Volume 6 Number 2 February 1979 Nucleic Acids Research

Metabolic stability of 2' 5'oligo (A) and activity of 2' 5'oligo (A)-dependent endonuclease in extracts of interferon-treated and control HeLa cells

Michael A.Minks, Susan Benvin, Patricia A.Maroney and Corrado Baglioni

Department of Biological Sciences, State University of New York at Albany, Albany NY 12222, USA

Received 23 October 1978

ABSTRACT

Extracts of interferon-treated HeLa cells adsorbed to poly(I) • poly(C)-agarose have been used to synthesize 2'5'oligo(A). This oligonucleotide has been characterized by enzymatic digestion with alkaline phosphatase, snake venom phosphodiesterase, T2 ribonuclease and chromatography on DEAE, and PEI-cellulose. The oligonucleotide inhibits protein synthesis in vitro and activates an endonuclease present in extracts of control and interferon-treated cells. The metabolic stability of 2'5'oligo(A) has been investigated in these cell extracts. The oligonucleotide undergoes rapid degradation, particularly in the absence of ATP and of an energy regenerating system. Furthermore, the 2'5'oligo(A)-activated endonuclease reverts to an inactive state under these conditions, but can be reactivated upon further addition of 2'5'oligo(A). A possible role for the degradation of 2'5'oligo(A) in the mechanism of interferon action is discussed.

INTRODUCTION

In animal cells exposed to interferon the accumulation and translation of viral mRNA is specifically inhibited by the activation of cellular defense mechanisms, the molecular basis of which is still unclear (1-2). Several investigators have compared biochemical characteristics of extracts prepared from interferon-treated cells with those of extracts from control cells in order to elucidate the molecular basis of these defense mechanisms. Among the many differences reported between these extracts, the most significant appear to be: 1) the enhanced inhibition of protein synthesis by double stranded RNA (dsRNA) (3-4); 2) the decreased methylation of the 5'-terminal guanosine of added viral mRNA (5-6); 3) an increase in three enzymatic activities, which are dependent on the addition of dsRNA to cell extracts. These are a protein kinase (7-9), an endonuclease (10-12) and an enzyme which utilizes ATP for the synthesis of 2'5'oligo(A), an oligonucleotide with the structure pppA(2'p5'A)$_n$ (where n is 2 to 6) (13-16).

The enzymatic activity which polymerizes ATP into 2'5'oligo(A) is here designated 2'5'oligo(A) polymerase. This activity has been detected in

© Information Retrieval Limited 1 Falconberg Court London W1V 5FG England

extracts of interferon treated L-cells (13-15) and chicken fibroblasts (16), and in reticulocyte lysate (17). The 2'5'oligo(A) activates an endonuclease, which degrades both viral and cellular mRNA (12,17) and inhibits protein synthesis in this way. The mechanism of endonuclease activation by 2'5'oligo(A) is not known but endonuclease activity is related to the amount of oligonucleotide added to cell extracts (12).

The 2'5'oligo(A) polymerase in cell extracts can be assayed by adsorbing the enzyme to poly(I)·poly(C) conjugated to agarose and incubating the bound enzyme with ATP (13). This method was used with HeLa cell extracts to synthesize an oligonucleotide tentatively identified as 2'5'oligo(A) (12). This identity has now been confirmed by chromatographic studies, enzymatic digestion and by showing that HeLa cell 2'5'oligo(A) inhibits protein synthesis in reticulocyte lysate. The present investigation was directed at studying the stability of 2'5'oligo(A) added to HeLa cell extracts. The nucleotide is rapidly degraded in interferon-treated and control cell extracts. The rate of RNA cleavage by the 2'5'oligo(A)-activated endonuclease decreases upon degradation of the oligonucleotide. However, the endonuclease can be reactivated by further addition of 2'5'oligo(A). The possible significance of the rapid turnover of 2'5'oligo(A) in the regulation of this endonuclease activity is discussed.

MATERIALS AND METHODS

Materials: Radioactive compounds were purchased from New England Nuclear; poly(I)·poly(C)-agarose from P-L Biochemicals; oligo(dT)-cellulose from Collaborative Research; and enzymes from Sigma.

Cell extracts: Extracts were prepared from HeLa cells grown in suspension culture as previously described (18). Interferon-treated cells were exposed to human fibroblast interferon (3×10^5 units/mg; obtained from the Interferon Working Group, National Cancer Institute, N.I.H.) at 100 N.I.H. reference units/ml for 17 hr prior to harvest.

