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pppA2′p5′A2′p5′A: An inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells

(cell-free systems/double-stranded RNA/low molecular weight inhibitor/oligomeric series/protein kinase)

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ABSTRACT **A low molecular weight inhibitor of cell-free protein synthesis effective at subnanomolar concentrations is formed on incubation of cytoplasmic extracts from interferon-treated cells with double-stranded RNA and ATP. It can be conveniently synthesized by incubating a poly(I)·poly(C)-Sepharose-bound enzyme fraction from such cells with [^{3}H]- or [α- or γ-^{32}P]ATP. The radioactive inhibitor has been characterized by its behavior on DEAE-Sephadex in the presence of urea and on the basis of the products obtained on enzymic, alkaline, and sequential degradation by periodate oxidation and β elimination. Its structure appears to be pppA2′p5′A2′p5′A. We have found no evidence for any modification or abnormality other than the 2′-5′ linkage. On occasion the inhibitor preparations have included what seems to be the corresponding dimer (pppA2′p5′A), tetramer [ppp(A2′p)$_3$A], pentamer [ppp(A2′p)$_4$A], and higher oligomers in decreasing amounts. The trimer, tetramer, and pentamer are similar in activity, but the dimer is less potent if active at all.**

Protein synthesis in extracts from interferon-treated cells shows an enhanced sensitivity to inhibition by double-stranded RNA (dsRNA) (1, 2). A protein kinase(s), a nuclease(s), and a low molecular weight inhibitor(s) seem likely to be involved (3–8). The kinase is thought to phosphorylate one of the initiation factors in protein synthesis, as appears to be the case in rabbit reticulocyte lysates on inhibition of protein synthesis by a number of agents, including dsRNA (9–13). The roles of the nuclease and low molecular weight inhibitor are not known. The latter is formed in appropriate cell-free systems in response to dsRNA and is effective at subnanomolar concentrations in the inhibition of protein synthesis in reticulocyte lysates or mouse L cell-free systems (3–5). From our previous work it appeared to be an oligonucleotide of unusual structure. It was shown to contain two or more AMP residues in alkali- and snake venom phosphodiesterase (SVPD)-labile linkage, but to be resistant to digestion with a variety of other nucleases (5). A further characterization of the inhibitor is presented, leading to the proposed structure pppA2′p5′A2′p5′A.

MATERIALS AND METHODS

Treatment of mouse L cells with low doses (5–30 effective units/ml) of highly purified ($\geq 10^7$ units/mg of protein) interferon, preparation of extracts from interferon-treated cells, synthesis and radioactive labeling of the oligonucleotide inhibitor, its initial purification on DEAE-cellulose and assay for inhibition of ^{14}C-labeled amino acid incorporation in the L cell-free system programmed with encephalomyocarditis virus RNA, were as previously described (2–5). SVPD (oligonucleate 5′-nucleotidohydrolase, EC 3.1.4.1) from Worthington, further purified by the method of Keller (14), was the generous gift of Beverley Griffin. Bacterial alkaline phosphate (BAP; orthophosphoric-monoester phosphohydrolase, EC 3.1.3.1) was "BAPF" from Worthington. Enzymic digestions were for 1–6 hr at 37° under the conditions of Brownlee (15). Periodate oxidation and β elimination with aniline were according to Frankel-Conrat and Steinschneider (16). DEAE-Sephadex A25 was from Pharmacia. Thin-layer plates of cellulose and polyethyleneimine cellulose (Machery Nagel, Polygram Cel 300, and Polygram Cel 300 PEI) were from Camlab, Cambridge, England. All nucleosides and nucleotides used as chromatographic markers were from either Sigma London or Boehringer Mannheim. All radiochemicals were from The Radiochemical Centre, Amersham, England.

