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A new and potent 2-5A analogue which does not require a 5′-polyphosphate to activate mouse L-cell RNase L

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Summary

In order to explore the possibility of supplanting the requirement of a 5′-triphosphate moiety for the activation of the 2-5A-dependent endonuclease (RNase L) of mouse L-cells, two new tetrameric analogues of 2-5A were synthesized. The first tetramer, obtained by both a modified prebiotic synthetic approach as well as a phosphite triester solid phase oligonucleotide synthesis method, was p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A). The second oligonucleotide was derived from the former by a sequence involving periodate oxidation, reaction with *n*-hexylamine, and cyanoborohydride reduction, resulting in conversion of the 2′-terminal adenosine residue to 9-(3′-aza-4′-hexyl-1′,2′,3′,4′-tetradeoxyhexopyranos-1′-yl)-8-bromoadenine. Both of these oligomers, bearing only 5′-monophosphate groups, were found to be as potent as 2-5A itself as activators of the RNase L of mouse L-cells.

Interferon; Antiviral agent; Protein synthesis inhibition

Abbreviations: 2-5A, ppp5′A2′p5′A2′p5′A; HPLC, high performance liquid chromatography; RNase L, 2-5A-dependent endonuclease. The 2-5A derivative in which the 2′-terminal adenosine has been replaced with 9-(3′-aza-4′-hexyl-1′,2′,3′,4′-tetradeoxyhexopyranosyl-1′-yl)-8-bromoadenine is abbreviated as p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$ where ahp stands for azahexapyranose.

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Introduction

2′,5′-Oligoadenylate (2-5A) synthetase is an enzyme which polymerizes ATP into 2-5A (Kerr and Brown, 1978). This enzyme can be induced by treatment of cells with a variety of agents including interferon, platelet-derived growth factor, nerve growth factor, epidermal growth factor, tumor necrosis factor, retinoic acid, theophylline, and dexamethasone (Torrence, 1985; Johnston and Torrence, 1984; Luxembourg, 1988). A variety of physiological situations also lead to elevation of levels of 2′,5′-oligoadenylate synthetase, including estrogen withdrawal from chick oviduct, hepatic regeneration, heat shock, inflammation, cell differentiation and viral and autoimmune disease (Torrence, 1985; Johnston and Torrence, 1984; Luxembourg, 1988). The 2-5A synthetase, 2-5A itself, and the ribonuclease, RNase L, which becomes activated by 2-5A, and an associated phosphodiesterase are collectively referred to as the 2-5A system. This 2-5A system has been postulated to be involved in interferon's antiviral action (Torrence, 1985; Luxembourg, 1988), hematopoiesis (Orlic et al., 1984), angiogenesis (Feldman et al., 1988), cell differentiation (Krause et al., 1985), cell growth (Kumar and Mendelsohn, 1989), and the expression of human immunodeficiency virus in latently infected cells (Schroeder et al., 1989).

Clearly, it is difficult to assign an exact role to the 2-5A system in the above contexts since the aforementioned agents and actions elicit sundry responses. Most useful would be a direct activator of the 2-5A-dependent ribonuclease (RNase L). The negatively charged highly polar 2-5A molecule itself will not enter cells readily (Torrence, 1985; Luxembourg, 1988). Various techniques to enhance 2-5A uptake such as calcium phosphate coprecipitation, hypertonic salt treatment, or liposomes have not been widely applicable (Johnston and Torrence, 1984; Luxembourg, 1988). An alternate approach to this uptake problem might seek to dispense with as much of the negative charge of the parent 2-5A structure as would be compatible with biological activity. A reasonable place to begin would be the 5′-terminal triphosphate moiety which appears to be a requirement for activation of the mouse RNase L. Herein, we describe the synthesis and behavior of a 2-5A analogue which is a potent activator of RNase L, but which bears only a monophosphate moiety at the 5′-terminus, resulting in a congener with a reduction of two in net nominal negative charge.

Materials and Methods

Except where otherwise indicated, HPLC was carried out using an Ultrasphere ODS column (4.6 × 250 mm, flow rate 1 ml/min), and any of the following solvent systems:

(A) linear gradient of 0 to 30% (in 30 min) of 50% aq. MeOH in 20 mM ammonium phosphate, pH 7.0 with two Beckman 110B solvent delivery modules and a 167 scanning UV detector with chromatograms recorded

simultaneously at 260 and 280 nm;

(B) linear gradient of 0 to 100% (in 30 min) of 75% aq. MeOH in 0.6 M potassium phosphate, pH 5.0 and 5 mM PIC A (Waters) and a Beckman system consisting of two 112 solvent delivery modules and a Knauer variable wavelength UV monitor operating at 260 nm;

(C) as in A, but the gradient was from 0 to 50%.

Proton NMR spectra were measured as approximately 2 mM solutions in D_2O. Samples were treated with Chelex and evaporated 3 × with D_2O.