Preparation and characterization of 2'5'oligo(A): Extracts (75 µl) from interferon-treated cells were passed over 0.2 ml columns of poly(I)·poly(C)-agarose thoroughly washed with 0.1 M KOAc, 2 mM $Mg(OAc)_2$, 2 mM dithiothreitol (DTT), 20% (v/v) glycerol and 20 mM Hepes-KOH, pH 7.4 (buffer K), and unbound proteins removed by washing with 5 ml of the same buffer. Columns were incubated with 0.15 ml buffer K containing 1 mM ATP and 20 µCi/ml [^{3}H]ATP (20 Ci/mmole) for 17 hr at 30°C, and eluted with 0.5 ml of buffer K. The eluted material was adsorbed to 0.2 ml columns of DEAE-cellulose (Whatman DE-52) equilibrated

with 90 mM KCl and 20 mM Hepes-KOH, pH 7.4 Residual ATP and less charged nucleotides were removed by washing with 25 ml of the same buffer. The 2'5' oligo(A) was eluted with 0.35 M KCl and 20 mM Hepes-KOH, pH 7.4. The material eluted with 0.35 M KCl was precipitated using 5 vol of acetone and then analysed by DEAE-cellulose chromatography in the presence of 7 M urea (16). This material was also characterized by digestion with bacterial alkaline phosphatase (BAP), snake venom phosphodiesterase (Crotalis adamanteus venom) and T2 ribonuclease. Digestions were performed according to Brownlee (19) and Rose (20). Digestion was for 1 hr in the case of BAP and two hours for phosphodiesterase and T2 ribonuclease. After digestion the samples were chromatographed on DEAE-cellulose columns or on thin-layer plates of polyethleneimine-cellulose (PEI plates) using 0.75 M KH_2PO_4, pH 3.4, 1 M LiCl, or 1 M acetic acid as solvent (16). Unlabeled markers were included and visualized under UV light. Labeled compounds were visualized by fluorography as described by Ball and White (16). The concentration of 2'5'oligo(A) was calculated from the known specific activity of [^{3}H]ATP, assuming all material eluted with 0.35 M KCl to be trinucleotide. (The true molarity is about 20% lower due to the presence of tetra and pentanucleotides in the reaction products; see Results.)

Protein synthesis assays: Protein synthesis in reticulocyte lysates was assayed as previously described (21) in incubations containing 120 mM KOAc, 1.1 mM $Mg(OAc)_2$, 50 µM hemin and 100 µCi/ml [^{3}H] lysine (73 Ci/mmole).

Nuclease assays: Exonuclease activity was determined by precipitating RNA with 10% (w/v) trichloracetic acid. Precipitates were collected on Millipore filters and counted in toluene based scintillant. Endonuclease activity was monitored by the loss of poly(A)-containing RNA using oligo(dT)-cellulose chromatography (22). Unless otherwise indicated, RNA degradation was determined in 25 µl incubations containing 0.6 parts cell extract, 120 mM KOAc, 20 mM Hepes-KOH, pH 7.4, 4 mM fructose 1,6-bisphosphate (Fru-P_2), 3.0 mM $Mg(OAc)_2$, 1 mM DTT and vesicular stomatitis virus (VSV) mRNA as indicated. The VSV mRNA was prepared from infected cells as previously described (12).

Degradation of 2'5'oligo(A): Degradation of 2'5'oligo(A) was determined in incubation mixtures identical to those used for the nuclease assays with either 0.2 or 0.6 parts of cell extract, omission of Fru-P_2, addition of 1.5 mM $Mg(OAc)_2$, 2'5'oligo(A), and other components as indicated in the text. Reactions were terminated by heating to 95°C for 3 min to denature protein and analysed by DEAE-cellulose chromatography as previously described (13).

RESULTS

Characterization of 2'5'oligo(A). The unusual oligonucleotide pppA $(2'p5'A)_n$, designated here 2'5'oligo(A), was first synthesized by Kerr and collaborators (13,15) using extracts from interferon-treated mouse L cells. The synthesis utilized poly(I) • poly(C)-agarose as an affinity column for and activator of 2'5'oligo(A)-polymerase (13-16). We have previously used the same procedure with extracts of interferon-treated HeLa cells to obtain an oligonucleotide tentatively identified as 2'5'oligo(A). Since this was the first report of 2'5'oligo(A) synthesis with a human cell extract, we have further characterized this oligonucleotide by procedures previously utilized in the analysis of 2'5'oligo(A) from other cell lines (14-16).