RESULTS

Throughout this work the inhibitor was assayed by its ability to inhibit the translation of encephalomyocarditis virus RNA in cytoplasmic extracts from mouse L cells. It was synthesized and radioactively labeled with a highly purified enzyme fraction from interferon-treated cells. The enzyme bound to poly(I)·poly(C)-Sepharose was washed extensively prior to synthesis of the inhibitor by incubation with 1 mM ATP. As much as 20% of the ATP was incorporated into the inhibitor and ATP appeared to be the only substrate required (4, 5). Inhibitor synthesized with [α- or γ-^{32}P or ^{3}H]ATP will be referred to as [α- or γ-^{32}P or ^{3}H]inhibitor.

DEAE-Sephadex Chromatography. To obtain an estimate of the charge and size of the inhibitor, it was analyzed on DEAE-Sephadex in the presence of 7 M urea (17) with and without prior treatment with BAP to remove terminal phosphate groups. The data in Fig. 1 are for [^{3}H]inhibitor. Similar results were obtained with [α-^{32}P]inhibitor. The inhibitor eluted just before the marker of charge −6 (Fig. 1*A*), and after BAP treatment it eluted just before ApApA of charge −2 (Fig. 1*B*). Inhibitory activity in the cell-free system eluted with the peak of radioactivity at approximately −6 (see below).

Enzymic and Alkaline Digestion. [α-^{32}P]Inhibitor of charge −6 was further characterized by analyzing the products of digestion with BAP and alkali (Fig. 2 *A* and *B*). The ratio of BAP-insensitive to BAP-sensitive radioactive phosphate (α-^{32}P) was 1.97:1 and digestion with BAP followed by alkali yielded ^{32}P-labeled A2′p, A3′p, and ^{32}P$_i$ in a ratio (A2′p + A3′p):P$_i$ of 1.96:1. Further analysis of the material by two-dimensional chromatography confirmed A2′p and A3′p as the only nucleotide products under conditions (same system as was used in Fig. 3*A*) in which the 2′- or 3′-phosphates of U, G, C, or I would have been easily detected. With [^{3}H]inhibitor, digestion with BAP followed by alkali yielded A in addition to A2′p and A3′p

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Abbreviations: dsRNA, double-stranded RNA; SVPD, snake venom phosphodiesterase; BAP, bacterial alkaline phosphatase; TU buffer, 20 mM Tris·HCl (pH 7.6)/7 M urea.

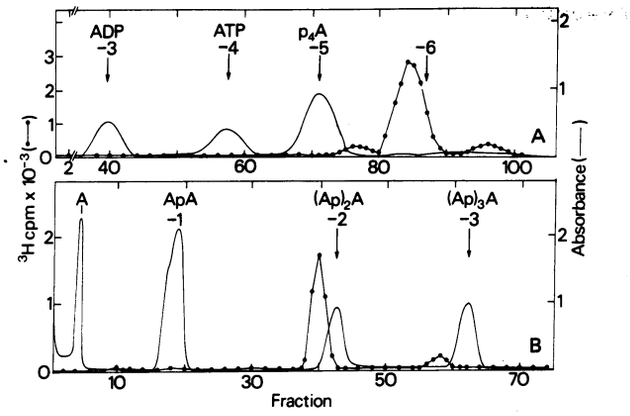

FIG. 1. DEAE-Sephadex chromatography of [^{3}H]inhibitor. (*A*) Inhibitor partially purified on DEAE-cellulose (4) was applied to a column of DEAE-Sephadex A25 (0.5 × 50 cm) equilibrated with 100 mM NaCl in 20 mM Tris·HCl (pH 7.6)/7 M urea (TU buffer). Elution (2.5-ml fractions, 5 ml/hr) was with a gradient of 100–350 mM NaCl (150 ml/150 ml) in TU buffer. ADP, ATP, and $(p)_4A$ were included as markers. The position of the −6 marker was by extrapolation from experiments in which a pancreatic ribonuclease digest of *Escherichia coli* tRNA (15) was employed as marker, as in Fig. 4*A*. (*B*). An aliquot of the inhibitor used in *A* was digested with BAP and applied to an identical column equilibrated in TU buffer. Elution (as above) was with a gradient of 0–300 mM NaCl (100 ml/100 ml) in TU buffer. A, A2′p5′A, A3′p5′A, $(Ap)_2A$, and $(Ap)_3A$ were included as markers. Approximately half as much A2′p5′A was loaded as A3′p5′A, and the shoulder on the ApA peak indicates their partial resolution. ●, [^{3}H]Inhibitor ± BAP (cpm/50-μl sample). —, Absorbance at 260 nm in arbitrary units.

