Cellular RNA is not degraded in interferon-treated HeLa cells after poliovirus infection

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A drastic inhibition of protein synthesis occurs in HeLa cells treated with human lymphoblastoid interferon and infected with poliovirus. At the time when this inhibition has been established no degradation of ^{32}P-labelled ribosomal RNA can be detected. Isolation of the mRNAs from poliovirus-infected cells plus or minus interferon treatment, followed by translation in a reticulocyte lysate indicates that cellular mRNAs remain active. These results suggest that gross degradation of cellular RNA does not occur in interferon-treated poliovirus-infected HeLa cells and that a non-specific nuclease induced by 2′–5′ A is not responsible for the inhibition of protein synthesis observed.

Interferon *Poliovirus* *Protein synthesis* *Shut-off* *2′–5′A Synthetase* *RNA degradation*

1. INTRODUCTION

Interferons are inducible proteins synthesised by mammalian cells in response to an array of different stimuli [1]. They interact with homologous cells and produce in them different biological effects [1–3]. Amongst these, the antiviral and antiproliferative activities of interferon have been most widely studied.

The interaction of interferon with a target cell induces the synthesis of a number of proteins. Some of these, like the 2′–5′ A synthetase [4–6], the protein kinase [4–6] and the major histocompatibility antigens [7,11], have been identified, but there are likely to be others [11,14]. The synthesis of these new proteins is required to establish the so-called 'antiviral state' and virus growth in such cells is inhibited. The step in virus development that is inhibited in IFN-treated cells is different for different virus species [6,15]. For the picornaviruses, there is evidence that the antiviral activity involves an inhibition of viral protein synthesis [16] and two different mechanisms to explain the inhibition have been proposed [5,17]. One involves an increase in a protein kinase activity that is activated by double-stranded (ds) RNA. The kinase phosphorylates an initiation factor, most probably the small subunit of eIF2, and results in the inhibition of its activity. As yet no evidence exists that the eIF2 becomes phosphorylated in virus-infected cells pretreated with interferon [18]. The other mechanism proposed to explain the inhibition of protein synthesis, involves an enzyme known as the 2′–5′-oligo(A) synthetase. This enzyme is also activated by dsRNA and catalyses the synthesis of a series of oligonucleotides known collectively as 2′–5′-oligo(A) [4,6]. These oligonucleotides, in turn, trigger the activation of an endogenous nuclease activity that non-specifically degrades RNA and leads to the inhibition of protein synthesis. Though the 2′–5′-oligo(A) has been detected in interferon-treated virus-infected cells [17], as yet there is very little evidence to indicate whether either mechanism operates in intact cells, and if so, which predominates.

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In contrast to the paucity of evidence in vivo, there is now overwhelming evidence obtained from cell-free systems that the 2′–5′ A system can inhibit the translation of viral and cellular mRNAs in vitro [19]. Moreover, translation is inhibited in cells permeabilized to 2′–5′-oligo(A), perhaps suggesting that this compound can activate a nuclease in the cytosol of the intact cell [20]. Evidence from EMC-infected cells indicates that 2′–5′-oligo(A) is synthesized in amounts considered sufficient to activate the nuclease [21]. However, it has been found [22] that the stability of early mRNA coding for SV40 T-antigen is unchanged in BSC-1 cells following treatment with interferon and the mRNA remains active in cell-free systems. Furthermore, mRNA from L cells treated with mouse interferon and infected with mengovirus is not degraded and can be translated in vivo [23]. These results argue against a non-specific nuclease as the basis of the antiviral effect.

2. MATERIALS AND METHODS

2.1. *Cells and virus*

HeLa cells were grown and propagated in Dulbecco's modified Eagle's medium (E4) supplemented with 10% calf serum. Poliovirus type I was grown on HeLa cells in Dulbecco's modified Eagle's medium supplemented with 1% calf serum. The fraction obtained after removal of cell debris by low-speed centrifugation was used as source of virus.

2.2. *Interferon*

Human lymphoblastoid interferon (HuIFN-α (Ly); 1.6×10^6 IU/mg protein) was a generous gift of Drs Finter, Fantes and Johnston, Wellcome Research Labs. (Beckenham).