Conditions of enzymatic digestions:

(a) Nuclease P_1: 1 A_{260} of the oligonucleotide in 50 mM sodium acetate, pH 5.0, 0.005 U of the enzyme (Pharmacia), 50 μl total volume, incubated for 90 min at 37°C. Under the same conditions, 3',5'-linked $A(pA)_4$ was completely digested in 30 min.

(b) Nuclease T_2: 1 A_{260} of the oligonucleotide in 50 mM sodium acetate, pH 4.5, 2 mM EDTA, 6 U of the enzyme (BRL), 50 μl total volume, incubated for 2 h at 37°C. Under the same conditions, 3',5'-linked $A(pA)_4$ was completely digested.

(c) Snake venom phosphodiesterase: 0.4 A_{260} of the oligonucleotide, 0.8 mM Tris/HCl, pH 7.5, 0.8 mM $MgCl_2$, 0.05 U of the enzyme (Cooper Biochemical), 100 μl total volume, incubated for 2 h at 37°C.

Extinction coefficient

The extinction coefficient of p5'A2'p5'A2'p5'(br^8A)2'p5'(br^8A) at 260 nm was determined by means of HPLC, by quantitation of the snake venom phosphodiesterase digestion products (5'-AMP and 8-bromo-5'-AMP) of the oligonucleotide sample of known optical density. The value of extinction coefficient at 260 nm was found to be 48,400.

Biochemical assays to evaluate the synthetic oligonucleotides were carried out as described earlier (Torrence and Friedman, 1979; Knight et al., 1981; Silverman, 1985; Torrence et al., 1988).

Preparation of Tetramer 5'-Monophosphoryladenylyl(2'->5')adenylyl(2'-5')-8-bromoadenylyl(2'->5')-8-bromoadenosine, p5'A2'p5'A2'p5'-(br^8A)2'p5'(br^8A)

(1) Preparation of Trimer, p5'A2'p5'(br^8A)2'p5'(br^8A)

The dinucleotide, p5'(br^8A)2'p5'(br^8A), described earlier (Lesiak and Torrence, 1987), was converted into the corresponding 5'-phosphoroimidazolidate in the usual manner (Lesiak and Torrence, 1987). The product, Imp5'(br^8A)2'p5'(br^8A) (1650 A_{max} units, 0.063 mmol) was reacted with MopA (adenosine 5'-phosphoromorpholidate (3500 A_{260} units, 0.23 mmol) in N-methylimidazolium nitrate buffer (0.2 M, 2.5 ml, pH 7.5) in the presence of the addition of 0.25 ml of lead nitrate (0.25 M). The reaction mixture was stirred at 4°C for 3 days. Chelex ion exchange resin was added to remove Pb^{2+} and the resulting solution was then applied to a DEAE Sephadex A-25 column

TABLE 1

Retention times of oligonucleotides in 3 different HPLC systems
See text for details

Conditions	Retention time (min)
A	21.0
B	21.5
C	16.8

which was eluted with a linear gradient of 0.05 M–0.50 M triethylammonium bicarbonate (TEAB). The yield of p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) was 550 A_{260} units or 22.2% based on input bromoadenosine dimer. Also obtained was starting dimer, p5′br^{8}A2′p5′br^{8}A(750 A_{260} units, 45.5%).

(2) Preparation of the Tetramer, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A)

The imidazolidate of p5′A2′p5′br^{8}A2′p5′br^{8}A was prepared by reaction of the trimer monophosphate with triphenylphosphine, imidazole, and dipyridyldisulfide according to previously described methodology (Lesiak and Torrence, 1987). The phosphoroimidazolidate trimer, Imp5′A2′p5′br^8-A2′p5′br^{8}A (400 A_{260} units, 0.011 mmol) was reacted with adenosine 5′-phosphoromorpholidate (1000 A_{260} units, 0.066 mmol) in N-methylimidazolium nitrate buffer (0.2 M, pH 7.5, 3.0 ml) to which 100 μl lead nitrate solution (0.25 M) was added. The reaction mixture was stirred for 2 days at 4°C. The mixture was then treated first with Chelex resin, then with acetic acid, and finally with nuclease P_1. The mixture of oligomers was separated on a DEAE-Sephadex A-25 column by elution with 0.05 M–0.60 M TEAB (1L + 1L). The yield of tetramer, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A), was 128 A_{260} units (22.5%). The starting trimer was recovered in 26.5% yield (106 A_{260} units). The tetramer product behaved identically to the product obtained from the solid phase synthesis. Both oligonucleotides had identical retention times in 3 different HPLC systems (vide ante) (see Table 1).

Only one peak (conditions A and B) was observed when oligonucleotide, obtained from solid-phase synthesis, was co-injected with the same compound obtained by the modified prebiotic synthesis approach. In addition, the A_{260}/A_{280} ratio (measured with the UV detector) was identical for these two products.