Extracts from interferon-treated HeLa cells were adsorbed to poly(I) • poly(C)-agarose and incubated with [^{3}H]ATP as described in Methods. The products were isolated by chromatography on DEAE-cellulose (13), and further analysed by chromatography on DEAE-cellulose with buffers containing 7 M urea (Fig. 1A). The resolution of multiple adenosine-containing oligonucleotides was incomplete. The first peak eluted with a net charge of -5.2 and was followed by a peak of net charge -5.7 (estimated by extrapolation of the mobilities of the indicated charge markers) and other peaks containing more highly charged nucleotides. Digestion of the labelled product with bacterial alkaline phosphatase (BAP), which removes 5' phosphate groups, resulted in a much simpler pattern (Fig. 1B). Three main peaks of net charge slightly less than -2, -3 and -4 were resolved. These peaks were eluted in the position of ApApA, ApApApA and ApApApApA respectively (16). The relatively greater

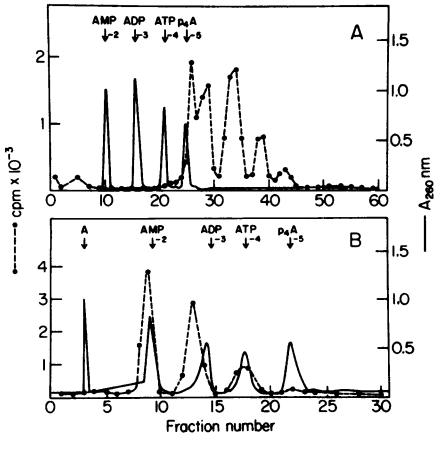

Figure 1: Chromatography of [^{3}H]2'5' oligo(A) and its bacterial alkaline phosphatase digest on DEAE-cellulose in the presence of 7 M urea. A) 2 µg of 2'5'oligo(A) and B) 4 µg of 2'5'oligo(A) digested with BAP as described in Methods were applied to a 0.7 x 25 cm column of DEAE-cellulose (Whatman DE-52) and eluted with a linear 300 ml gradient of 50 to 300 mM NaCl in 20 mM Tris—HCl, pH 7.6, and 7 M urea. The elution was at a flow rate of 20 ml/hr and fractions of 5 ml were collected. The indicated markers were included with 2'5'oligo(A); the nominal charge of each marker is indicated.

complexity of elution pattern prior to BAP digestion could be due to the presence of oligonucleotides lacking the γ-phosphate group. A phosphatase activity in HeLa cell extracts may be responsible for the removal of the γ-phosphate (see below). This situation is analogous to that observed with the oligonucleotides made by chick fibroblast extracts, although the charge heterogeneity of 2'5'oligo(A) is greater in the HeLa product.

The labelled oligonucleotides were further characterized by chromatographic analysis of the products of digestion with different nucleases on PEI-cellulose plates (see Methods). Figure 2 shows that the oligonucleotides are resistant to ribonuclease T2. Under these conditions an internal standard of poly(A) is completely degraded (data not shown). Therefore, the oligonucleotides do not contain adenosine residues linked by the normal 3'5'phosphodiester bonds. The oligonucleotides are completely degraded to AMP by venom phosphodiesterase, indicating that the adenosine residues are linked by phosphodiester bonds and that the terminal 2' and 3' OH groups are free. The partial hydrolysis of oligonucleotides by BAP (Fig. 1B) was confirmed on thin-layer plates developed with 1 M acetic acid; this result is consistent with the presence of an oligomeric series $A(pA)_n$. The data presented indicate that these oligonucleotides have the general structure $(p)ppA(pA)_n$ where n = 2 to 5

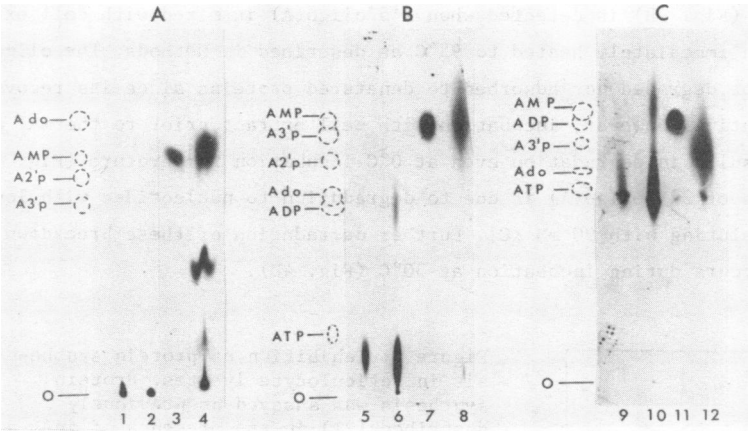

Figure 2: Chromatography of [^{3}H] 2'5'oligo(A) and its digestion products on PEI-cellulose. Labeled 2'5'oligo(A) prepared as described in Methods was chromatographed on thin-layer PEI-cellulose plates (EM Laboratories) using (A) 1 M acetic acid, (B) 1 M LiCl or (C) 0.75 M KH_2PO_4 as solvents. 1,5,9: 2'5'oligo(A); 2,6,10:T2 nuclease digest; 3,7,11:venom phosphodiesterase digest; 4,8,12:BAP digest. The position of marker nucleotides is indicated.

and the phosphate linkage is not 3'5'. These properties are shared with previously characterized 2'5'oligo(A) (15). Furthermore, we have analyzed the effect of 2'5'oligo(A) from HeLa cells on endogenous protein synthesis in reticulocyte lysates, since preparations of 2'5'oligo(A) are potent inhibitors of protein synthesis in extracts of mouse L-cells (13) and chicken fibroblasts (16), and in reticulocyte lysates (13,17). As shown in Fig. 3, protein synthesis is inhibited by subnanomolar concentrations of HeLa cell 2'5'oligo(A). It is probable that this inhibition of protein synthesis results from the activation of an endonuclease by 2'5'oligo(A) (12,17).