(figure 4 of ref. 5) in accord with the structure ApApA for the BAP-resistant "core" of the molecule. The charge of −2 (Fig. 1*B*) and simplicity of the pattern obtained on alkaline digestion (Fig. 2*B*) excludes di- or polyphosphate or inverted (5′-5′) linkages in the BAP core. The results of a double-label experiment in which [^{3}H]- and [α-^{32}P]AMP were incorporated from ATP in the ratio 1:1 similarly excludes any incorporation of adenine in non-nucleotide linkage.

Nature of the Termini. The inhibitor is sensitive to periodate oxidation, which excludes 2′- or 3′-terminal phosphates. Moreover, it can be labeled with [γ-^{32}P]ATP. In a double-label experiment, 2.9 mol of [^{3}H]AMP were incorporated for each mol of [γ-^{32}P]ATP. All of the γ-^{32}P was BAP sensitive. This, together with the shift in charge from −6 to −2 on BAP treatment (Fig. 1), suggests the presence of a 5′-terminal triphosphate. The release of all of the [γ-^{32}P] from the inhibitor as $^{32}PP_i$ on digestion with SVPD (Fig. 2*C*) provides direct evidence for this and the structure pppApApA. (Digestion of [^{3}H or α-^{32}P]inhibitor with SVPD yielded [^{3}H or α-^{32}P]AMP as the only detectable produce—see below.)

Resistance to Nuclease Digestion. If pppApApA were the structure, the nuclease resistance of the molecule (5) might be explained by the presence of 2′-5′ rather than 3′-5′ linkages (e.g., see ref. 22). In accord with this, commercial A2′p5′A showed the same resistance to P1, T1, T2, U2, pancreatic, spleen, and micrococcal nucleases and sensitivity to SVPD as did the inhibitor or its BAP-resistant core (ref. 5; unpublished results).

Sequential Degradation. Direct evidence for 2′-5′ linkages was provided by analysis of the products formed on sequential degradation of the [α-^{32}P]inhibitor by periodate oxidation and β elimination and digestion with BAP or SVPD carried out according to the following schemes:

$$\text{pppApApA} \xrightarrow{1} \text{pppApAp} \xrightarrow{2} \text{ApA} + P_i \xrightarrow{3} \text{Ap} + P_i$$
$$\text{pppApAp} \xrightarrow{4} \text{pAp} + \text{pA} + PP_i$$

Periodate oxidation followed by β elimination (step 1) and BAP digestion (step 2) yielded A2′p5′A, which was readily distinguished from A3′p5′A by chromatography in three different systems (Fig. 2 *D* tracks 1 and 2, *E*, and *F*). It was further iden-

