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2′5′Oligo(A) Polymerase Activity and Inhibition of Viral RNA Synthesis in Interferon-Treated HeLa Cells†

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ABSTRACT: A quantitative assay for 2′5′oligo(A) polymerase, based on the adsorption of cell extract to poly(I)·poly(C)–agarose and incubation with [^{3}H]ATP, has been developed. The [^{3}H]2′5′oligo(A) synthesized is resolved from ATP by chromatography on DEAE-cellulose. The 2′5′oligo(A) polymerase activity has been measured in extracts of control HeLa cells and cells incubated with different concentrations of human fibroblast interferon or for different lengths of time with 100 units/mL of interferon. An increased enzymatic activity can be detected in cells treated with 12.5 units/mL or higher concentrations for 17 h and in cells treated with 100 units/mL longer than 3 h. The synthesis of encephalomyocarditis virus (EMC) RNA is inhibited in these cells in parallel with the increase in polymerase activity. Treatment of HeLa cells with interferon followed 2 h later by actinomycin D prevents the increase in 2′5′oligo(A) polymerase and the inhibition of viral RNA synthesis. After 4-h treatment with interferon, actinomycin D does not show this effect, whereas cycloheximide prevents the increase in polymerase activity. Active RNA synthesis 2 h after the start of interferon treatment and active protein synthesis 4 h afterward is necessary for the increase in 2′5′oligo(A) polymerase activity. Another enzymatic activity, previously reported to be increased in interferon-treated cells, is a double-stranded RNA activated protein kinase, which phosphorylates two polypeptides associated with ribosomes. Assays of this protein kinase have shown an increase in activity in cells treated with 12.5 units/mL or higher interferon concentrations. However, the increase in kinase activity can only be detected in cells treated for at least 10 h. In particular, HeLa cells treated for 7 h with 100 units/mL of interferon do not show increased protein kinase activity; upon such treatment, EMC RNA synthesis is already significantly inhibited. Therefore, an increased protein kinase activity may not be required for an inhibition of EMC RNA synthesis.

Hovanessian et al. (1977) have recently reported that, when double-stranded RNA (dsRNA)[1] is added to extracts of interferon-treated L cells, an enzymatic activity forms an inhibitor of protein synthesis from ATP. The inhibitor has been characterized by Kerr & Brown (1978) as pppA-(2′p5′A)$_{1-4}$2′p5′A$_{OH}$ or 2′5′oligo(A). Cell extracts adsorbed

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[1] Abbreviations used: ds, double stranded; pfu, plaque-forming units.

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to poly(I)·poly(C)–agarose have been shown to polymerize ATP into 2′5′oligo(A), with the predominant product being the trinucleotide (Kerr & Brown, 1978). We designate the corresponding enzymatic activity to 2′5′oligo(A) polymerase. Its presence in other cell types has been shown by Ball & White (1978) in interferon-treated chick embryo cells, by Hovanessian & Kerr (1978) in rabbit reticulocytes, and by Baglioni et al. (1978) in interferon-treated and untreated HeLa cells. The picture emerging from these studies is that 2′5′oligo(A) polymerase is present at some basal level in cells not treated with interferon and also in cells not susceptible to the antiviral effect of interferon-like reticulocytes. However, a greatly increased 2′5′oligo(A) polymerase activity is found in interferon-treated cells.

The 2′5′oligo(A) inhibits protein synthesis. Kerr & Brown (1978) first suggested that 2′5′oligo(A) may not be directly inhibitory for protein synthesis but that it may regulate an inhibitory event. This was subsequently shown by Clemens & Williams (1978), who demonstrated that 2′5′oligo(A) activates a nuclease, which prevents mRNA from being utilized for protein synthesis. Similar conclusions were reached by Baglioni et al. (1978). The nuclease activated by 2′5′oligo(A) is present in extracts of interferon-treated and control cells. It is an endoribonuclease since it cleaves mRNA bound to ribosomes which are blocked by inhibitors of ribosome movement (Clemens & Williams, 1978; Baglioni et al., 1978) and does not cleave DNA (Nilsen and Baglioni, unpublished observations).