Proton NMR provided the following relevant data used to corroborate structure (δ in ppm); 8.27, 8.13, 7.99, 7.95, 7.89, 7.81 (each s, 1H, base aromatic protons); 6.07 (d, 1H, $J_{1'2'}$ = 3.3 Hz), 5.83 (d, 1H, $J_{1'2'}$ = 2.9 Hz), 5.77 (d, 1H, $J_{1'2'}$ = 1.7 Hz), and 5.75 (d, 1H, $J_{1'2'}$ = 6.4 Hz), all anomeric protons.

Synthesis of 'tailed' p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A), 5′-monophosphoryladenylyl(2′->5′)adenylyl(2′->5′)-8-bromoadenylyl(2′->6′)(3′-aza-4′-hexyl-1′,2′,3′,4′-tetradeoxy-hexopyranosyl-1′-yl)-8-bromoadenosine

Sodium periodate solution (0.1 M, 35 μl) was added to an aqueous solution

of p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) (135 A_{260} units, 0.0025 mmol), and the resulting mixture was kept in the dark at 0°C for 30 min.

Ethylene glycol in water (1 M, 3.5 μl) was added to destroy remaining periodate. After an additional 5 min at 0°C, an aqueous *n*-hexylamine solution (0.05 M, 270 μl) was added and the pH of the mixture was adjusted to 8.5 with 0.1% acetic acid. After an additional 1.5 h at 0°C, 10 μl of a solution (0.5 M) of sodium cyanoborohydride was added and the pH was lowered to 6.5 with acetic acid. After 3 h, the reaction mixture was diluted 10 × with water, and the resultant solution was applied to a column of DEAE-Sephadex A-25 which was eluted with a gradient of 0.05 M/0.50 M TEAB, pH 7.5, 500 ml + 500 ml. The yield was 106 A_{260} units (78%). This product was purified further by HPLC on a column (7.8 × 300 mm) of μBondapak-C_{18} using a program of 0–50% buffer B in buffer A in 20 min, where A was 50 mM ammonium phosphate, pH 7, and buffer B was 50% methanol in H_2O. The flowrate was 2 ml/min. The tetramer product was desalted on a DEAE Sephadex column using a 0.2 M–0.6 M gradient of TEAB (pH 7.5, total of 500 ml). The final yield was 40 A_{260} units.

Synthesis of triphosphates

(a) pppA2′p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) The triethylammonium salt of p5′A2′p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) (30 A_{260} units, 6 μmol) was dried by repeated addition and evaporation of dry DMF and then finally dissolved in a mixture of DMF (1 ml) and DMSO (0.1 ml). Triphenylphosphine (5 mg, 0.011 mmol), imidazole (3 mg, 0.044 mmol) and 2,2′-dipyridyldisulfide (3 mg, 0.014 mmol) were added to this solution, and the reaction mixture was stirred at room temperature for 1.5 h. Subsequently, the solution was poured into a sodium iodide/acetone solution (0.1 M). The precipitate of sodium salt was collected by centrifugation and washed several times with fresh acetone. This product was dried under vacuum, and then it was dissolved in tri-*n*-butylammonium pyro-phosphate in DMF (150 μl of a 0.5 M solution). After a reaction time of 20 h at room temperature, the mixture was diluted with water and applied to a column of DEAE-Sephadex A25. Elution was accomplished with a gradient of 0.1 M/0.6 M TEAB, pH 7.5 (250 ml + 250 ml). The final yield was 13.5 A_{260} units of ppp5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) (45%) with 8 A_{260} units of recovered tetramer monophosphate.

(b) ppp5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$ In a similar manner to the previous preparation, 40 A_{260} units (0.75 μmol) of p5′A2′p5′A2′p5′(br^{8}A)-2′p5′(br^{8}ade)$_{ahp}$ was converted to the corresponding 5′-triphosphate in 75% yield (20 A_{260} units).

Results

Preparation of the 2′,5′-oligonucleotides

Synthesis of the tetramer, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A), was based on

the approach developed previously (Imai and Torrence, 1985; Lesiak and Torrence, 1986, 1987). The dimer imidazolidate, Imp5′(br^{8}A)2′p5′(br^{8}A), was coupled to the morpholidate of adenosine, Mop5′A, using lead ion catalysis. After acidic hydrolysis, the trimer, p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) was produced. This trimer was in turn converted to the imidazolidate which, in a second cycle. was coupled to Mop5′A to yield, after hydrolysis, the desired tetramer. The reaction sequence of oxidation with sodium periodate, condensation with hexylamine, and reduction with sodium cyanoborohydride, gave the 'tailed' tetramer, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$. Both of these 5′-monophosphates were readily converted to the corresponding 5′-triphosphates by reaction of pyrophosphate with the 5′-phosphoroimidazolidates.