2.3. *Labelling and analysis of protein in vivo*

Cells were grown in 30-mm diam. dishes and pulsed with 0.5 ml E4 medium without methionine containing 1% calf serum and 5.4 μCi [^{35}S]methionine (1000 Ci.mmol^{-1}). At the end of the labelling period the medium was removed. Cell monolayers were washed with phosphate buffer solution and dissolved in 200 μl 0.02 N NaOH plus 1% SDS and 200 μl of buffer [62.5 mM Tris (pH 6.8), 2% SDS, 0.1 M dithiotreitol, 17% glycerol and 0.024% Bromophenol blue as indicator]. Samples were sonicated to reduce viscosity and heated at 90°C for 5 min; 10 μl were precipitated with 10% trichloroacetic acid and filtered through GF/C filters. The radioactivity retained in the filters was estimated in an Intertechnique spectrometer using a toluene–PPO–POPOP cocktail. Other 10 μl samples were applied to a 15% polyacrylamide gel and run overnight at 30 V. Fluorography of the gels was done with 2,5-diphenyloxazole/dimethylsulfoxide (20%, w/w). The dried gels were exposed to X-ray films.

2.4. *Analysis of [^{32}P]RNA*

Cells were grown in 100-mm diam. dishes. When they reached confluence the medium was removed and the monolayers were washed with E4 medium without phosphate for 15 min. Then 6 ml of E4 medium containing 0.1 mM phosphate and 250 μCi/ml carrier-free [^{32}P]phosphate and 2% fetal calf serum dialyzed against Eagles saline without phosphate, were added; 6 h later 200 IU/ml HuIFN-α (Ly) was added to the corresponding dishes, and 18 h after this the medium was removed and the cells were infected with poliovirus (20 PFU/cell). After 1 h at 37°C the cells were placed in E4 medium containing 2% calf serum and 4 h later the RNA was extracted. For this purpose the medium was removed and cell monolayers were washed 3-times with phosphate saline salution, scraped off in E4 medium using a rubber policeman, washed 3-times and resuspended in 2 ml 10 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 10 mM Tris–HCl (pH 7.4), 50 μg dextran sulfate/ml and 220 μl 10% NP40. Nuclei were removed by centrifugation at 900 × *g* for 5 min at 2°C. The supernatant was made of 2 M LiCl and 1% SDS and left overnight at −20°C. RNAs were collected by centrifugation at 8000 × *g* for 30 min at 2°C, resuspended in distilled water and stored at −80°C; 20 000 cpm of each sample were applied to a 1.5% agarose gel and run for 2.5 h at 25 mA. The gel was stained with ethidium bromide (10 μg/ml) for 10 min and then dried and exposed to X-ray film.

2.5. *Purification and cell-free translation of mRNAs*

The medium was removed 5 h after poliovirus infection and the cell monolayers were washed with phosphate saline solution and chilled Tris–

saline; 2 ml/dish of Tris–saline were added and cells scraped off with a rubber policeman.

Cells were collected into siliconised tubes by centrifugation at 9000 × *g* for 5 min at 0°C, washed 3-times with Tris–saline and resuspended in 10 vol. NDS buffer [20 mM Tris (pH 7.5), 100 mM NaCl, 1 mM EDTA, pH 7.5, 1% naththalene-1,5-disulphonic acid–disodium salt] and 1/10th vol. of 10% NP40. Nuclei were pelleted by centrifugation at 10 000 × *g* for 5 min at 0°C. The supernatant was collected and 1/30th vol. 15% SDS, 1/30th vol. 3 mg/ml potassium polyvinyl sulphate and 1 vol. phenol:chloroform:isoamyl alcohol (50:50:1, by vol.) were added. The phenol extraction was repeated 3-times at room temperature and was followed by extraction with chloroform:isoamyl alcohol (50:1, v/v). The final aqueous phase was made 0.1 M NaCl and left overnight at −20°C with 2.5 vol. ethanol. The RNAs were collected by centrifugation at 10 000 × *g* for 30 min at 0°C, resuspended in distilled water and reprecipitated 3-times to remove phenol and finally resuspended in 0.7 M NaCl, 10 mM EDTA (pH 7.5), 25% formamide and 50 mM Tris (pH 7.5). The poly(A)$^+$ fractions were then removed from the total cellular RNAs by affinity chromatography through 250-μl poly(U)–Sepharose 2B columns. The retained RNA was eluted with a buffer containing 90% formamide, 10 mM EDTA (pH 7.5), 0.2% SDS and 10 mM Tris (pH 7.5) and precipitated overnight with 0.1 M NaCl and 2.5 vol. −20°C ethanol at this temperature. mRNAs were collected and washed as before and finally their purities checked using a 190–450 nm scan.