Relatively little is known about the substrate specificity of the nuclease activated by 2′5′oligo(A). Baglioni et al. (1978) reported that both cellular and viral mRNAs are cleaved by this enzyme and that free and polysome-bound mRNAs are cleaved equally well. Clemens & Williams (1978) found that globin mRNA was cleaved by the 2′5′oligo(A)-activated nuclease. However, Ratner et al. (1977) had previously reported that a nuclease activated by dsRNA and ATP—presumably the same enzyme—cleaves different mRNAs at different rates. Whether the nuclease attacks viral templates in interferon-treated virus-infected cells and participates in the inhibition of virus replication in this way has not yet been established.

A protein kinase activity is also activated by dsRNA and ATP in extracts of interferon-treated L cells (Lebleu et al., 1976; Roberts et al., 1976; Zilberstein et al., 1976). A similar protein kinase activity is also present in rabbit reticulocytes (Farrell et al., 1977) and in HeLa and L cells not treated with interferon (Lenz & Baglioni, 1978). The dsRNA-activated protein kinase may therefore be present at a basal level in these cells, but its activity is increased several-fold by treatment with interferon. The dsRNA-activated protein kinase is associated with ribosomes and phosphorylates two polypeptides, one of M_r about 38000 and the other of M_r 70000 to 80000 in different cells (Farrell et al., 1977; Shaila et al., 1977; Lenz & Baglioni, 1978). The smaller polypeptide has been identified with a subunit of the initiation factor eIF-2; phosphorylation of this polypepetide leads to loss of Met-tRNA$_f$ binding to 40S ribosomal subunits and to inhibition of protein synthesis (Farrell et al., 1977). This observation explains why initiation of protein synthesis is markedly inhibited by dsRNA in extracts prepared from interferon-treated cells (Cooper & Farrell, 1977). However, the role of the dsRNA-activated protein kinase in the inhibition of virus replication and its relationship, if any, to the 2′5′oligo(A) polymerase and the endonuclease are not yet understood.

We have measured 2′5′oligo(A) polymerase activity and synthesis of viral RNA in HeLa cells exposed to different doses of interferon or at different times after exposure to a standard dose. An increase in 2′5′oligo(A) polymerase activity has been demonstrated after an initial 3-h lag. This increase is strikingly correlated with an inhibition of encephalomyocarditis virus (EMC) RNA synthesis. Treatment of the cells with actinomycin D prevents the increase in 2′5′oligo(A) polymerase activity and the inhibition of viral RNA synthesis. The activity of the ribosome-bound dsRNA-activated protein kinase has also been determined under the same experimental conditions. The increase in this protein kinase activity is delayed relative to that of 2′5′oligo(A) polymerase but is elicited by the same interferon concentrations which are effective in inducing the polymerase. However, the increase in protein kinase activity follows by several hours the inhibition of viral RNA synthesis, suggesting that an increased protein kinase activity may not be necessary for this inhibition.

Experimental Procedures

Cell Growth, Treatment, and Preparation of Cell Extracts. HeLa cells were grown in suspension cultures in minimum Eagle's medium with 5% horse serum. The cells were treated with human fibroblast interferon (3×10^5 NIH units/mg; obtained from the Interferon Working Group of the National Cancer Institute, NIH) while in logarithmic growth at a density of about 3×10^5 cells/mL. The cells were harvested by centrifugation and washed with spinner salts for the preparation of cell extracts as described by Weber et al. (1975). One volume of packed cells was resuspended in 2 vol of homogenization buffer in this procedure. This resulted in a rather uniform concentration of cellular components, as determined by measurements of protein concentration and A_{260} of the cell extracts.

2′5′Oligo(A) Polymerase Assay. The assay is a modification of the method used by Hovanessian et al. (1977) to prepare 2′5′oligo(A). Poly(I)·poly(C)–agarose was purchased from P-L Biochemicals or prepared according to the procedure of Wagner et al. (1971), by covalently binding poly(I) to cyanogen bromide activated Sepharose 4B and then annealing poly(C). Poly(I)·poly(C)–agarose (0.25 mL) was dispensed in a 0.5-cm diameter column and washed with 10 mL of 1 M KCl and 10 mL of 0.1 M KOAc, 5 mM $Mg(OAc)_2$, 2 mM dithiothreitol, 20% glycerol, and 20 mM Hepes–KOH, pH 7.4 (buffer K). Cell extract (20–75 μL) was diluted with 0.2 mL of buffer K and passed through the column, which was then washed with 10 mL of buffer K. Three 1-mL aliquots of 1 mM ATP in buffer K were then passed through the column, followed by three 0.1-mL aliquots of the same solution containing 2 μCi/mL of [^{3}H]ATP. This solution was allowed to pass through the column until no liquid was left above the top of the agarose. The column was then sealed at the bottom and incubated in a water bath at 30 °C. The column was eluted with two 0.5-mL aliquots of buffer K. The columns can be reused a few times by washing with 10 mL of water, 10 mL of 0.5% sodium dodecyl sulfate in 1 M LiCl, 10 mL of water, and again with 1 M KCl and buffer K.