Corroboration of structure for the synthetic products was obtained both by enzyme digestion and by NMR. The oligomer p5′A2′p5′A2′p5′(br^{8}A)2′-p5′(br^{8}A) was resistant to the action of nuclease P_1 and RNase T_2, but was degraded by snake venom phosphodiesterase to give only two detectable products upon HPLC analysis; namely, 5′AMP and 8-bromo-5′-AMP in the expected 1:1 ratio. The 'tailed' tetramer, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$, was degraded by the snake venom enzyme to yield 3 products, 5′-AMP, 8-bromo-5′-AMP, and a third nucleotide corresponding to p5′(br^{8}ade)$_{ahp}$. The ratio of the 3 degradation products was 2:1:0.8, respectively. Snake venom digestion of the corresponding 5′-triphosphates gave similar results to the monophosphates. Bacterial alkaline phosphatase digestion of p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) and its corresponding 5′-triphosphate, provided the same product which possessed an HPLC retention time characteristic of 'core' (5′-unphosphorylated) tetramer. Likewise alkaline phosphatase treatment of p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$ and its triphosphate produced the same oligomer product, different from the above product obtained from (pp)p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A). In the proton NMR, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) displayed a total of 6 singlet resonances from 7.81 ppm to 8.27 ppm, each integrating to one proton. These correspond to the purine C2 protons of the two 8-bromoadenosine moieties and the C2 and C8 hydrogens of the two adenosine residues. The anomeric protons were accounted for as 4 doublets at 6.07, 5.83, 5.77 ppm and 5.75 ppm.

In a wholly different and independent approach, the above tetrameric oligomer, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A), was prepared by the solid-phase phosphite-triester method of oligonucleotide synthesis as modified for the synthesis of oligoribonucleotides with 2′-5′ internucleotide bonds (K. Lesiak, B. Uznanski and P.F. Torrence, manuscript in preparation). The synthesis was carried out on a controlled pore long chain alkylamine glass support with N_6-protected 8-bromoadenosine attached through a 3′-O-succinyl linkage. Synthesis was in the 2′ to 5′ direction using 2′-O-[5′-O-dimethoxytrityl-3′-O-tert-butyldimethylsilyl-N^6-benzoyladenosine (or di-*n*-butylaminomethylene-8-bromoadenosine)]-2-cyanoethyl-*N*,*N*-diisopropyl phosphoramidites as building blocks for chain elongation. Before deprotection, the oligonucleotide was 5′-end phosphorylated with *bis*-(O-2-cyanoethyl)-*N*,*N*-diisopropyl phosphorami-

dite. The product, obtained in 47% yield, was purified by HPLC and identified by means of analyses of its enzymatic hydrolysis products (snake venom phosphodiesterase, nuclease P_1 and ribonuclease T_2) and proton NMR spectra. This product was also identical, as determined by HPLC using 3 different solvent systems, with the oligonucleotide synthesized by controlled 'prebiotic synthesis' approach (Imai and Torrence, 1985) as outlined above.

Inhibition of translation by the bromoadenylate triphosphates

Both of the synthetic oligobromoadenylate 5'-triphosphates were evaluated for their capacity to act as inhibitors of protein synthesis in an EMCV RNA-directed mouse L-cell-free system. 2-5A trimer triphosphate was employed as a control. The newly synthesized oligomers were potent inhibitors of translation. In a series of experiments, the following mean values for the concentration necessary to effect half maximal inhibition were obtained: ppp5'A2'p5'A2'p5'A, $6 \pm 2 \times 10^{-10}$ M; ppp5'A2'p5'A2'p5'(br^8A)2'p5'(br^8A), 2×10^{-10} M; ppp5'-A2'p5'A2'p5'(br^8A)2'p5'(br^8ade)$_{ahp}$, $3 \pm 1 \times 10^{-10}$ M. Thus both of the analogues were at least as potent as 2-5A trimer triphosphate itself as inhibitors of protein synthesis. Moreover, in contrast to the behavior of the corresponding 5'-monophosphates described below, both of the bromoadenylate triphosphates caused the same degree of maximal translational inhibition as 2-5A itself (data not presented).

Inhibition of protein synthesis by bromoadenylate 5'-monophosphates

Fig. 1 presents the results of experiments in which varying concentrations of 3 different oligonucleotide 5'-monophosphates were examined for their ability to block protein synthesis in the EMCV RNA-programmed mouse L-cell-free system (Torrence and Friedman, 1979). All 3 compounds, ppp5'A2'p5'A2'p5'A, p5'A2'p5'A2'p5'(br^8A)2'p5'(br^8A), and the 'tailed' bromoadenylate monophosphate achieved their maximal effect at about 10^{-8} M, and brought about a half-maximal inhibition of translation at about 5×10^{-10} M. These experiments were repeated a number of times to yield the following mean values for the concentration to effect one half of the maximum observed inhibition (for that agent): ppp5'A2'p5'A2'p5'A, $6 \pm 2 \times 10^{-10}$ M; p5'A2'p5'-A2'p5'(br^8A)2'p5'(br^8ade)$_{ahp}$, $7 \pm 4 \times 10^{-10}$ M; p5'A2'p-5'A2'p5'(br^8A)2'-p5'(br^8A), $3 \pm 1 \times 10^{-10}$ M. The inset of Fig. 1 depicts the behavior of the parent tetramer p5'A2'p5'A2'p5'A2'p5'A under the identical conditions. It was evident that this material was incapable of inhibiting protein synthesis at any tested concentration. Also evident from the experiments of Fig. 1 was the somewhat anomalous observation that the various oligomers differed in the extent to which they could reduce protein synthesis. Thus the trimer triphosphate caused about a maximum 70% reduction in translation, the bromoadenylate p5'A2'p5'A2'p5'(br^8A)2'p5'(br^8A) effected only a 35% maximum inhibition, and the 'tailed' bromo-adenylate brought about a 50% maximum inhibition. The basis for this behavior was examined in the experiments reported below.

