed" 5'-monophosphate were consistent with the assigned structure (Tables 1 and 2). Bacterial alkaline phosphatase treatment of the triphosphate gave a "core" oligomer identical to that obtained from the 5'-monophosphate.

Biological Studies

The ability of the analogs to bind to RNase L was determined using a competition assay that relied upon displacement of the radioligand, ppp5'A2'p5'A-

TABLE 2

Chemical and Enzymatic Characterization of Oligonucleotides[a]

Oligomer	BAP digest product (retention time)	SVP digest (mol product)	IO_4/base/BAP product (retention time)
p5'U2'p5'A	U2'p5'A (16.24)	1.0 p5'U + 0.95 p5'A	ND
p5'A2'p5'U2'p5'A	A2'p5'U2'p5'A (18.28)[b]	1.0 p5'U + 1.9 p5'A	A2'p5'U (14.84)[b]
ppp5'A2'p5'U2'p5'A	A2'p5'U2'p5'A (18.28)[b]	ND	ND
p5'U2'p5'A2'p5'A	U2'p5'A2'p5'A (12.38)[b]	1.0 p5'U + 2.0 p5'A	U2'p5'A (12.40)[b]
ppp5'U2'p5'A2'p5'A	U2'p5'A2'p5'A (12.38)[b]	ND	ND
p5'A2'p5'A2'p5'U	A2'p5'A2'p5'U (12,38)[b]	1.0 p5'U + 2.2 p5'A	A2'p5'A (19.03)[b]
ppp5'A2'p5'A2'p5'U	A2'p5'A2'p5'U (12.38)[b]	ND	ND
p5'A2'p5'U2'p5'A2'p5'A	A2'p5'U2'p5'A2'p5'A (30.87)[b]	1.0 p5'U + 2.7 p5'A	
ppp5'A2'p5'U2'p5'A2'p5'A	A2'p5'U2'p5'A2'p5'A (30.87)[b]	ND	
p5'A2'p5'U2'p5'A2'p5'ahp(ade)	A2'p5'U2'p5'A2'p5'ahp(ade) (29.10)[c]	1.0 p5'U + 1.9 p5'A + 1.0(ahp)ade	
ppp5'A2'p5'U2'p5'A2'p5'ahp(ade)	A2'p5'U2'p5'A2'p5'ahp(ade) (29.10)[c]	ND	

[a] Alkaline phosphatase or snake venom phosphodiesterase digestion of monophosphates and triphosphates to dephosphorylated linkage isomer products was performed as described under Experimental. HPLC was performed on two Beckman system Model 110A pumps and a 421 controller. The detector was an ISCO Model UA-5 absorbance/fluorescence monitor. The integration of the chromatograph was determined with an Altex Model C-RIA integrator. The column employed was μBondapak C_{18}(Waters).

[b] Buffer A (50 mM ammonium phosphate, pH 7.0) in 0 to 50% Buffer B (methanol:water, 1 : 1) over 30 minutes at 1 ml/min.

[c] Buffer A (50 mM ammonium phosphate, pH 7.2) in 70% Buffer B (methanol:water, 1 : 1) at 1 ml/min.

2'p5'A3'[^{32}P]p5'C3'p from the L_K cell endonuclease in a nitrocellulose filter binding assay. The results are presented in Table 3 and are reported as IC_{50} values (molar concentrations of oligomer needed to displace 50% of the labeled probe from endonuclease) and C_{REL} (relative concentration, as compared to 2-5A trimer, of analog needed to displace 50% of the probe). The uracil for adenine substitution effect on binding was position-dependent. When ade_1 (the 5'-terminal base) was replaced by uracil as in ppp5'U2'p5'A2'p5'A and p5'U2'p5'A2'p5'A, the greatest decrease in binding was observed since it required 70–100x more analog to displace radiolabeled probe as compared to 2-5A itself. When ade_2, or the middle base of 2-5A trimer was replaced by uracil, a 30-fold decrease in binding was observed for the triphosphate. The monophosphate showed a 50-fold decrease in binding. A decrease (10–30x) in binding to RNase L was found with the analogs, ppp5'A2'p5'A2'p5'U and p5'A2'p5'A2'p5'U, in which ade_3 of 2-5A trimer was replaced by uracil. When the tetrameric analogs were examined, both ppp5'A-2'p5'U2'p5'A2'p5'A and its 2'-modified congener were bound to RNase L almost as well as 2-5A trimer itself (previous studies (*1–10*) have shown that 2-5A trimer and tetramer are bound to RNase L with similar efficiencies). For all analogs examined, the triphosphates were bound to the 2-5A-dependent endonuclease slightly better than were the corresponding 5'-monophosphates.