The column eluate is applied to a 0.5-cm column containing 1 cm^3 of swollen DEAE-cellulose equilibrated with 90 mM KCl, 20 mM Hepes–KOH, pH 7.4. The column is washed with 25 mL of this solution and then eluted with 2 mL of 0.35 M KCl, 20 mM Hepes–KOH, pH 7.4. Calibration of this chromatographic procedure with AMP, ADP, and ATP showed these nucleotides to be eluted in this order from DEAE-cellulose with the first 25 mL of eluant. It was verified

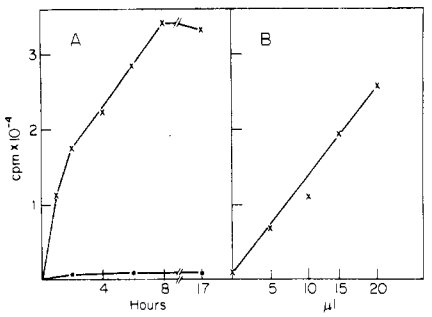

FIGURE 1: Assay for 2'5'oligo(A) polymerase with extracts of control and interferon-treated cells. The preparation of cell extracts is described under Experimental Procedures. Twenty microliters of extract from control cells (●) or from cells treated with 100 units/mL of human fibroblast interferon (X) was applied to a column of poly(I)·poly(C)–agarose and incubated with [^{3}H]ATP (200000 cpm) as described under Experimental Procedures. Panel A shows the time course of 2'5'oligo(A) synthesis. The [^{3}H]ATP was incubated with column-bound 2'5'oligo(A) polymerase for the times indicated and [^{3}H]2'5'oligo(A) measured by chromatography on DEAE-cellulose. Panel B shows the relationship between amount of extract from interferon-treated cells applied to the poly(I)·poly(C)–agarose columns and amount of [^{3}H]2'5'oligo(A) synthesized in a 17-h incubation. To each column was applied 20 μL of cell extract containing either control cell extract only or increasing amounts of extract from interferon-treated cells. The abscissa indicates the microliters of interferon-treated cell extract used for each determination. The conditions of incubation were the same as for panel A.

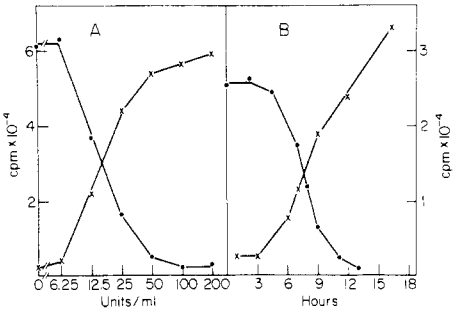

FIGURE 2: 2'5'Oligo(A) polymerase activity and inhibition of encephalomyocarditis virus RNA synthesis in interferon-treated HeLa cells. (A) Effect of treatment with increasing interferon concentrations and (B) effect of the time of treatment with interferon on 2'5'oligo(A) polymerase activity (X) and on EMC virus RNA synthesis (●). Two hundred milliliters of cells was treated with the indicated interferon concentration for 17 h or for the indicated time with 100 units/mL of interferon. A 10-mL aliquot was infected with EMC virus and viral RNA synthesis assayed after 5 h, while the rest of the culture was used for the preparation of cell extract as described under Experimental Procedures. Cell extracts (40 μL) were applied to poly(I)·poly(C)–agarose columns and incubated for 17 h with [^{3}H]ATP as described in Figure 1. The cpm of [^{3}H]2'5'oligo(A) synthesized are indicated. EMC RNA synthesis was assayed (A) by infecting the cells at the end of the 17-h treatment with interferon and (B) by infecting an aliquot of the cells 5 h prior to harvesting the rest of the culture. EMC RNA synthesis is expressed as cpm incorporated by 0.2 mL of cell culture in a 30-min incubation (see Experimental Procedures); the incorporation in 0.2 mL of a cell culture treated in the same way but uninfected was 1600 cpm.