The in vitro assay for protein synthesis was carried out in micrococcal nuclease-treated reticulocyte lysates prepared as in [29]. Each tube contained (in 30 μl): 20 μl reticulocyte lysate, 165 μM of each of the unlabelled L-amino acids minus methionine, 30 μCi [^{35}S]methionine (1000 Ci/mmol), 5 mM 2-aminopurine and the indicated quantity of mRNA of each origin. After 90 min incubation at 34°C, 2 μl of each sample were analyzed by polyacrylamide gel electrophoresis followed by fluorography and autoradiography as above. Other samples (2 μl) were incubated at 34°C for 15 min with 20 vol. H_2O_2 and precipitated with 10% trichloroacetic acid. The radioactivity incorporated into proteins was estimated after filtration on nibrocellulose filters.

3. RESULTS AND DISCUSSION

We have studied HeLa cells infected with poliovirus and treated with human lymphoblastoid interferon and asked two questions:

(i) Is there inhibition of protein synthesis in poliovirus-infected HeLa cells when they are pretreated with interferon?

(ii) Is the 2′–5′ A activated nuclease involved in this inhibition?

To answer the first question HeLa cells were treated with a high dose of interferon (50 IU/ml) sufficient to block the production of infectious virus by almost 99%, and then infected with poliovirus at a high multiplicity (20 PFU/cell). Protein synthesis was followed throughout infection by estimating the amount of [^{35}S]methionine incorporated into trichloroacetic acid precipitable material and by analysis of the proteins synthesized on polyacrylamide gels. Fig.1 shows that a drastic inhibition of translation takes place in poliovirus-infected HeLa cells, and this is even more marked when the cells are pre-treated with the HuIFN-α (Ly). Analysis of the proteins synthesized indicated that interferon treatment blocks the appearance of detectable amounts of poliovirus proteins.

To know whether the shut-off of host protein synthesis that occurs in IFN-treated cells is mediated by the non-specific degradation of RNA, we analyzed the RNAs from poliovirus-infected HeLa cells, plus or minus IFN-treatment. In the first experiment the cellular RNAs were labelled overnight with [^{32}P]phosphate. The label was removed and the cells were infected with poliovirus. The RNA was extracted 4 h after infection at the time when shut-off of protein synthesis was maximal. The extracted RNA was analyzed by agarose gels, stained with ethidium bromide and autoradiographed. No degradation of ribosomal RNA was apparent (fig.2). Contrary to the results using cell-free systems, where the 2′–5′-oligo(A)-activated nuclease causes extensive non-specific degradation of RNA [4–6], this experiment indicates that no gross degradation of cellular RNAs occurs in intact cells.

The experiment in fig.2 cannot exclude the possibility that cellular mRNAs are specifically modified or degraded in the interferon-treated, infected cells, and rendered inactive in protein synthesis. To test this possibility the mRNA was extracted from untreated cells and cells treated with interferon 5 h