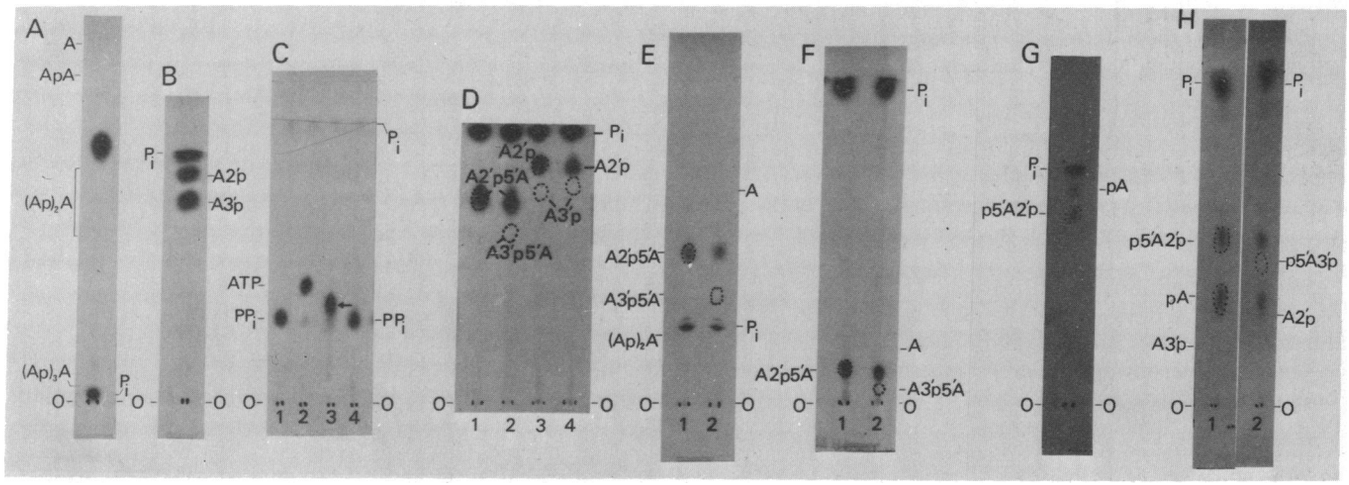

FIG. 2. Enzymic and alkaline digestion and sequential degradation of the inhibitor. [α- or γ-^{32}P]Inhibitors were synthesized as previously and purified on DEAE-cellulose (4). Analysis of the digestion products was by thin-layer chromatography on PEI-cellulose in (*A*) 1 M acetic acid; (*B–D, G*) 0.75 M potassium phosphate at pH 3.4; or (*E*) 0.1 M ammonium bicarbonate; or (*F* and *H*) on cellulose in saturated ammonium sulfate/1 M sodium acetate/isopropanol (80:18:2, vol:vol:vol) (18–21). [α-^{32}P]Inhibitor (from −6 peak, Fig. 4*B*): (*A*) BAP digest; (*B*) BAP digest followed by 0.3 M KOH for 17 hr at 37°; (*C*) [γ-^{32}P]inhibitor (arrowed track 3) and SVPD digest of [γ-^{32}P]inhibitor (track 4). [γ-^{32}P]ATP (track 2) and a SVPD digest of [γ-^{32}P]ATP (track 1) are included for comparison. [α-^{32}P]Inhibitor *D* to *H*: (*D*) tracks 1 and 2; (*E* and *F*) products of one round of periodate oxidation and β elimination followed by BAP digestion. (*D*) tracks 3 and 4, product of a second round of periodate oxidation and β elimination on the same material (BAP was inactivated at 95° for 10 min in 10 mM EDTA prior to the second periodate treatment). (*G* and *H*) tracks 1 and 2: products of one round of periodiate oxidation and β elimination followed by digestion with SVPD. Autoradiographs of the chromatograms are presented. Dotted circles indicate the positions of markers included in the samples analyzed. Other markers were run in parallel. Markers were detected under UV light. For quantitation the chromatograms were cut and their radioactivities were measured in a scintillation counter.