Binding of the ade_2-modified analogs (trimer and tetramers) also was explored using RNase L from mouse liver extracts. Under these assay conditions, the

TABLE 3

Radioligand Binding Assay[a]

Oligomer	IC_{50}(M)	C_{rel}
p5'U2'p5'A2'p5'A	6×10^{-8}	100
ppp5'U2'p5'A2'p5'A	4×10^{-8}	70
p5'A2'p5'U2'p5'A	3×10^{-8}	50
ppp5'A2'p5'U2'p5'A	2×10^{-9}	3
p5'A2'p5'A2'p5'U	2×10^{-8}	30
ppp5'A2'p5'A2'p5'U	6×10^{-9}	10
ppp5'A2'p5'U2'p5'A2'p5'A	1×10^{-9}	2
ppp5'A2'p5'U2'p5'A2'p5'(ahp)ade	1×10^{-9}	2
ppp5'A2'p5'A2'p5'A	6×10^{-10}	1

[a] IC_{50} was defined as the molar concentration of oligomer required to displace 50% of the specific binding of the labeled probe from the endonuclease. C_{rel} was defined as the relative concentration of analog to displace 50% of the probe. The greater the value, the less effective the analog was at competing with probe. 2-5A itself was arbitrarily set equal to 1.

following IC_{50} values were determined: p5'A2'p5'U2'p5'A, 3×10^{-9} M; ppp5'A-2'p5'U2'p5'A, 4×10^{-10} M; ppp5'A2'p5'U2'p5'A2'p5'A, 4×10^{-10} M; ppp5'A-2'p5'U2'p5'A2'p5'Aahp, 8×10^{-10} M; ppp5'A2'p5'A2'p5'A, 4×10^{-10} M. These results paralleled quite closely those obtained with mouse L_K cell RNase L within a factor of 2.5 or less. The only exception was the trimer triphosphate, ppp5'A-2'p5'U2'p5'A, which was somewhat more avidly bound to the liver enzyme source than to RNase L of L_K cells.

Activation of the 2-5A-dependent endonuclease by the synthetic analogs was ascertained by two different functional assays: a protein synthesis inhibition assay and a core-cellulose assay that measures RNA degradation (Table 4). In these cases, the ability to activate RNase L is presented in terms of the IC_{50} (molar concentration of oligomer needed to bring about a 50% reduction of maximal inhibition of protein synthesis or maximal RNA degradation) and P_{rel}, which is the relative (compared to 2-5A trimer) potency of the analogs as nuclease activators. Substitution of either ade_1 or ade_2 of 2-5A trimer by uracil gave oligonucleotides with 0.5–2% of the RNase L activation of parent 2-5A trimer. The substitution which produced the greatest loss of activity was uracil for ade_3 to give ppp5'A-2'p5'A2'p5'U, which possessed no detectable RNase L activation ability. Activation ability of the tetrameric analogs showed some variation depending upon the assay. In the protein synthesis assay both ppp5'A2'p5'U2'p5'A2'p5'A and its 2'-terminus-modified congener displayed 20–30% of the activity of 2-5A. However, in the core cellulose assay, both analogs possessed only 3% of the activity of 2-5A.

One facet of the biological behavior of certain of these analogs needs to be emphasized. This refers to the previously observed (*7, 20*) behavior of submaximal inhibition of translation or degradation of poly(U)pCp as compared to 2-5A itself. The parent material 2-5A, in the currently employed translational inhibition

TABLE 4

RNase L Activation Assay[a]

Oligomer	Protein synthesis		Core cellulose	
	IC_{50}(M)	P_{rel}	IC_{50}(M)	P_{rel}
ppp5'U2'p5'A2'p5'A	2×10^{-7}	0.01	2×10^{-8}	0.01
ppp5'A2'p5'U2'p5'A	4×10^{-7}[b]	0.005	9×10^{-9}[b]	0.02
ppp5'A2'p5'A2'p5'U	$>5 \times 10^{-5}$	$<4 \times 10^{-5}$	$>5 \times 10^{-5}$	$<4 \times 10^{-6}$
ppp5'A2'p5'U2'p5'A2'p5'A	1×10^{-8}[b]	0.2	8×10^{-9}[b]	0.03
ppp5'A2'p5'U2'p5'A2'p5'(ahp)ade	7×10^{-9}[b]	0.3	6×10^{-9}[b]	0.03
ppp5'A2'p5'A2'p5'A	2×10^{-9}	1	2×10^{-10}	1