that no 2'5'oligo(A) eluted from DEAE-cellulose with 90 mM KCl buffer by analyzing this fraction on DEAE-cellulose in the presence of 7 M urea as described by Kerr & Brown (1978) and Ball & White (1978). Rechromatography of the material eluted with 0.35 M KCl showed that it was completely retained on the column during the elution with the first 25 mL of 90 mM KCl and again quantitatively eluted when the KCl concentration was raised to 0.35 M.

Encephalomyocarditis Virus RNA Synthesis. A large stock of EMC virus was prepared in mouse ascites cells as previously described (Shafritz et al., 1976). This stock of EMC virus containing about 10^9 pfu/mL was used for all the experiments. EMC RNA synthesis was assayed in HeLa cells by measuring RNA-dependent RNA synthesis (Baltimore, 1969). The cells were collected by centrifugation and washed once with MEM minus serum and resuspended at 4.4×10^6 cells/mL in the same medium with additional 2 mM glutamine. The pH was adjusted by incubation in 5% CO_2, and 0.13 mL of the virus stock was added to 1 mL of cells for 30 min at room temperature; 1.1 mL of minimum Eagle's medium containing 10% fetal calf serum and 2 mM glutamine were then added and the cultures were incubated at 37 °C. Duplicate 0.2-mL aliquots were taken at the indicated times from the infected cultures, and 0.8 mL of minimum Eagle's medium containing 5% fetal calf serum and enough actinomycin D to have a final concentration of 5 μg/mL were added. After 10 min, 0.1 mL of the same medium containing 10 μCi of [^{3}H]uridine was added and the incubation continued for 30 min. The incubation was stopped by the addition of 10 mL of cold spinner salts, and the cells were collected by centrifugation. The pellet was resuspended in 0.5 mL of 0.5% sodium dodecyl sulfate and precipitated with 0.5 mL of 20% trichloroacetic acid onto glass fiber filters for counting.

Double-Stranded RNA-Dependent Protein Kinase Assay. Ribosomes were prepared from HeLa cell extracts and incubated with 0.5 μg/mL poly(I)·poly(C) and [γ-^{32}P]ATP as previously described by Lenz & Baglioni (1978). Samples were fractionated on 8.5-cm polyacrylamide slab gels which were made with 10% acrylamide and 0.1% bisacrylamide and run and autoradiographed as described by Lenz & Baglioni (1978).

Results

The procedure devised by Hovanessian et al. (1977) to synthesize 2'5'oligo(A) on poly(I)·poly(C)–agarose has been adapted to a quantitative assay for 2'5'oligo(A) polymerase (see Experimental Procedures). Figure 1A shows that, upon addition of [^{3}H]ATP to columns containing polymerase adsorbed from extracts of interferon-treated HeLa cells, 2'5'oligo(A) is synthesized for at least 8 h. Upon a 17-h incubation, an approximately equal amount of 2'5'oligo(A) is recovered as upon an 8-h incubation. The efficiency of conversion of ATP into 2'5'oligo(A) is extremely high; Figure 1A shows that about 15% of the input radioactivity is recovered as 2'5'oligo(A). This 2'5'oligo(A) behaved identically with oligonucleotides synthesized with interferon-treated L cell or chicken fibroblast extracts when analyzed according to Kerr & Brown (1978) and Ball & White (1978) by chromatography before and after digestion with nucleolytic enzymes (Minks et al., 1979).

Figure 1A shows that a column containing control cell extract synthesizes a very small amount of 2'5'oligo(A), in this experiment about 1/40 of the amount synthesized with interferon-treated cell extract. However, the presence of control cell extract does not interfere with the synthesis of 2'5'oligo(A). Figure 1B shows that, when mixtures of control and interferon-treated cell extract are applied to poly(I)·poly(C)–agarose, the synthesis of 2'5'oligo(A) is directly proportional to the microliters of interferon-treated cell extract used. This observation allowed us to measure 2'5'oligo(A) polymerase when this activity progressively increased upon treatment with graduated interferon concentrations or with the time of exposure of cell cultures to interferon.