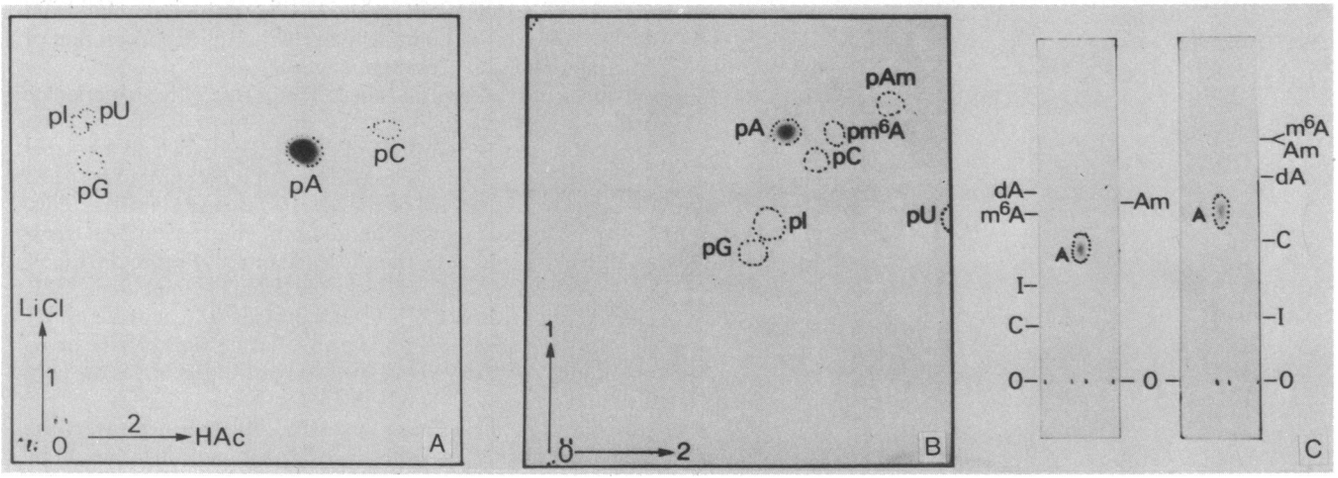

FIG. 3. Products of digestion with SVPD. SVPD digestion of (*A*) [α-^{32}P]inhibitor: two-dimensional chromatography on PEI-cellulose with 1 M LiCl and 1 M acetic acid as solvents (18) and (*B*) [^{3}H]inhibitor: two-dimensional chromatography with (dimension 1) isobutyric acid/0.5 M NH_4OH (5:3, vol/vol) and (dimension 2) isopropanol/concentrated HCl/H_2O (70:15:15, vol/vol/vol) as solvents (23). (*C*) SVPD plus BAP digestion of [^{3}H]inhibitor: chromatography on cellulose was with (*Left*) amyl alcohol/methyl ethyl ketone/acetonitrile/ethyl acetate/water/formic acid (40:20:15:20:15:1.8 by volume), (*Right*) acetonitrile/ethyl acetate/1-butanol/isopropanol/1 M NH_4OH (35:10:5:5:13.5, by volume) (24). An autoradiograph (*A*) and fluorographs are presented. Markers were as in Fig. 2.

tified as A2′p5′A by two-dimensional chromatography (using the system employed in Fig. 3*B*) and by a second round of periodate oxidation and β elimination (step 3) yielding A2′p (Fig. 2*D* tracks 3 and 4). Confirmation of this product as A2′p rather than A3′p was provided by analysis in two additional systems giving good resolution of these components (those used in figure 4*B* of ref. 5 and Fig. 2*H* here). These results identify the 5′-proximal linkage as 2′-5′. That the 3′-proximal linkage is also 2′-5′ was suggested by the nuclease resistance of the molecule and the resistance of the first periodate and β elimination product pppApAp to S1 nuclease employed as a 3′-nucleotidase as described by Schibler and Perry (18). Direct evidence was provided by SVPD digestion (step 4) of the putative pppA2′p5′A2′p (Fig. 2 *G* and *H*). The product p5′A2′p, originating from the 3′-terminus of the molecule, was readily distinguished from p5′A3′p included as an internal marker (Fig. 2*H*). The release of significant amounts of $^{32}P_i$ (Fig. 2 *G* and *H*) reflects the requirement with the 2′ phosphate-terminated molecule for overnight digestion at 37° with 400 times the concentration of SVPD employed for complete digestion in $\leq$1 hr of the untreated inhibitor. In accord with the proposed structure, all of the phosphate in this SVPD digest (step 4) was BAP sensitive.

Absence of Additional Modifications. The presence of 2′-5′ linkages is sufficient to explain the nuclease resistance of the inhibitor but does not preclude additional modifications. We have been unable, however, to detect any such modifications. For example, on digestion of [α-^{32}P or ^{3}H]inhibitor with SVPD $\pm$ BAP, the only detectable products analyzed on a variety of systems known to resolve modified nucleotides and nucleosides (23, 24) were AMP or A (Fig. 3).