[a] For both the protein synthesis assay and the core cellulose assay, IC_{50} was defined as the molar concentration of oligonucleotide that was required to effect a 50% reduction of the maximal inhibition (protein synthesis) or maximal degradation (core cellulose assay) observed. P_{rel} was defined as the relative potency of the analogs as RNase L activators. The greater the value, the greater the potency as a nuclease activator. These were calculated by setting the value for pppAAA equal to 1; analog values were calculated from the quotient (IC_{50} pppAAA)/(IC_{50} analog).

[b] These oligomers produced, even at the highest concentrations tested, less inhibition of translation or less degradation of RNA than 2-5A or pppU2'p5'A2'p5'A. For instance, under conditions in which 2-5A gave 80–90% inhibition of protein synthesis of 90% degradation of RNA, these analogs showed a maximal inhibition of translation of 40–50% and a maximal RNA degradation of 60–70% except for pppA2'p5'U2'p5'A2'p5'A, which produced a maximum of 25–30% degradation of labeled poly(U).

assay, is able to cause up to a 90% inhibition of protein synthesis at maximum concentration ($\sim 10^{-6}$ M). Likewise it can affect the degradation of 90% of the input-labeled poly(U) as judged by trichloroacetic acid soluble radioactivity. Several analogs in this study were not able to achieve these same high levels of inhibition and degradation despite the fact that they most definitely activated the endonuclease. These included (Table 4) ppp5'A2'p5'U2'p5'A, ppp5'A2'p5'U-2'p5'A2'p5'A, and ppp5'A2'p5'U2'p5'A2'p5'(ahp)ade.

To assess the stability of the various analogs to 2',5'-phosphodiesterase activity, the 5'-monophosphates were used as potential substrates under conditions of the radioligand binding assay, the protein synthesis assay, and the core cellulose assay. Under protein synthesis assay conditions, and at an initial concentration of $2–3 \times 10^{-4}$ M, the half-life times of the analogs were as follows (Fig. 1): p5'A-2'p5'U2'p5'A, 49 min; p5'A2'p5'A2'p5'U, >120 min; p5'U2'p5'A2'p5'A, 100 min; p5'A2'p5'A2'p5'A, 120 min; p5'A2'p5'U2'p5'A2'p5'A, 75 min; p5'A2'p5'A-2'p5'A2'p5'A, 120 min; p5'A2'p5'U2'p5'A2'p5'(ahp)ade, $\gg$120 min. The latter analog with the 2'-terminus modified was almost totally resistant to degradation over a 2-h incubation at 30°C. The analog most sensitive to degradation, namely p5'A2'p5'U2'p5'A, was examined for its stability under radioligand binding assay conditions: after 90 min incubation, 83% of p5'A2'p5'A2'p5'A remained whereas 55% of p5'A2'p5'U2'p5'A was left (data not illustrated). Under conditions of the core cellulose assay, no apparent degradation of either ppp5'A2'p5'U2'p5'A or ppp5'A2'p5'A2'p5'A could be detected during a 2-h incubation at 30°C (data not shown).

DISCUSSION

The purpose of the current work was to attempt to illuminate further the role of the second nucleotide of 2-5A trimer in determining binding to and activation of RNase L. Since earlier studies (*6–8, 19*) suggested the lack of a crucial role for the N1/N6 and N7 determinants of ade_2 of 2-5A in these processes, a series of 2-5A analogs was constructed in which each adenine ring of 2-5A trimer was sequentially replaced by uracil.