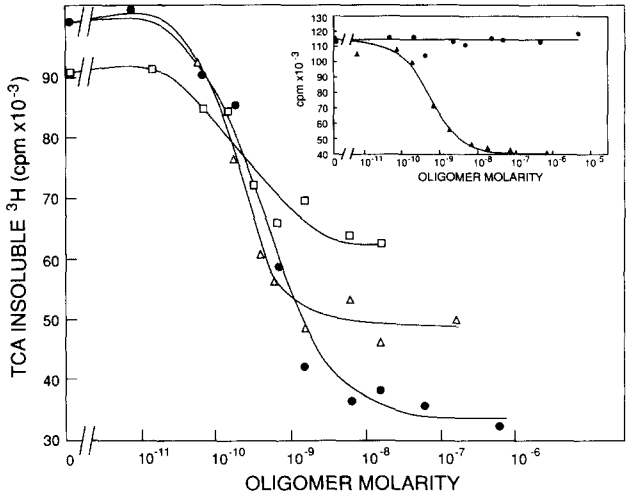

Fig. 1. Inhibition of protein synthesis in extracts of mouse L-cells programmed with encephalomyocarditis virus RNA (10). At time 0, oligonucleotide in the indicated concentrations was added to the reaction mixtures containing [^{3}H]leucine. After 120 min incubation, aliquots were withdrawn and analyzed for trichloroacetic acid-insoluble radioactivity after filtration on glass fiber discs. Controls were prepared containing water in place of oligonucleotide. The compounds evaluated were: ●, ppp5′A2′p5′A2′p5′A; □, p5′A2′p5′A2′p5(br^{8}A)2′p5′(br^{8}A); △, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$. The inset shows the effect of p5′A2′p5′A2′p5′A2′p5′A (●) on translation under identical conditions with ppp5′A2′p5′A2′p5′A (▲) reference.

Kinetics of protein synthesis inhibition

To explore the basis for the inability of p5′A2′p5′A2′p5′(br^{8}A)2′p5′br^{8}A) to bring about the same extent of protein synthesis inhibition as 2-5A trimer triphosphate itself, a study was made of the kinetics of protein synthesis in the presence or absence of the oligonucleotides in question. In this experiment, the 2′-5′-linked oligonucleotides were added to protein synthesis reaction mixtures at a final concentration of 6.5×10^{-7} M. Aliquots were removed at the indicated times and TCA-insoluble radioactivity was determined. The results are presented in Fig. 2. Under these conditions, it was clear that the difference in the final extent of translational inhibition was due to differing times of onset of protein synthesis shutdown. For at least 15 min, no inhibition of synthesis was observed to be caused by any inhibitor. However, at 30 min, the effect of 2-5A trimer was clearly seen and shutdown was total by 45 min. In contrast, the oligomer p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) did not effect complete shutdown of translation until after 90 min. Noteworthy also was the fact that the tetramer monophosphate p5′A2′p5′A2′p5′A2′p5′A had absolutely no effect on translation at any time. Similar data were obtained for the 'tailed' derivative of the bromo tetramer, except that the kinetic curve was intermediate between 2-5A trimer and the unmodified bromoadenylate tetramer (data not shown).

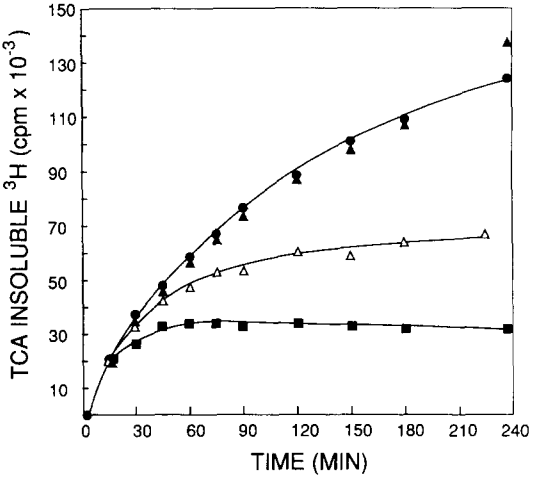

Fig. 2. Kinetics of protein synthesis in the system of Fig. 1. A series of reaction mixtures were composed containing the indicated oligomer at a concentration of 6.5×10^{-7} M. At the indicated times aliquots were removed and analyzed for TCA-insoluble radioactivity. The oligomers were as follows: ▲, water control; ●, p5′A2′p5′A2′p5′A2′p5′A; ■, ppp5′A2′p5′A2′p5′A; △, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A).