Definitive proof of the identity of the oligonucleotides must await comparison with synthetic 2'5'oligo(A). However, the oligonucleotides synthesized with extracts of interferon-treated HeLa cells are probably identical to those produced with extracts of other cells (13,15,16) since their behavior towards nucleases, their size and biological activities are the same.

Stability of 2'5'oligo(A) in cell extracts. The synthesis of 2'5'oligo(A) and endonuclease activation may play an important role in the mechanism of interferon action (12). We have therefore investigated the stability of 2'5'oligo(A) in cell extracts. Degradation of 2'5'oligo(A) was monitored as a loss of material eluting from DEAE-cellulose with 0.35 M KCl (see Methods). Figure 4A shows the elution patterns of 2'5'oligo(A) and marker AMP, ADP and ATP. Only 2'5'oligo(A) elutes with 0.35 M KCl, and no difference in elution pattern (Fig. 4B) is detected when 2'5'oligo(A) is mixed with cell extract and then immediately heated to 95°C as described in Methods. The oligonucleotide is not degraded nor adsorbed to denatured proteins since its recovery is quantitative. However, incubation with cell extract prior to thermal denaturation results in degradation even at 0°C incubation temperature (Fig. 4C). The loss of 2'5'oligo(A) is due to degradation to nucleotides with lower net charge eluting with 90 mM KCl. Further degradation of these breakdown products occurs during incubation at 30°C (Fig. 4D).

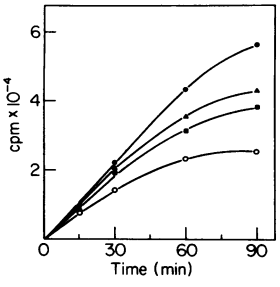

Figure 3: Inhibition of protein synthesis in reticulocyte lysates. Protein synthesis was assayed as previously described (21) in the presence of increasing concentrations of 2'5'oligo(A). Incorporation is expressed as cpm/5 µl incubation. ●—●, control; ▲—▲, 0.3 nM 2'5'oligo(A); ■—■, 1.5 nM 2'5'oligo(A); o—o, 7.5 nM 2'5'oligo(A).

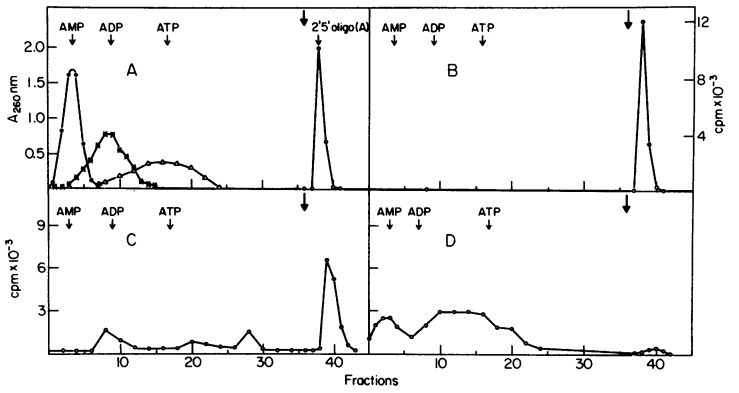

Figure 4: Chromatography of 2'5'oligo(A) on DEAE cellulose. The [^{3}H]2'5'oligo(A) was analyzed in A) after addition of AMP, ADP and ATP markers (the elution patterns of these markers are superimposed); in B) after addition of cell extract; in C) after 15 min incubation at 0°C with cell extract; and in D) after 30 min incubation with cell extract at 30°C. The concentration of 2'5'oligo(A) was 7 µM in all the experiments except D, where it was 14 µM. DEAE-cellulose chromatography was on columns of 0.3 x 3 cm at a flow rate of 3 ml/hr. o—o, cpm; other symbols, A_{260}. The arrows indicate the position of the markers and the change in elution buffer from 90 to 350 mM KCl.

In similar experiments designed to follow the degradation of γ-^{32}P labelled 2'5'oligo(A) we have been unable to detect any ^{32}P-containing breakdown product eluting after fraction 3 (result not shown). This indicates that the breakdown products lack the 5'-terminal γ-phosphate group. This may be due either to phosphatase activity, which is also presumably responsible for the appearance of partially dephosphorylated 2'5'oligo(A) (Fig. 1), or to γ-phosphate cleavage as an early event in pppA(2'p5'A)$_n$ degradation.