Figure 2A shows that treatment of HeLa cells with concentrations of interferon higher than 6.25 units/mL causes a progressive increase of the activity of 2'5'oligo(A) polymerase.

Table I: Effect of Actinomycin D and Cycloheximide on the Induction of 2′5′Oligo(A) Polymerase Activity and on the Synthesis of EMC Virus RNA in Cells Treated with Interferon[a]

expt	cell treatment	2′5′oligo(A) synthesized (cpm)	EMC RNA synthesis (cpm)		
			−interferon	+interferon	% inhibition
1	none	16780	54030	38640	31
	Act. D after 2 h	3550	45820	39410	5
	Act. D after 4 h	13880	57950	39410	32
	cycloheximide after 4 h	3810			
2	none	21520	56790	27230	52
	Act. D after 2 h	5060	26660	21810	18
	Act. D after 4 h	14600	37635	16720	56

[a] In experiment 1, the cells were treated for 7 h and in experiment 2 for 8 h with 100 units/mL of interferon. Cell extracts were prepared and assayed for 2′5′oligo(A) polymerase activity as described in Figure 1. Aliquots of these cells and of cells not treated with interferon but incubated under the same conditions with 5 μg/mL of actinomycin D were tested for EMC virus RNA synthesis as described under Experimental Procedures. The cpm of [^{3}H]2′5′oligo(A) eluted with 0.35 M KCl are reported. The [^{3}H]uridine incorporated in a 30-min incubation 5 h after EMC virus infection is also indicated. An extract from cells not treated with interferon synthesized 2020 cpm of 2′5′oligo(A).

This increase seems to be directly proportional to the interferon concentration between 6.25 and 25 units/mL, whereas it becomes relatively smaller with higher concentrations of interferon. Treatment with 3 units/mL or lower concentrations of interferon has no effect on 2′5′oligo(A) polymerase activity or on viral RNA synthesis (data not shown). Figure 2B shows the time course of the increase of 2′5′oligo(A) polymerase activity upon cell treatment with 100 units/mL of interferon. No increase was detected up to 3 h. Afterward, a rapid increase was observed, which was followed up to 17 h.

The increase in 2′5′oligo(A) polymerase activity was correlated with an inhibition of viral RNA synthesis in interferon-treated cells infected with EMC virus. Figure 2A shows that EMC RNA synthesis was not inhibited in cells exposed to interferon concentrations which did not result in increased 2′5′oligo(A) polymerase activity. As the polymerase activity increased upon treatment with higher interferon concentrations, viral RNA synthesis was inhibited. Similarly, in the experiments shown in Figure 2B, as the polymerase activity increased with the duration of interferon treatment, viral RNA synthesis was inhibited. For a few hours after addition of interferon, the synthesis of EMC RNA was not impaired. After about 10 h of treatment with interferon, viral RNA synthesis was almost completely inhibited.

In these experiments, EMC RNA synthesis was measured by a 30-min pulse with [^{3}H]uridine in the presence of actinomycin D at 5 μg/mL. This assay of RNA-dependent viral RNA synthesis offered several advantages. The main one was that the assay could be carried out within a few hours of virus infection. It is relevant to point out that, in cells treated with 40 units/mL or higher interferon concentrations and infected with EMC virus, the time course of viral RNA synthesis is not appreciably changed (Figure 3 and data not shown). This allowed us to measure viral RNA synthesis 5 h after infection in both control and interferon-treated cells. However, when the effect of short incubations with interferon on viral RNA synthesis was determined, it was necessary to add interferon after virus infection. Since EMC virus shuts off host protein synthesis (Baltimore, 1969), expression of the antiviral state, which requires active protein synthesis (Friedman, 1977), may be prevented by this phenomenon. However, the experiment of Figure 2B shows that, even after 1 h exposure to interferon, cells washed and infected with EMC virus manifest after 5 h inhibition of viral RNA synthesis. Therefore, even under these conditions, the increase in 2′5′oligo(A) polymerase activity parallels the inhibition of viral RNA synthesis.