Complexity. In some experiments a more complex pattern of inhibitory products has been obtained with the poly(I)·poly(C)-Sepharose-bound enzyme, particularly when synthesis has been continued for several days. From analyses on DEAE-Sephadex in the presence of urea it seems likely that the additional products constitute an oligomeric series, consisting of the corresponding dimer (pppA2′p5′A, rarely seen in significant amounts), tetramer [ppp(A2′p)$_3$A] and pentamer [ppp(A2′p)$_4$A] (Fig. 4 *A* and *B*). Traces of putative hexamer and heptamer have also been detected. The results of analyses similar to the analysis in Fig. 1*B* of the BAP-resistant cores of these complex preparations of either [α-^{32}P]- or [^{3}H]inhibitor were consistent with this interpretation. The inhibitory activity of the different components (dimer to pentamer, Fig. 4 *A* and *B*) is shown in Fig. 4*C*. The trimer, tetramer, and pentamer have similar specific activities, whereas the dimer appears to be approximately one-tenth as active. pppA2′p5′A2′p produced from highly purified trimer (Fig. 4*B*) by periodate oxidation and β elimination had a residual activity very similar to that of the putative dimer. One cannot be certain, however, that the activity of these dimer preparations does not reflect some contamination with higher oligomers.

DISCUSSION

A number of products were obtained on synthesis of the inhibitor with the column-bound enzyme (Fig. 4). These are thought to represent an oligomeric series from the dimer to the pentamer with occasional traces of hexamer and heptamer. The predominant, or essentially exclusive species in many preparations, however, was the trimer pppA2′p5′A2′p5′A (Fig. 1). This appears to be the major species made on incubation of crude extracts from interferon-treated cells with ATP and dsRNA (ref. 5; unpublished results).

To the best of our knowledge a 2′-5′ linkage has not been previously demonstrated for biologically synthesized material, but there can be little doubt that the basic structure of the trimer is pppA2′p5′A2′p5′A (Fig. 2). It was synthesized using a highly purified enzyme fraction with radioactive ATP as the only added substrate. There was no evidence for inclusion of unlabeled material or for any modification of the AMP residues incorporated (Figs. 2 and 3). In addition, the presence of the 2′-5′ linkages is sufficient to explain the known unusual characteristics of the molecule. Rigorous proof of the structure will, however, only be provided by chemical synthesis and characterization of the biologically active molecule.

The products other than the trimer have not been extensively characterized. Nevertheless, they are similar to the trimer in their resistance to a variety of nucleases and the BAP-resistant core of the dimer chromatographed with A2′p5′A. After conversion of the tetramer to trimer by periodate oxidation and β