Binding of the oligonucleotides to RNase L

The ability of the two bromoadenylate tetramer monophosphates to bind to the 2-5A-dependent RNase was examined using the standard assay that measures the capacity of an analogue to displace a radiolabelled probe, ppp5′A2′p5′A2′p5′A2′p5′A3′p5′C3′p, from the RNase L (Knight et al., 1981). As could be expected from the results of the protein synthesis inhibition experiments, the brominated analogues were bound to RNase L with about the same affinity as was 2-5A trimer triphosphate itself. The experimentally determined IC_{50}'s (concentration to displace 50% of bound probe), ± standard deviations were as follows: ppp5′A2′p5′A2′p5′A, $6 \pm 1 \times 10^{-10}$ M; p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A), $3 \pm 1 \times 10^{-10}$ M; p5′A2′p5′A2′-p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$, $1 \pm 0.2 \times 10^{-10}$ M. Similar results were obtained for p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) when mouse liver was the source of RNase L (data not shown).

Ability of the oligonucleotides to activate RNase L as determined by their ability to stimulate the degradation of RNA

Another method to determine how effectively a given 2-5A analogue can activate RNase L is due to Silverman (1985). In this approach, the RNase L was adsorbed from a crude extract onto 2-5A core cellulose (i.e., A2′p5′A2′p5′A2′p5′A linked to cellulose). This resulted in a substantial purification of the RNase. Activator (2-5A or an analogue) was then added to the adsorbed enzyme and the degradation of an RNA substrate (poly(U)[^{32}P]pCp) then could be ascertained. Typical results obtained for the bromoadenylate monophosphate are given in Fig. 3. It was clear that both the

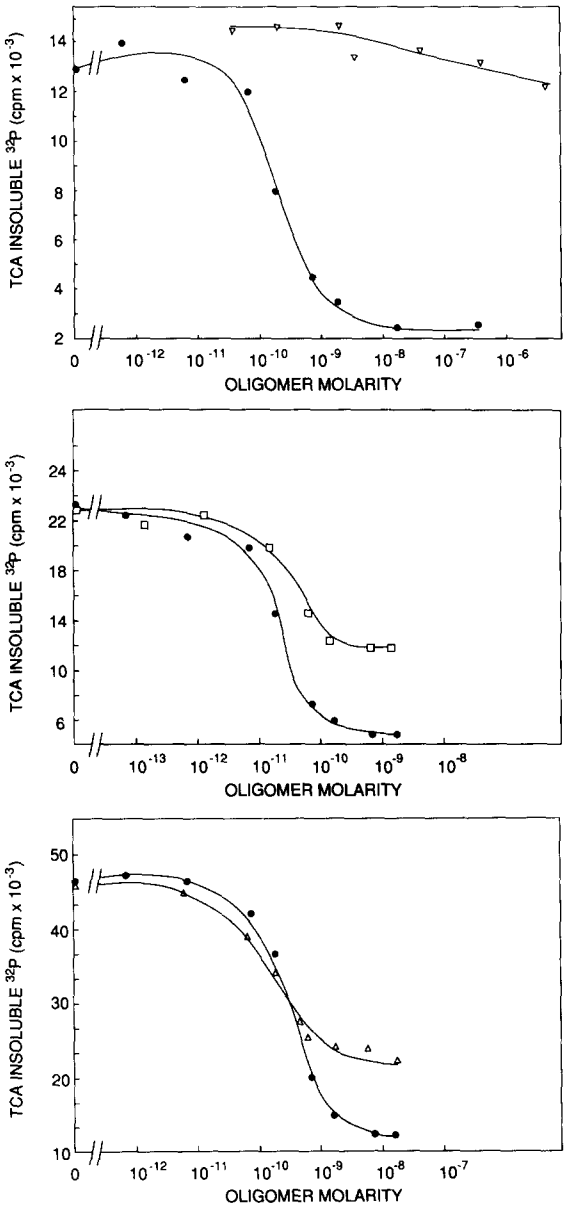

Fig. 3. Ability of oligonucleotides to stimulate the degradation of RNA. Oligomers were added, in the indicated concentrations, to reaction mixtures containing mouse L-cell RNase L absorbed to 2-5A cellulose and containing the nuclease substrate poly(U)[^{32}P]pCp. After a 2-h incubation, aliquots were analyzed for TCA-insoluble radioactivity. Top: ▽, p5′A2′p5′A2′p5′A2′p5′A vs ppp5′A2′p5′A2′p5′A (●) reference; middle: □, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) vs ppp5′A2′p5′A2′p5′A (●) as reference; bottom: △, p5′A2′p5′2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$ vs ppp5′A2′p5′A2′p5′A (●) as reference.