A further correlation between increase in 2′5′oligo(A) polymerase activity and inhibition of viral RNA synthesis was

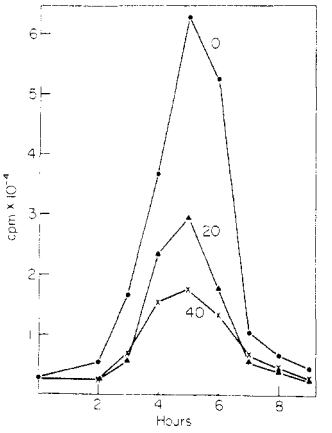

FIGURE 3: Encephalomyocarditis virus RNA synthesis in control cells and in interferon-treated cells. The cells were not treated with interferon (0) or treated with 20 and 40 units/mL for 17 h. The cultures were infected with EMC virus as described under Experimental Procedures. At the indicated times, aliquots of the cultures were incubated for 10 min with 5 μg/mL of actinomycin D and for 30 min with [^{3}H]uridine. The cpm incorporated in each assay are indicated. The procedures for lysing the cells and for counting are described under Experimental Procedures. On the abscissa are indicated the hours after infection with EMC virus.

provided by experiments with actinomycin D. When added 2 h after the beginning of interferon treatment, actinomycin D prevented a subsequent increase in polymerase activity to a large extent; however, after 4 h, actinomycin D had relatively less effect on the increase in polymerase activity (Table I). These results suggest that the increase in 2′5′oligo(A) polymerase requires RNA synthesis from 2 to 4 h after interferon addition. It seems possible that during this time period cells treated with interferon begin to synthesize an RNA required for the increase in 2′5′oligo(A) polymerase. That the increased activity is due to de novo protein synthesis is suggested by an experiment utilizing cycloheximide to block protein synthesis 4 h after addition of interferon. In this case very little increase in 2′5′oligo(A) polymerase activity was observed (Table I).

Viral RNA synthesis was measured in cells treated with interferon and actinomycin D or in parallel in cells treated with actinomycin D only. Prolonged treatment with actinomycin D reduced viral RNA synthesis in control cells. Therefore, the inhibition of EMC RNA synthesis due to interferon treatment was calculated with respect to control cells incubated with actinomycin D only (Table I). Addition of the antibiotic 2 h after interferon resulted in little or no inhibition of viral RNA synthesis. However, when added after 4 h actinomycin D had no effect on the inhibition of viral RNA synthesis.

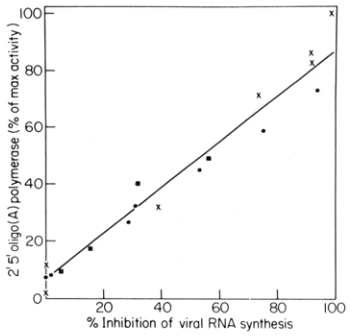

FIGURE 4: Correlation between 2′5′oligo(A) polymerase activity and inhibition of viral RNA synthesis. Each symbol represents the result of an experiment measuring 2′5′oligo(A) polymerase and viral RNA synthesis. The data are taken from Figures 2A (X) and 2B (●) and Table I (■). The 2′5′oligo(A) polymerase activity is expressed as percent of the maximal activity obtained with the highest interferon concentration tested (see Figure 2A) after 17-h treatment with interferon (Figure 2B) or relative to cells treated for 17 h with interferon (Table I). The inhibition of viral RNA synthesis is measured relative to control cells not incubated with interferon but infected with EMC virus and pulsed with [^{3}H]uridine under the same conditions.

These results and those of Figure 2 demonstate a striking correlation between 2′5′oligo(A) polymerase activity in cell extracts and inhibition of viral RNA synthesis in intact cells (Figure 4).

Increase in dsRNA-Activated Protein Kinase in Interferon-Treated Cells. The assay for dsRNA-activated protein kinase described by Farrell et al. (1977) and by Lenz & Baglioni (1978) has been used to follow the increase in this enzymatic activity upon cell treatment with interferon. This assay is based on the incubation of isolated reticulocyte or HeLa cell ribosomes with [γ-^{32}P]ATP and dsRNA and analysis of the phosphorylated products by electrophoresis and autoradiography. Several polypeptides are phosphorylated during an incubation without added dsRNA, but two polypeptides become markedly phosphorylated upon addition of dsRNA to the incubations (Farrell et al., 1977). The smaller polypeptide (M_r = 38 000) has been identified with a subunit of the initiation factor eIF-2, whereas the larger polypeptide has not yet been unequivocally identified (Farrell et al., 1977). It has been suggested, however, that this polypeptide may correspond to the dsRNA-activated protein kinase (Farrell et al., 1977).