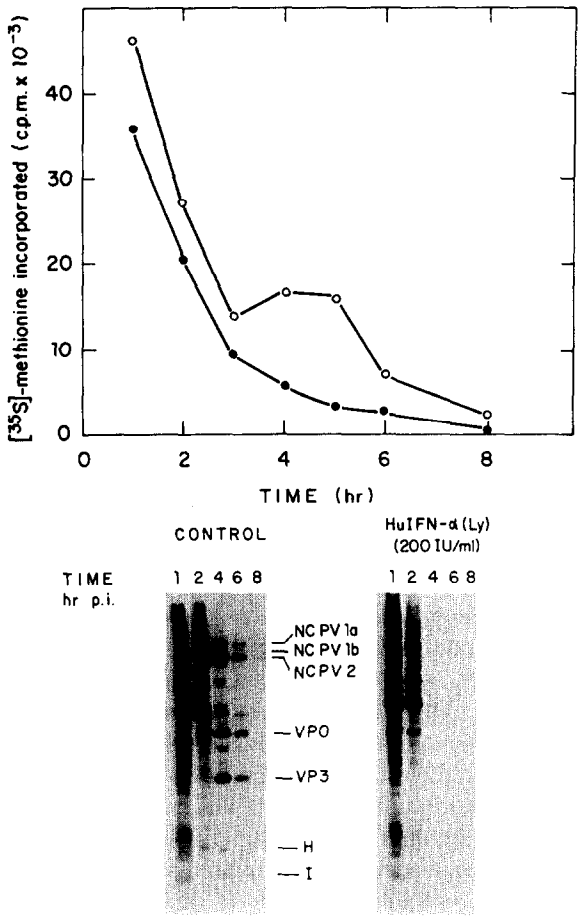

Fig.1. Effect of human lymphoblastoid interferon in poliovirus-infected HeLa cells. Cells were grown in 30-mm diam. dishes containing 2 ml E4 medium with 10% calf serum. One series of dishes was treated with 50 IU/ml HuIFN-α (Ly) 18 h before the infection with poliovirus (20 PFU/cell). After 1 h at 37°C the virus was removed and 1 ml E4 medium containing 2% calf serum was added. At the indicated times medium was removed and cells were pulsed for 1 h as in section 2: (○) control cells; (●) interferon-treated cells.

after poliovirus infection, and translated in a rabbit reticulocyte lysate. The mRNAs from poliovirus-infected cells that were not treated with IFN direct the synthesis of cellular and viral proteins in vitro. This demonstrates that active cellular mRNAs can be detected after infection and suggests that such mRNA is not degraded. It also demonstrates that viral RNA is present and can be detected. This is in agreement with other reports obtained using picornavirus-infected cells [24,25].

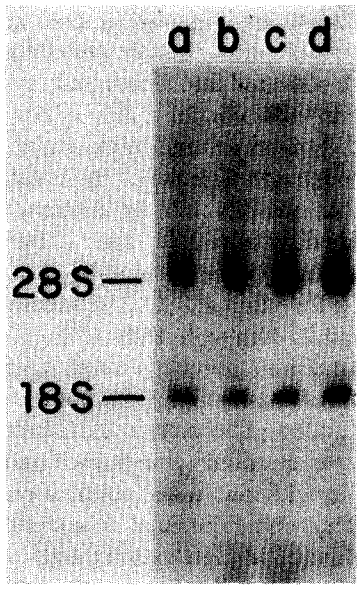

Fig.2. Stability of the total cellular RNA in interferon-treated poliovirus-infected HeLa cells. Cells were grown in 100-mm diam. dishes containing 10 ml E4 medium with 10% calf serum and labelled with [^{32}P]phosphate as in section 2. The labelled RNA was extracted 5 h after infection: (a) uninfected untreated cells; (b) uninfected IFN-treated cells; (c) poliovirus-infected untreated cells; (d) poliovirus-infected IFN-treated cells.

The mRNAs extracted from IFN-treated cells after poliovirus infection direct the synthesis exclusively of cellular proteins (fig.3). This suggests that the cellular mRNAs remain active in cell-free protein synthesis, when maximal shut-off of cellular protein synthesis has occurred in vivo, and this indicates that extensive degradation of cellular mRNA has not taken place. Because no poliovirus RNA-directed proteins are detected, this experiment also suggests that the block to poliovirus replication that occurs in HeLa cells treated with HuIFN-α (Ly) leads to an inhibition of viral RNA production. Such a conclusion contrasts with the results in [23], where it was found that some mengovirus RNA can be extracted and translated in vitro from IFN-treated mouse L cells infected with mengovirus.