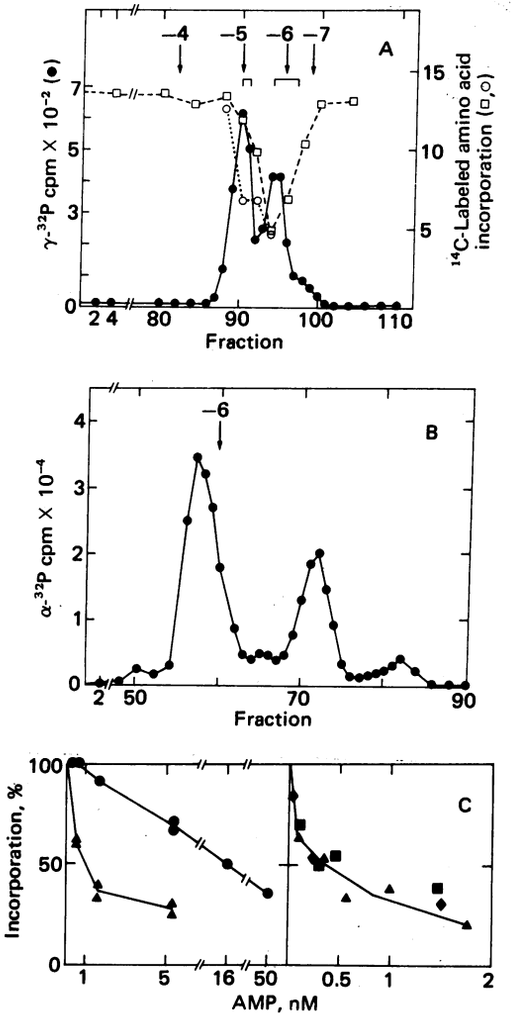

FIG. 4. Complexity of the inhibitor. [α- or γ-^{32}P]Inhibitors partially purified on DEAE-cellulose (4) were applied to columns of DEAE-Sephadex A25 (0.5 × 50 cm) equilibrated with TU buffer (Fig. 1). (*A*) [γ-^{32}P]Inhibitor (●, cpm/50-μl sample). Individual fractions were assayed at final dilutions of 1/1250 (○) and 1/3750 (□) for inhibitory activity in an encephalomyocarditis virus RNA-programmed L cell-free system. The right-hand ordinate gives the incorporation of ^{14}C-labeled amino acids in the cell-free system (cpm/10-μl sample). Elution (1.8-ml fractions, 3.6 ml/hr) was with a gradient of 0–400 mM NaCl (100 ml/150 ml) in TU buffer (Fig. 1). Arrows indicate the positions at which the tri- (−4), tetra- (−5), penta- (−6), and hexa- (−7) nucleotide products of a pancreatic RNase digest of *E. coli* tRNA (15), included as marker, eluted. The predominance of the dimer in this inhibitor preparation (fractions 90–91) was unusual and has only been observed once. (*B*) [α-^{32}P]Inhibitor (●, cpm/50-μl sample). A gradient of 0–400 mM NaCl (150 ml/150 ml) in TU buffer was used and the elution rate was reduced from 4 to 2 ml/hr at fraction 60 (45-min fractions were collected). Fractions 56–61 were pooled, dialyzed, lyophilized, and further characterized as putative pppA2′p5′A2′p5′A in Fig. 2. Further appropriate fractions from A and B were similarly but less extensively characterized as the corresponding dimer, trimer, tetramer, and pentamer. (*C*) Specific inhibitory activity in the L cell-free system. (*Left*) [γ-^{32}P]Inhibitor: (●) dimer and (▲) trimer (fractions 90–91 and 94–96, respectively, from *A*). (*Right*) [α-^{32}P]-Inhibitor: (▲) trimer, (■) tetramer, and (♦) pentamer (fractions 56–61, 69–74, and 80–84 from *B*). The concentration of the inhibitor in AMP equivalents in the cell-free system assay was calculated from the known specific activity of the [α- or γ-^{32}P]ATP used in its synthesis, the cpm in the sample, and the dilution used in the assay (5). For the [γ-^{32}P]inhibitor, allowance was made for the fact that only one (terminal) phosphate per molecule was labeled. ^{14}C-Labeled amino acid incorporation in the cell-free system for assays in the presence of different dilutions of the inhibitor is expressed as a percent of the control (no inhibitor) value, which was 8000 and 12,000 cpm/10-μl sample in duplicate experiments.

elimination, the BAP-resistant core of the converted molecule appeared identical to that of the original trimer (i.e., A2′p5′-A2′p5′A) and was clearly different from 3′-5′ linked ApApA. It seems reasonable to conclude that the products form an oligomeric series of 2′-5′ linked molecules.

The inhibitor was synthesized here with an enzyme fraction from interferon-treated mouse L cells. An apparently identical inhibitor can be similarly synthesized using an enzyme(s) from rabbit reticulocytes (25) and from interferon-treated primary chick embryo cells (26). The ability to synthesize inhibitors of this type is not, therefore, restricted to mouse extracts or exclusively to interferon-treated cell material. This, the inhibitors' activity at subnanomolar concentrations, and their possible correlation with protein kinases (3, 6–8) make it tempting to speculate that the inhibitors may be of wider significance in the control of cell metabolism. We assay the inhibitor by its effect on cell-free protein synthesis, but it is unlikely that it is directly inhibitory. It seems more probable that it, in turn, regulates the formation of an inhibitor or subsequent inhibitory events (5).