Phosphorylation of the larger polypeptide is easily detected when HeLa cell ribosomes (Lenz & Baglioni, 1978) or extracts (Shaila et al., 1977) are incubated with dsRNA. We have confirmed the large increase in the phosphorylation of this polypeptide in HeLa cells treated with interferon (Shaila et al., 1977). Ribosomes from cells treated with 100 units/mL of interferon for 6 or 10 h and from cells not treated with interferon were incubated with or without poly(I)·poly(C). Figure 5 shows that a markedly phosphorylated band of M_r about 75 000 was detected only in cells treated with interferon for 10 h. The phosphorylation of the 38 000-M_r polypeptide was much less evident in the present experiments, because only one-half the amount of ribosomes was used relative to previous experiments (Lentz & Baglioni, 1978). Addition of purified eIF-2 (a gift of William C. Merrick of NIH) resulted in an enhanced phosphorylation of the 38 000-M_r band (data not shown). However, addition of poly(I)·poly(C) decreased the phosphorylation of a polypeptide of M_r about 110 000. The reason for this effect of dsRNA is not clear. Figure 5 also shows the results of a second experiment assaying the phosphorylation in the presence of poly(I)·poly(C) of ribosomes obtained from cells treated for various times with interferon.

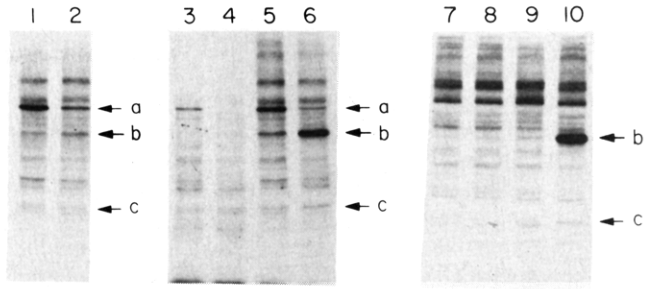

FIGURE 5: Phosphorylation of ribosome-associated proteins of cells treated with interferon for different lengths of time. Approximately 30 μg of ribosomes was incubated at 30 °C for 7 min with 0.1 mM ATP and 4 μCi of [γ-^{32}P]ATP (2690 Ci/mmol) in the absence (1, 3, 5) or presence (2, 4, 6–10) of 0.5 μg/mL poly(I)·poly(C) as described by Lenz & Baglioni (1978). The reactions were stopped by the addition of electrophoresis buffer and then analyzed as described under Experimental Procedures. Ribosomes are from control cells (1 and 2) or from cells treated with 100 units/mL interferon for 1 (7), 3 (8), 6 (3 and 4), 7 (9), or 10 h (5, 6, 10). Arrows indicate phosphorylated proteins of 110 000 (a), 75 000 (b), and 38 000 M_r (c).

Phosphorylation of the larger polypeptide is only evident with ribosomes of cells treated for 10 h with interferon.

The dsRNA-activated protein kinase was also assayed with ribosomes from cells treated for 17 h with different interferon concentrations. Phosphorylation of the larger polypeptide was only detected after treatment with 12.5 units/mL or higher interferon concentrations (data not shown). The lowest interferon concentration shown to inhibit viral RNA synthesis and to increase 2′5′oligo(A) polymerase activity was 12.5 units/mL (Figure 2). Therefore, the same interferon concentrations are effective in promoting an increase in these enzymatic activities and the inhibition of viral RNA synthesis. However, the increase in protein kinase activity is clearly detected only in cells incubated with interferon for at least 10 h, whereas inhibition of viral RNA synthesis can be shown in cells treated for 6 h (Figures 2 and 5). In particular, in cells treated with interferon for 7 h there is significant inhibition of viral RNA synthesis, whereas no increase in protein kinase activity can be detected. A significant increase in dsRNA-dependent protein kinase activity does not seem to be required for the inhibition of EMC RNA synthesis under the conditions of our experiments.