Our results show that although viral and cellular protein synthesis is drastically blocked in interferon-treated cells after poliovirus infection, active cellular RNA can still be detected by cell-free translation. This argues that degradation of cellu-

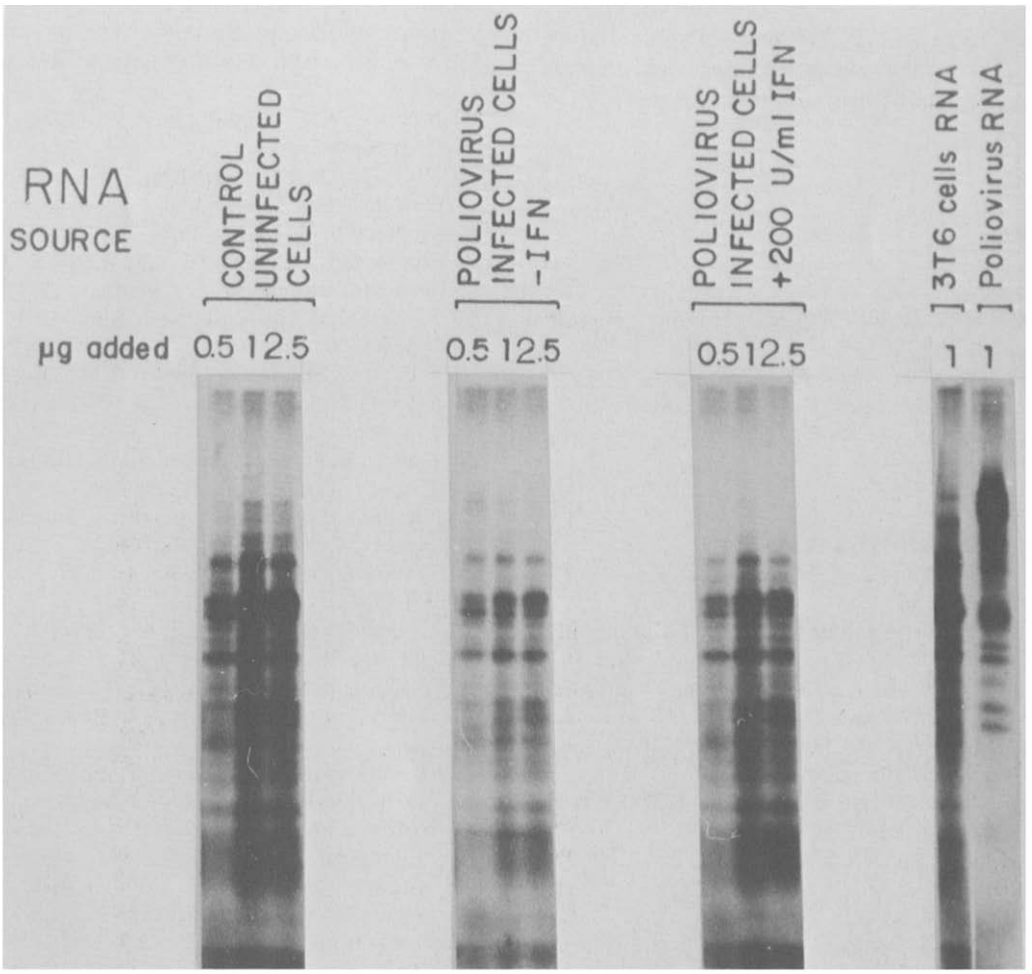

Fig.3. In vitro translation of mRNAs extracted from interferon-treated poliovirus-infected HeLa cells. Total cellular RNA of 8×10^8 cells was extracted and in vitro translated as in section 2 from either control untreated, or poliovirus-infected, or IFN-treated (200 IU/ml) and poliovirus-infected cells 5 h post-infection with 20 PFU/cell. Incorporation with 0.5 µg RNA after substraction of background was: control 110 805 cpm; poliovirus-infected 71 098 cpm; IFN-treated poliovirus-infected 90 456 cpm; incorporation without RNA, 18 553 cpm.

lar RNA is not involved in the inhibition of protein synthesis. Other evidence obtained using intact cells showed that there is no correlation between the levels of 2′–5′ A synthetase and the antiviral effect of interferon [26–28]. Besides, there is now firm evidence indicating that 2′–5′-oligo(A) is present in interferon-treated virus-infected cells [21]. In addition, authors in [30] have suggested on the basis of in vitro experiments that a selective degradation of viral mRNAs mediated by the 2′–5′ A system could occur in the intact cell. The possibility still remains open that such a selective degradation of viral mRNA is responsible for the antiviral effects of interferon.

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