'tailed' and unmodified bromoadenylate tetramer 5′-monophosphates were as

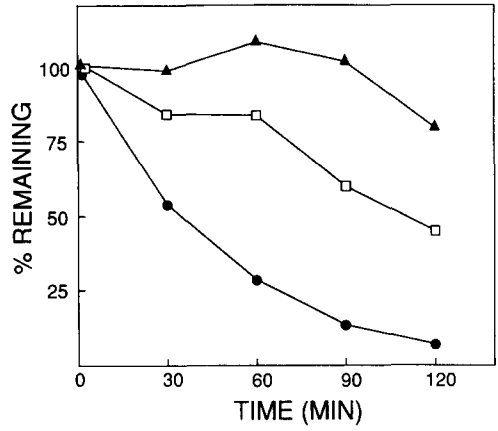

Fig. 4. Degradation of oligonucleotides in extracts of mouse L-cells under conditions of protein synthesis except that no mRNA or [^{3}H]leucine were added. Oligomers were added to reaction mixtures in a concentration of 5×10^{-5} M. Incubation was at 30°C. Aliquots were withdrawn at the indicated times, heated at 100°C to denature proteins, and then, after centrifugation, the supernatant was analyzed by HPLC. The oligonucleotides were: ●, p5′A2′p5′A2′p5′A2′p5′A; □, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A; ▲, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A)$_{ahp}$.

effective as 2-5A trimer triphosphate itself as activators of RNase L. In a series of experiments, the values (± standard deviation) for the concentration of oligomer needed to effect half-maximal degradation of RNA were as follows: ppp5′A2′p5′A2′p5′A, $3 \pm 0.5 \times 10^{-10}$ M; p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A, $4 \pm 1 \times 10^{-10}$ M; p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$, $2.5 \pm 0.5 \times 10^{-10}$ M, p5′A2′p5′A2′p5′A2′p5′A, $\gg 4 \times 10^{-6}$ M. As was true in the experiments with inhibition of translation, the two bromo analogues failed to cause as extensive an effect as did 2-5A itself. Thus, both analogues effected about a 50% increase in TCA-soluble radioactivity whereas 2-5A caused a 70–80% increase.

Not unexpectedly, the corresponding 5′-triphosphates of the bromoadenylates were potent activators of RNA degradation. Thus, the concentrations for half-maximum degradation of poly(U)[^{32}P]pCp were: $5 \times 10^{-11} \pm 3 \times 10^{-11}$ M for ppp5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) and $8 \times 10^{-11} \pm 3 \times 10^{-11}$ M for ppp5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$ (data not illustrated). Both of these triphosphates, in distinct contrast to their monophosphate precursors, brought about the same extent (70–80%) of RNA degradation as did 2-5A trimer triphosphate. These data suggest that the 5′-triphosphates can effect an additional increase in binding/activation efficiency as compared to the corresponding 5′-monophosphates of the brominated analogues.

The oligonucleotide, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A), was evaluated for its ability to activate RNase L of mouse liver, human Daudi cells, and human CEM cells, using the same type of assay. Under such conditions, it was found consistently to be at least as potent as 2-5A itself (data not presented).

Degradation of the analogues under protein synthesis conditions

The stabilities of the two bromoadenylate monophosphates were determined under the above protein synthesis assay conditions wherein 2-5A itself is most labile due to action of a 2′,5′-phosphodiesterase activity (Fig. 4). In accord with earlier studies (Imai et al., 1982), the half-life of the unmodified 2′,5′-oligoadenylate, in this case p5′A2′p5′A2′p5′A2′p5′A, was approximately 30 min under these conditions. Modification of adenosine to 8-bromoadenosine significantly extended the lifetime ($t_{1/2}$ ~120 min) of the resulting congener as had been observed previously (Lesiak and Torrence, 1987). Finally, the 'tailed' oligonucleotide, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$, was the most stable with a $t_{1/2}$ considerably in excess of 120 min.

Discussion

The RNase L of mouse L-cells generally seems to have a distinct requirement for the presence of a 5′-terminal triphosphate moiety in order to become an activated endonuclease (Johnston and Torrence, 1984; Luxembourg, 1988). The 5′-diphosphate group has been reported to be able to substitute for this triphosphate (Johnston and Torrence, 1984; Luxembourg, 1988), but the report that the 5′-diphosphate and 5′-triphosphate forms of 2′,5′-oligoadenylates can interconvert under certain conditions complicates interpretation of such findings (Lesiak and Torrence, 1985). Apparently, the 5′-polyphosphate affects only activation of the RNase L since binding of the monophosphate is of the same high affinity as that of the corresponding oligonucleotide 5′-triphosphate. In fact, the 5′-monophosphates p5′A2′p5′A2′p5′A2′p5′A and p5′A2′p5′A2′p5′A, which are at best poor activators of the mouse L-cell RNase L, can be used as antagonists of 2-5A action since they bind to, but do not activate, the enzyme (Torrence et al., 1981; Miyamoto et al., 1983). The 5′-unphosphorylated 2′,5′-oligoadenylates, such as A2′p5′A2′p5′A, bind very poorly to RNase L (Torrence et al., 1984). These requirements regarding the nature of the phosphate residue at the 5′-terminus of RNase L also hold for the enzyme of rabbit reticulocytes (P.F. Torrence, unpublished observations). The enzyme from human cells, however, behaves significantly differently since the activity from extracts of HeLa or Daudi cells does not seem to require a 5′-di- or 5′-triphosphate for effective activation (Haugh et al., 1983; Imai et al., 1985). A 5′-monophosphate alone will suffice.