The breakdown at 30°C is quite rapid (Fig. 5). A 50% degradation of 250 nM 2'5'oligo(A) occurs within 2 min. This rate of degradation has been observed over a wide range of concentrations, from 7 µM to 25 nM, and is similar in extracts of both control and interferon-treated cells (data not shown). The breakdown of 2'5'oligo(A) measured by DEAE-cellulose chromatography is also reflected in a loss of biological activity (Fig. 5). This was established by assaying samples of oligonucleotides incubated with cell extract as described above, for stimulation of endonuclease activity. This stimulation changed slightly untill a critical concentration of 2'5'oligo(A) was reached and then decayed rapidly (Fig. 5). This effect of 2'5'oligo(A) concentration on endonuclease activity has been described previously (12).

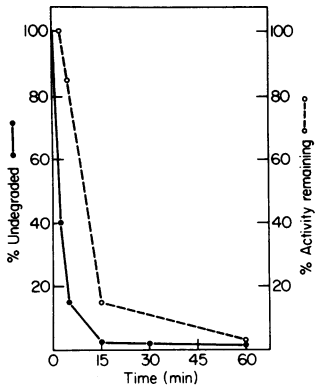

Figure.5: Time course of 2'5'oligo(A) degradation. The 0.25 ml incubation contained 0.6 parts of cell extract, 2.5 mM $Mg(OAc)_2$, 250 nM [^{3}H]2'5'oligo(A) and the other components indicated in Methods. At the times shown, 40 µl samples were withdrawn and the amount of 2'5'oligo(A) remaining determined by chromatography on DEAE-cellulose and by stimulation of endonuclease activity in HeLa cell extracts as described in Methods. The final dilution of 2'5'oligo(A) in the endonuclease assay was 1:10. Results are expressed as % of an unincubated sample.

When 7 µM [^{3}H] 2'5'oligo(A) was incubated 15 min with cell extract, heated to 95°C, digested with alkaline phosphatase and analyzed by chromatography on DEAE-cellulose as described in Fig. 1, at least 95% of the radioactivity cochromatographed with adenosine (not shown). This indicates that the 2'5'phosphodiester bond is hydrolyzed by enzymes present in both control and interferon-treated cells.

Factors affecting 2'5'oligo(A) stability. In light of the rapid degradation of 2'5'oligo(A) in cell extracts we have investigated factors affecting its stability. In these experiments the concentration of cell extracts was reduced 3-fold relatively to the experiments shown above, in order to detect more accurately differences in the rate of degradation of 2'5'oligo(A). The effect of an energy regeneration system based on the use of Fru-P_2 as an energy source (23) was first tested (Table 1). This system has the advantage that no additional enzymes are added to the incubation. There is a marked increase in the stability of 2'5'oligo(A) in the presence of Fru-P_2, and the effect is enhanced with the simultaneous addition of ATP. But other nucleotides can also stabilize 2'5'oligo(A); among the most effective are ADP and ADPCP. The exact nature of the protection afforded by these nucleotides is not clear, but under such conditions the biological activity of 2'5'oligo(A) (as determined by stimulation of endonuclease activity) is also preserved (data not shown). The degradation is also affected by magnesium concentration (Fig. 6). When extracts prepared in a buffer containing 1.5 mM Mg^{2+} are supplemented with additional magnesium, the rate of 2'5'oligo(A) degradation is enhanced by up to 5-fold, whilst chelation of Mg^{2+} by EDTA prevents degradation of 2'5'oligo(A).

In light of the 2'5'oligo(A) requirement for endonuclease activity (12) we have attempted to correlate the stability of 2'5'oligo(A) in cell extracts

Table 1

Effect of Added Nucleotides on the Degradation of 2'5'oligo(A)

Treatment	% Degradation of 2'5'oligo(A)
No addition	68
4 mM Fru-P_2	28
1 mM ATP	38
5 mM ATP	20
4 mM Fru-P_2 + 1 mM ATP	17
4 mM Fru-P_2 + 5 mM ATP	15
1 mM ADP	59
5 mM ADP	26
1 mM ADPCP	57
5 mM ADPCP	43
1 mM CTP	65
5 mM CTP	63
1 mM GTP	68
5 mM GTP	45

Degradation of 2'5'oligo(A) was monitored in 50 µl incubations (see Methods) containing 7 µM [^{3}H]2'5'oligo(A), and the indicated components. Incubation was at 30°C for 15 min. Degradation is expressed as % of samples not incubated. The values indicated are averages of between 2 and 5 experiments, ADPCP is βγ-methylene adenosine 5' triphosphate.