Discussion

The 2′5′oligo(A) polymerase activity can be determined by adsorbing cell extracts on poly(I)·poly(C)–agarose and measuring the labeled 2′5′oligo(A) formed upon incubation with [^{3}H]ATP (Hovanessian et al., 1977; Figure 1). The level of this enzymatic activity is determined by the treatment of the cells with interferon. Exposure of HeLa cells to low concentrations, below 6 units/mL of interferon, does not result either in increased 2′5′oligo(A) polymerase activity or in an inhibition of EMC virus RNA synthesis (Figure 2 and data not shown). Similarly, treatment with 100 units/mL of interferon for 3 h or less does not result in increased enzymatic activity or inhibition of viral RNA synthesis. Therefore, these two phenomena are concomitantly manifested after exposure of cells to interferon.

Experiments utilizing actinomycin D and cycloheximide suggest that the increase in 2′5′oligo(A) polymerase activity requires both RNA and protein synthesis. When RNA synthesis is inhibited within 2 h of interferon addition, only a small increase in polymerase activity and no inhibition of viral RNA synthesis are subsequently observed. Inhibition of RNA synthesis after 4 h of interferon treatment does not

prevent the expression of these two phenomena. The most significant observation is that inhibition of RNA synthesis prevents the increase in 2′5′oligo(A) polymerase activity since several investigators have previously reported that RNA synthesis is required for the establishment of the antiviral state (see Friedman, 1977). We have also shown that inhibition of protein synthesis by cycloheximide after a 4-h treatment with interferon prevents the increase in polymerase activity. Unfortunately, viral RNA synthesis cannot be measured in cells treated with cycloheximide since translation is required for the synthesis of viral replicase. In any case, this experiment suggests that synthesis of 2′5′oligo(A) polymerase or of another protein necessary for the increase in this enzymatic activity is initiated in HeLa cells approximately 4 h after exposure to interferon.

The striking parallelism between increase in 2′5′oligo(A) polymerase and inhibition of viral RNA synthesis suggests a relationship between these phenomena, but it is not possible to establish a cause–effect relationship with the present data, since we do not yet know whether 2′5′oligo(A) interferes with viral RNA synthesis in intact cells. Baglioni et al. (1978) have previously suggested that 2′5′oligo(A) polymerase may be activated in interferon-treated virus-infected cells by dsRNA of viral origin. However, dsRNA is not an intermediate in RNA virus replication, though partially double-stranded structures called replicative intermediates are involved in replication of picornaviruses (Bishop & Levintow, 1971).

The role of the dsRNA-activated protein kinase in the establishment of the antiviral state is obscure. An increase in the activity of this enzyme is detected only after 10 h of interferon treatment in HeLa cells. At this time EMC virus RNA synthesis is already markedly inhibited in interferon-treated virus-infected cells (Figure 2B). This may suggest that an increased protein kinase activity is not necessary for the inhibition of EMC RNA synthesis. However, the failure to detect enhanced protein kinase activity earlier than 10 h after interferon addition does not exclude a role for this enzyme in the early antiviral effects of interferon. Since no information is available on the level of kinase activity required for a biological effect (whatever this may be in intact cells), it is possible that the phosphorylation observed in vitro may be in excess of that effective in vivo. Thus, phosphorylation of the 38 000-M_r polypeptide may contribute, along with synthesis of 2′5′oligo(A), to the inhibition of protein synthesis in interferon-treated, virus-infected cells.

It must be pointed out that our understanding of the mechanism of interferon action is rather limited. The involvement of dsRNA in the activation of the 2′5′oligo(A) polymerase and protein kinase, for example, is somewhat puzzling. Interferon prevents replication of DNA as well as RNA viruses (Friedman, 1977), and even though there are reports that some DNA viruses, like vaccinia, synthesize a small amount of dsRNA (Colby & Duesberg, 1969; Duesberg & Colby, 1969), this is not necessarily a characteristic feature of DNA viruses replication.

In conclusion, we have established a correlation between an increase in 2′5′oligo(A) polymerase in interferon-treated cells and the inhibition of viral RNA synthesis in these cells infected by EMC virus. Further work is necessary to establish whether the polymerase is in fact activated by double-stranded replicative intermediates of RNA viruses and whether this activation results in cleavage of viral templates. The increase in 2′5′oligo(A) polymerase activity and the activation of an endonuclease by the product of this enzyme may represent one of the mechanisms which prevent virus replication in interferon-treated cells.

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