The observation (Fig. 2) that there was a difference in the kinetics of RNA degradation induced by p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) as compared to 2-5A (ppp5′A2′p5′A2′p5′A) itself suggests the following possibility. If the ability of 2-5A or some analogue to activate the latent ribonuclease activity of RNase L is related to an induced conformational change in the protein, then perhaps different oligomers may be able to effect the required conformational change, albeit not always achieving the optimal state for maximum catalytic activity. In this view, there may exist some range of RNase L enzyme conformations

capable of ribonucleolytic activity, but differing in their relative catalytic efficiency.

Earlier studies have shown that dramatic variations in the RNase L binding and activating abilities of 2′,5′-oligoadenylates can be achieved by replacement by bromine of the purine 8 hydrogen in one or more adenylate residues (Lesiak and Torrence, 1986, 1987; Krause et al., 1986). When 5′-di- or 5′-triphosphates were examined, it was found that substitution of 8-bromoadenosine for adenosine in either the first or second (from the 5′ terminus) residue of (p)pp5′A2′p5′A2′p5′A led to greater than 100-fold losses of biological activity related to a decrease in binding to RNase L. These effects have been related to oligonucleotide conformation (Van den Hoogen et al., 1989). Replacement by bromine at the purine 8 position of the third nucleotide of ppA2′p5′A2′p5′A resulted in a 10-fold *increase* in RNase L activating ability (Lesiak and Torrence, 1987). Furthermore, in contrast to the situation for unsubstituted adenylates, a number of such brominated oligoadenylates proved able to activate the RNase L of mouse L-cells when they were added to assays as their 5′-monophosphates (Lesiak and Torrence, 1986, 1987; Krause et al., 1989). At this time. the mechanism of this effect has not been solidly established. Such bromoadenylate-substituted oligomers activate RNase L that has been partially purified by an affinity absorption procedure, and β,γ-methylene ATP did not inhibit the activity of the bromo-analogues, suggesting that they may directly activate the nuclease rather than first being phosphorylated to the di- or triphosphate (Krause et al., 1986). More recently, 2′,5′-oligonucleotides containing 8-hydroxy-adenosine also have been shown to possess RNase L activation ability as their 5′-monophosphates (Kanou et al., 1990, 1991).

Regardless of the mechanism underlying the activity of these bromoadenylate analogues of 2-5A monophosphate, they do represent an important step in defining structural characteristics that may be used to obtain a 2-5A derivative that would possess the ability to cross the cell membrane and activate RNase L in intact cells. For this reason, we chose to pursue some of the earlier findings somewhat further to discover if the activity of these bromoadenylates might be improved. One reasonable possibility was to increase the resistance of such congeners to degradation by the 2′,5′-phosphodiesterase. This has been accomplished previously (Imai et al., 1982) by modifying the 2′-terminal ribose of 2-5A tetramer to an *N*-hexylmorpholine-derivative by means of a periodate oxidation, cyclic hemialdal formation with *n*-hexylamine, and then cyanoborohydride reduction. This modification must be carried out on a tetrameric rather than a trimeric species to preserve the maximum RNase L binding and activating ability. We chose the bromo-analogue p5′A2′p5′A2′p5′(br^{8}A) as the logical candidate for this experiment since previously it had been shown to possess significant, albeit reduced compared to 2-5A, activity as an inhibitor of translation. We extended the length of this trimer by adding an 8-bromoadenylate to its 2′-terminus as this provided the most expeditious access using the modified prebiotic synthesis route (Imai and Torrence, 1985) (vide ante).

We found that p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A) itself was an extremely potent inhibitor of protein synthesis and activator of RNase L. In either assay it was as active as 2-5A trimer itself. No significant increase in translational inhibitory potency or ability to enhance RNA degradation was seen when the phosphodiesterase blocking modification was introduced in the analogue, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}ade)$_{ahp}$. It may be noteworthy that the unmodified tetramer itself was significantly more resistant to degradation than parent oligomer, although it was less resistant than the 'tailed' analogue. The oligonucleotide, p5′A2′p5′A2′p5′(br^{8}A)2′p5′(br^{8}A), as a 2-5 analogue that is equipotent with 2-5A but does not possess a 5′-terminal di- or triphosphate moiety, represents a valuable lead to the further manipulation of the 2-5A system. The reduction of overall negative charge achieved by such 8-bromoadenosine-substituted modifications coupled with the 2′-phosphodiesterase-stabilizing and more lipophilic 3′-terminal alterations may presage intact cell experiments.

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