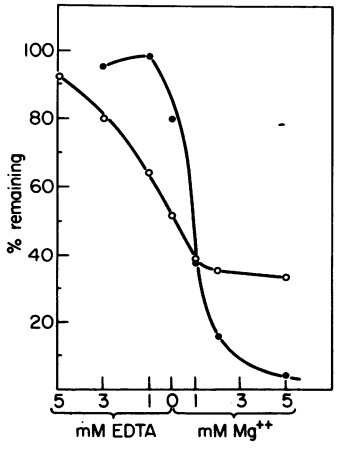

Figure 6: Magnesium dependence of RNA and 2'5'oligo(A) degradation. o—o, oligo(A) stimulated degradation of poly(A)-containing RNA; ●—●, 2'5'oligo(A) degradation. RNA degradation was determined over a 30 min incubation period whereas 2'5'oligo(A) degradation was monitored over a 15 min period, as described in Table 1. The abscissa indicates the concentration of EDTA or Mg^{2+} added to the incubations. The amount of undegraded 2'5'oligo(A) and of poly(A)-containing RNA remaining after incubation is expressed as % of an unincubated sample.

with the activity of the 2'5'oligo(A)-dependent endonuclease. This enzyme also shows a magnesium requirement (Fig. 6). Therefore, it is not possible to study the relationship between 2'5'oligo(A) stability and endonuclease activity by manipulating the magnesium concentration. We have followed instead endonuclease activity during prolonged incubations of cell extracts with 2'5'oligo(A).

Previously, we had observed a decline in the rate of loss of poly(A)-containing RNA in cell extracts incubated with 2'5'oligo(A) (12). This was attributed to a decline in loss of poly(A)-containing RNA as the size of the RNA fragment linked to poly(A) decreases with successive cleavages (12). However, other causes for this pattern of RNA degradation could not be ruled out. The experiments shown in Table 2 were conducted to investigate factors affecting the endonuclease activity. In these experiments master incubations with or without added 2'5'oligo(A) were set up and aliquots withdrawn at the indicated times. These aliquots were supplemented with labelled VSV mRNA at the time of withdrawal and the degradation of the RNA determined over a one hour period. In this assay both VSV mRNA and cellular mRNA are degraded at

Table 2

Degradation of Vesicular Stomatitis Virus mRNA by 2'5'oligo(A) Activated Nuclease

Experiment	Incubation time (minutes)	cpm A^+-RNA	$\frac{cpm_t}{cpm_{t_o}}$
-2'5'oligo(A)	0- 60	1091	0.89
	60-120	1099	0.89
	120-180	1072	0.87
+2'5'oligo(A) added at t_o	0- 60	503	0.41
	60-120	984	0.80
	120-180	1134	0.92
+2'5'oligo(A) added at t_o and readded at t_{60} and t_{120}	0- 60	534	0.43
	60-120	750	0.61
	120-180	835	0.68

Each 50 µl incubation contained the components described in Methods, 28 nM 2'5'oligo(A) where indicated, and 3 µl of vesicular stomatitis virus [^{3}H]mRNA solution containing 1230 cpm of mRNA retained by oligo(dT)-cellulose (A^+-RNA). The mRNA was added at the beginning of each 60 min incubation. In the third experiment an additional 28 nM 2'5'oligo(A) was added at the beginning of each incubation.

similar rates (12). The viral mRNA added to each aliquot allows assay of nuclease activity after 2'5'oligo(A) has been incubated with cell extract for different lengths of time. This assay also allows us to distinguish between loss of nuclease activity and the previously described decline in loss of poly(A)-containing RNA as the size of the poly(A)-containing fragments is reduced. From Table 2 it can be seen that the degradation of RNA in the absence of added 2'5'oligo(A) is 12% per hour, and that this rate remains constant throughout the incubation. When 2'5'oligo(A) is added to the master incubation mix at t_o there is a greatly enhanced degradation of RNA (59% degradation in the first hour). This stimulation of nuclease activity declines with time, so that the sample withdrawn at t_{60} shows only slightly greater nuclease activity than the control incubated without 2'5'oligo(A). By the end of the second hour there is no significant difference between the activities of samples exposed to 2'5'oligo(A) at t_o and the control samples. From these results it is evident that the activation of endonuclease by 2'5'oligo(A) is a transient phenomenon, with most of the activity lost within one hour. In order to elucidate the reasons for the loss of activity with time, samples withdrawn from a master mix containing 2'5'oligo(A) added at t_o were supplemented after withdrawal with both VSV mRNA and 2'5'oligo(A) and the rate of RNA degradation during the following hour determined. The results show that further additions of 2'5'oligo(A) are effective in enhancing the rate of RNA degradation. This suggests that the decline in RNA cleavage is due to degradation of 2'5'oligo(A) during the incubation rather than to an inactivation of the endonuclease. Furthermore, it is evident that the active endonuclease returns reversably to an inactive state when 2'5'oligo(A) is degraded. It should be pointed out that the initial rate of RNA degradation is not completely restored by further addition of 2'5'oligo(A). We cannot exclude that this is caused by a partial inactivation of the endonuclease. However, it seems more likely that catabolism of components involved in stabilizing 2'5'oligo(A) during the prolonged incubations involved in these experiments explains this observation; a rapid degradation of the 2'5'oligo(A) added after 1 or 2 hr incubation may result in decreased activation of the endonuclease. This is also suggested by experiments in which endonuclease activity was determined after incubation of 2'5'oligo(A) with or without added ATP and Fru-P_2. In the absence of these components, the endonuclease activity decayed at about twice the rate observed in the presence of these components (data not shown), in agreement with the higher stability of 2'5'oligo(A) under these conditions (Table 1). Therefore, an important factor in determining the