When ade_1 of 2-5A was replaced by uracil, binding to RNase L was diminished by 70- to 100-fold. This finding was consistent with earlier work (*6*) which implicated the adenine N1/N6 functionality in endonuclease binding since both an inosine-substituted (p5′I2′p5′A2′p5A) and a guanosine-substituted [p5′G2′p-5′(c^7A)2′p5′(d^7A)][8] analog of 2-5A trimer underwent at least a 100-fold loss in binding ability compared to 2-5A itself. Conversely, other substituents in the 5′-terminal nucleotide base or sugar (tubercidin or 3′-deoxyribose) produced minor or no changes in RNase L binding (*7, 9*). One exception, however, was the substitution of 8-bromoadenosine at the 5′-terminus (*20*). Such an analog showed considerably diminished binding most probably due to an unfavorable base–sugar torsion angle which prevented optimal contacts of the ade_1 N1/N6 determinant with appropriate domains in RNase L (*20, 21*).

Even though the ade_1 to uracil transformation effected a major loss in binding to RNase L, it did not effect activation of the enzyme beyond that expected due to loss of binding. Table 5 presents the combined data of Tables 3 and 4 in a different form which permits a relative assessment of activation and binding parameters. If, arbitrarily, it is assumed that 2-5A trimer itself activates as well as it binds (and this is approximately true) then the value R_n (binding IC_{50}/core cellulose IC_{50}) would provide an estimate of how effectively other analogs can activate even if they bind to RNase L less avidly than does 2-5A. From Table 5, it is seen that ppp5′U2′p5′A2′p5′A activated nearly as well as it bound, compared to 2-5A. Thus, we conclude that ade_1 does not play a major role in activation of RNase L although it represents a critical component for maximum binding.

TABLE 5

Normalized Binding/Activation Ratios[a]

Oligomer	R_n
ppp5′U2′p5′A2′p5′A	0.6
ppp5′A2′p5′U2′p5′A	0.07
ppp5′A2′p5′A2′p5′U	<0.00003
ppp5′A2′p5′U2′p5′A2′p5′A	0.04
ppp5′A2′p5′U2′p5′A2′p5′(ahp)ade	0.06
ppp5′A2′p5′A2′p5′A	1

[a] R_n was calculated from the quotient (radioligand binding IC_{50})/(core cellulose IC_{50}). The quotient for 2′,5′-pppAAA was then set equal to 1, and all other quotients were normalized to that value.

The situation with the analog wherein the second or middle adenine, ade_2, was substituted by uracil, was somewhat different from the instance of 5′-terminal substitution. The oligomers, p5′A2′p5′U2′p5′A and its 5′-triphosphate showed, respectively, a 50-fold and a 3-fold decrease in RNase L binding, and the triphosphate was only 0.5–2% as effective as 2-5A in activation of the endonuclease. It would appear that the presence of a 5′-triphosphate moiety can, to some extent, compensate for a loss of binding affinity seen in the 5′-monophosphate, p5′A-2′p5′U2′p5′A. The normalized binding/activation ratio, R_n (Table 5) was 0.07, which indicated that even though this analog did bind to the endonuclease, there seemed to be some role for ade_2 in RNase L activation. This result also was consistent with previous work which demonstrated that substitution of either inosine of 7-deazaadenosine for ade_2 led to only a 2- to 3-fold drop in RNase L binding (*6, 8*). Usually, such analogs could also activate RNase L effectively. When, however, the sugar moiety of the middle nucleotide of 2-5A trimer was altered to 3′-deoxyribose, RNase L binding decreased nearly 10-fold and activation dropped about 500- to 1000-fold (*9*). One apparent anomaly is the case in which ade_2 was replaced by 8-bromoadenine (*20*). The resultant oligonucleotide, p5′A2′p5′(br^8A)2′p5′A, bound to RNase L with but 1% of the avidity of 2-5A. Yet the disubstituted analog, p5′A2′p5′(br^8A)2′p5′(br^8A), was bound to RNase L very effectively. While it has been established that such 8-bromoadenosine-substituted 2′,5′-linked oligoadenylates have *syn* as opposed to the more usual *anti* glycosidic torsion angles and accompanying alterations in sugar conformation (*21*), it is presently not possible to sort out how such modifications may act in a cooperative process in the phenomenon of oligonucleotide binding to RNase L.

The most dramatic result was obtained when the 2′-terminal nucleotide base, ade_3, was replaced by uracil. Binding was diminished only 10- to 30-fold, but the ability of ppp5′A2′p5′A2′p5′U to activate RNase L was virtually undetectable. Thus, even though ppp5′A2′p5′A2′p5′U could bind to the 2-5A-dependent endonuclease more effectively than any other analog studied in the current trimer series, the binding was nonfunctional. This finding is entirely consistent with an earlier study which showed that the simple substitution of inosine for adenosine in the 2′-terminal nucleotide of 2-5A trimer gave rise to a 3-fold decrease in binding but a >10,000-fold drop in RNase L activation ability (*6*).