The possible involvement of a viral dsRNA-mediated inhibition of protein synthesis in the sequence of events following virus infection in intact, interferon-treated cells has already been discussed (1, 2). The roles of the oligonucleotide inhibitor(s), the kinase(s), and nuclease(s) in such inhibition remain to be established and their relationship and respective roles in the inhibition of protein synthesis in the cell-free system are not yet clear. The knowledge of the characteristics and the structure of the inhibitor provided by this work should, however, considerably facilitate the further analysis of its mechanism of action and role in the inhibitions observed.

1. Kerr, I. M., Brown, R. E. & Ball, L. A. (1974) *Nature* **250**, 57–59.
2. Kerr, I. M., Brown, R. E., Clemens, M. J. & Gilbert, C. S. (1976) *Eur. J. Biochem.* **69**, 551–561.
3. Roberts, W. K., Hovanessian, A. G., Brown, R. E., Clemens, M. J. & Kerr, I. M. (1976) *Nature* **264**, 477–480.
4. Hovanessian, A. G., Brown, R. E. & Kerr, I. M. (1977) *Nature* **268**, 537–540.
5. Kerr, I. M., Brown, R. E. & Hovanessian, A. G. (1977) *Nature* **268**, 540–542.
6. Lebleu, B., Sen, G. C., Shaila, S., Cabrer, B. & Lengyel, P. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3107–3111.
7. Sen, G. C., Lebleu, B., Brown, G. E., Kawakita, M., Slattery, E. & Lengyel, P. (1976) *Nature* **264**, 370–373.
8. Zilbertstein, A., Federman, P., Shulman, L. & Revel, M. (1976) *FEBS Lett.* **68**, 119–124.
9. Farrell, P. J., Balkow, K., Hunt, T. & Jackson, R. J. (1977) *Cell* **11**, 187–200.
10. Levin, D. H., Ranu, R. S., Ernst, V. & London, I. M. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 3112–3116.
11. Kramer, G., Henderson, A. B., Pinphanichakarn, P., Wallis, M. H. & Hardesty, B. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 1445–1449.
12. Traugh, J., Tahara, S. M., Sharp, S. B., Safer, B. & Merrick, W. C. (1976) *Nature* **263**, 163–165.
13. Datta, A., de Haro, C., Sierra, J. M. & Ochoa, S. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 3326–3329.
14. Keller, E. B. (1964) *Biochem. Biophys. Res. Commun.* **17**, 412–415.
15. Brownlee, G. G. (1972) in *Laboratory Techniques in Biochemistry and Molecular Biology* eds. Work, T. S. & Work, E. S. (North Holland Publishing Co., Amsterdam), Vol. 3, pp. 1–265.
16. Frankel-Conrat, H. & Steinschneider, A. (1967) in *Methods in*

Enzymology, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12, pp. 243–246
17. Tener, G. M. (1967) in *Methods in Enzymology*, eds. Grossman, L. & Moldave, K. (Academic Press, New York), Vol. 12, pp. 398–404.
18. Schibler, U. & Perry, R. P. (1976) *Cell* **9**, 121–130.
19. Cashel, M., Lazarini, R. A. & Kalbacher, B. (1969) *J. Chromatogr.* **40**, 103–109.
20. Barzilay, I., Sussman, J. L. & Lapidot, Y. (1973) *J. Chromatogr.* **79**, 139–146.
21. Markham, R. (1957) in *Methods in Enzymology*, eds. Colowick, S. P. & Kaplan, N. O. (Academic Press, New York), Vol. 3, pp. 743–745.
22. Michelson, A. M. (1963) *The Chemistry of Nucleosides and Nucleotides* (Academic Press, New York), p. 429.
23. Nishimura, S. (1972) *Prog. Nucleic Acid Res. Mol. Biol.* **12**, 50–53.
24. Faust, M. & Millward, S. (1977) *Anal. Biochem.* **79**, 16–22.
25. Hovanessian, A. G. & Kerr, I. M. (1978) *Eur. J. Biochem.*, in press.
26. Ball, L. A. & White, C. N. (1978) *Proc. Natl. Acad. Sci. USA* **75**, in press.