rate at which active endonuclease returns to an inactive state may be the catabolism of 2'5'oligo(A).

DISCUSSION

Extracts from interferon-treated HeLa cells polymerize ATP into oligonucleotides capable of activating an endonuclease (12). These oligonucleotides have been shown: 1) to inhibit protein synthesis in reticulocyte lysates at sub-nanomolar concentrations; 2) to be resistant to T2 ribonuclease digestion, but to be completely degraded by snake venom phosphodiesterase; and 3) to consist of an oligomeric series (p)ppA(pA)$_n$, where n = 2 to 5. These oligonucleotides appear to be identical to 2'5'oligo(A) prepared from interferon-treated mouse L cells (13,15) and chicken fibroblasts extracts (16). The structure pppA(2'p5'A)$_n$2'p5'A$_{OH}$ has been proposed for these oligonucleotides by Kerr and Brown (15).

These unusual oligonucleotides are rapidly degraded in HeLa cell extracts, with a halflife of 2 min at 30°C. This rate of degradation is similar in extracts of control and interferon-treated cells. Thus, interferon treatment increases several fold the enzymatic activity responsible for 2'5'oligo(A) synthesis, but does not seem to significantly affect the level of 2'5'oligo(A)-dependent endonuclease (12) or of the enzymes which degrade 2'5'oligo(A).

The degradation of 2'5'oligo(A) is slowed down upon addition of an ATP generating system, of ADP and ADPCP (Table 1). The enzymes which degrade 2'5'oligo(A) require Mg^{2+}, since in the absence of free Mg^{2+} the oligonucleotides are almost completely stable (Fig. 6). The biological significance of these observation is not clear.

The experiments of Table 2 suggest that the activity of the 2'5'oligo(A)-dependent endonuclease is correlated with the stability of the 2'5'oligo(A). It seems likely that the activation of the endonuclease by 2'5'oligo(A) is a transient phenomenon, and that this enzyme returns reversably to an inactive form when 2'5'oligo(A) is degraded; the endonuclease may therefore require the continuous presence of 2'5'oligo(A) for activity.

An endoribonuclease activated by dsRNA and ATP has been reported by Shaila et al. (24) in extracts of HeLa cells treated with human interferon. This activity was detected by faster degradation of reovirus mRNA and it may correspond to the 2'5'oligo(A)-dependent endoribonuclease activity. This is also supported by a recent report on the separation and partial purification from interferon-treated Ehrlich ascites tumor cells of two components involved in the endonuclease system (25). One macromolecular component generates a low

molecular weight product upon incubation with dsRNA and ATP, whereas the other component has nuclease activity dependent on the addition of the low molecular weight compound (25). A nucleolytic activity was also partially purified from the ribosomal salt-wash fraction of mouse L cells (26). This nucleolytic activity, however, apparently did not require activation by dsRNA and ATP. Since reovirus mRNA was used as substrate, presence of reovirus genome dsRNA could not be excluded (26). Addition of 2'5'oligo(A) prepared according to Hovanessian et al. (13) did not enhance the activity of this already "activated" nuclease and $Mg(OAc)_2$ was inhibitory at all concentrations tested (27).This behavior of the nucleolytic activity is in striking contrast with that described for the 2'5'oligo(A)-dependent endonuclease reported in this manuscript. It seems possible that these are two completely different nucleases, though it cannot be excluded that the nuclease observed by Eppstein and Samuel (27) is "activated" by the formation of an activator during incubation with reovirus RNA and that Mg^{2+} has an effect on the catabolism of this hypothetic activator. Further studies with purified enzymes will be necessary to clarify this point.