The 2′,5′-linked oligonucleotide degradation study provided a result that paralleled those of earlier studies (*17, 22*); specifically, the trimer, p5′A2′p5′U2′p5′A, with the uracil substitution in the penultimate position, was the most readily degraded akin to the instances of 3′-deoxyadenosine/adenosine analogs and xyloadenosine/adenosine analogs. This result thus reinforces the previous speculation (*17, 22*) that the 2′,5′-phosphodiesterase may be able to recognize determinants on the penultimate AMP unit of a 2′,5′-oligonucleotide.

Because p5′A2′p5′U2′p5′A was markedly more sensitive to degradation than p5′A2′p5′A2′p5′A or the other trimeric analogs, we became concerned that this relative instability may influence our results even though HPLC studies (at relatively high concentrations of substrate) demonstrated persistence of the material under both radioligand binding and core cellulose assay conditions. Since we have previously shown that 2′,5′-oligoadenylates can be stabilized against 2′,5′-phos-

phodiesterase activities by modification of the 2'-terminal ribose moiety (*18*) we applied this procedure to a tetrameric analog containing the ade_2 to uracil transformation. We chose to execute the necessary reactions of periodate oxidation, cyclic hemialdal formation, and cyanoborohydride reduction on p5'A2'p5'U-2'p5'A2'p5'A rather than trimer, in order to maximize binding to the endonuclease. The resultant product, ppp5'A2'p5'U2'p5'A2'p5'(ahp)ade, was extremely stable ($t_{1/2} \gg 2$ h) under the most demanding conditions, those of the protein synthesis assay. Both the 2'-terminally modified product and its parent tetramer, p5'A2'p5'U2'p5'A2'p5'A, were considerably more effective as inhibitors of translation (20–30x), both bound to RNase L somewhat more effectively, but neither showed a significant improvement in nuclease activation ability in the core cellulose assay. Parenthetically it may be noted that while the unmodified tetramer, p5'A2'p5'U2'p5'A2'p5'A, was virtually as active as the 2',5'-phosphodiesterase-resistant congener even though its $t_{1/2}$ was only 75 min. This may be related to the observation (not shown) that the first degradation production of p5'A2'p5'U-2'p5'A2'p5'A was the active (as triphosphate) trimer p5'A2'p5'U2'p5'A. The conclusion to be reached by study of these tetrameric analogs of 2-5A is that even when phosphodiesterase degradation is minimal, such analogs still show only 3–30% of the nuclease activation ability of 2-5A itself.

This conclusion, reached on the basis of comparison of IC_{50} values, is further reinforced when an additional behavior of the adenine-modified oligomers is considered. This pertains to the inability of such analogs (ppp5'A2'p5'U2'p5'A, ppp5'A2'p5'U2'p5'A2'p5'A, and ppp5'A2'p5'U2'p5'A2'p5'(ahp)ade) to effect the same maximal level of RNA degradation or protein synthesis inhibition as 2-5A and the other analog with activity (pppU2'p5'A2'p5'A). The exact basis of this differential behavior has yet to be ascertained, but it may be related to the rate of RNA degradation. Certain derivatives of 2-5A may not be able to bring about an optimal conformational change to achieve full catalytic activity of RNase L. In any case, such submaximal activation further implies that the ade_2 to uracil conversion results in a suboptimal interaction with RNase L.

The data presented herein demonstrated that while the adenine ring (ade_2) of the second nucleotide from the 5'-terminus of 2-5A trimer is not a *vital* requirement for activation of RNase L, it does play a significant role. Even though the adenine to uracil modification did not lead to a significant drop in binding of the *triphosphates* to RNase L, ppp5'A2'p5'U2'p5'A and its congeners were an order of magnitude less effective at nuclease activation than 2-5A itself. Although replacement of ade_2 of 2-5A by uracil does lead to an analog with diminished biological activity, it is clear that such derivatives, ppp5'A2'pU2'p5'A2'p5'A and ppp5'A-2'p5'U2'p5'A2'p5'(aph)ade, still possess considerable activity since they can inhibit translation at concentrations in the range of 10^{-8} M. This information may prove of use in the future design of novel analogs of 2-5A.

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