The observed behavior of 2'5'oligo(A) and of active endonuclease in extracts is compatible with a previously proposed mechanism of interferon action (12), based on the localized activation of endonuclease limiting the degradation of RNA. An essential prerequisite for such mechanism is a high turnover of 2'5'oligo(A) and of active endonuclease. The 2'5'oligo(A) added to cell extracts is indeed degraded very rapidly (Fig. 4). It seems likely that 2'5'oligo(A) synthesized in vivo is also rapidly degraded. It should be pointed out, however, that neither synthesis nor degradation of 2'5'oligo(A) have yet been shown in intact cells. In the proposed mechanism of localized cleavage of viral RNA (12), the synthesis of 2'5'oligo(A) would take place at the level of viral replicative intermediates containing dsRNA. A rapid degradation of 2'5'oligo(A) would produce a sharp concentration gradient of the oligonucleotides as the distance from their site of synthesis increases. Consequently, the endonuclease would be activated preferentially near the site of 2'5'oligo(A) synthesis. Discrimination by 2'5'oligo(A)-dependent endonuclease between viral and cellular RNA may be a consequence of this localized activation. This hypothetical mechanism is highly speculative, but provides a working model to design experiments to test its validity.

AKNOWLEDGEMENTS

This work was supported by grant AI-17710 from the National Institute of

Health.

REFERENCES

1 Finter, N.B. (1973) Interferon and Interferon Inducers (2nd edn) (North Holland, Amsterdam).
2 Friedman, R.M. (1977) Bact. Rev. 41, 543-567.
3 Kerr, I.M., Brown, R.E. and Ball, L.A. (1974) Nature 250, 57-59.
4 Kerr, I.M., Brown, R.E., Clemens, M.J. and Gilbert, C.S. (1976) Eur. J. Biochem. 69, 551-561.
5 Sen, G.C., Lebleu, B., Brown, G.E., Rebello, M.A., Furuichi, Y., Morgan, M., Shatkin, A.J. and Lengyel, P. (1975) Biochem. Biophys. Res. Comm. 65, 427-434.
6 Sen, G.C., Shaila, S., Lebleu, B., Brown, G.E., Desrosiers. R.C., and Lengyel, P. (1977) J. Virol. 21, 69-83.
7 Lebleu, B., Sen, G.C., Shaila, S., Cabrer, B. and Lengyel,P.(1976) Proc. Natl.Acad. Sci. USA 73, 3107-3111.
8 Zilberstein, A., Federman, P., Shulman, L. and Revel, M. (1976) FEBS Letters 68, 119-124.
9 Roberts, W.K. Hovanessian, A.K., Brown, R.E., Clemens, M.J. and Kerr, I.M. (1976) Nature 264, 477-480.
10 Sen, G.C., Lebleu, B., Brown, G.E., Kawakita, M., Slattery, E. and Lengyel, P. (1976) Nature 264, 370-373.
11 Ratner, L., Sen, G.C., Brown, G.E., Lebleu, B., Kawakita, M., Cabrer, B., Slattery, E. and Lengyel, P. (1977) Eur. J. Biochem. 79, 565-577.
12 Baglioni, C., Minks, M.A. and Maroney, P.A. (1978) Nature 273, 684-687.
13 Hovanessian, A.R., Brown, R.E. and Kerr, I.M. (1977) Nature 268, 537-540.
14 Kerr I.M.,, Brown, R.E. and Hovanessian, A.G. (1977) Nature 268, 540-542.
15 Kerr, I.M. and Brown, R.E. (1978) Proc. Natl. Acad. Sci. USA 75, 256-260
16 Ball, A.L. and White, C.N. (1978) Proc. Natl. Acad. Sci. USA 75, 1167-1171.
17 Clemens, M.J. and Williams, B.R.G. (1978) Cell 13, 565-572.
18 Weber, L.A., Feman, E. and Baglioni, C. (1975) Biochemistry 14, 5315-5321.
19 Brownlee, G.C. (1972) in Laboratory Techniques in Biochemistry (North Holland, Amsterdam), vol 3, pp 1-265.
20 Rose, J.K. (1975) J. Biol. Chem. 250, 8098-8104.
21 Weber, L.A., Hickey, E.D., Maroney, P.A. and Baglioni, C. (1977) J. Biol. Chem. 252, 4007-4010.
22 Hickey, E.D., Weber, L.A. and Baglioni, C. (1978) Biochem. Biophys. Res. Commun. 80, 377-383.
23 Baglioni, C. and Weber, L.A. (1978) FEBS Letters 88, 37-40.
24 Shaila, S., Lebleu, B., Brown, G.E., Sen, G.C. and Lengyel, P. (1977) J. Gen. Virol. 37, 535-546
25 Ratner, L., Wiegand, R.C., Farrell, P.J., Sen, G.C., Cabrer, B. and Lengyel P. (1978) Biochem. Biophys. Res. Commun. 81, 947-954.
26 Eppstein, D.A. and Samuel, C.E. (1977) Biochem. Biophy. Res. Commun. 79, 145-153.
27 Eppstein, D.E. and Samuel, C.E. (1978) Virology 89, 240-251