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EFFECT OF INTERFERON ON TRANSIENT SHUT-OFF OF CELLULAR RNA AND PROTEIN SYNTHESIS INDUCED BY MENGO VIRUS INFECTION

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Infection of mouse L929 cells with Mengo virus resulted in a rapid shut-off of cellular RNA synthesis followed within the first hours post infection by a gradual decrease in host protein synthesis Pretreatment of the cells with high doses of interferon, blocking viral multiplication, did not affect the virus-induced shut-off of host macromolecular synthesis In these interferon-treated cells the 2',5'A-activated nuclease may account for the degradation of viral RNA, soon after its replication However, the inhibition of host protein synthesis could not be explained by this mechanism Poly(A)-containing RNA, present in interferon-treated and infected cells, amounted to as much as 70% of that present in interferon-treated, non-infected cells. On the other hand, extracted cytoplasmic RNA was efficiently translated in a reticulocyte lysate, showing that extensive mRNA degradation was not involved in the inhibition of host protein synthesis.

In the continued presence of interferon, the virus-induced shut-off was found to be transient. Late in infection, RNA synthesis was found to recover, followed by recovery of protein synthesis and survival of the cells.

interferon Mengo virus shut-off

INTRODUCTION

Infection of animal cells by picornaviruses results in rapid shut-off of cellular RNA and protein synthesis. This inhibition can be detected within the first hours post infection and precedes viral RNA and protein synthesis [25]. Finally, late in infection, cell death ensues. When the cells are pretreated with interferon, virus replication is inhibited, but shut-off of host protein synthesis is not prevented [18, 23]. However, in the continued presence of interferon, the shut-off is transient and the cells survive [9].

Treatment of cells with interferon induces at least two dsRNA-dependent enzymatic modifications, as shown by experiments with cell-free translating systems: a protein kinase which phosphorylates the small subunit of initiation factor eIF-2 and could be responsible for the inhibition of protein synthesis at the initiation level [14, 22, 30, 32, 39] and an oligonucleotide polymerase which synthesizes from ATP a series of oligo-

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nucleotides containing unusual 2′,5′-phosphodiester bonds, commonly designated as 2′,5′A [13, 20]. These are capable of activating an endonuclease responsible for the degradation of RNA [2, 5, 33, 37]. By these two pathways, virus multiplication would be inhibited in interferon-treated cells, provided that the infecting virus synthesizes some dsRNA. In fact, we have reported that the viral replicative form synthesized during Mengo virus infection can trigger, in interferon-treated L cells, the enzymatic modifications described in the in vitro experiments [1].

Since the 2′,5′A-activated nuclease was shown, in vitro, to degrade different species of RNA indiscriminately [29, 37], the obvious question was whether the inhibition of cellular protein synthesis that is observed early during viral infection in interferon-treated cells could be a consequence of the degradation of host mRNA. Here we show that this is not the case. We report also results on the sequence of events leading to the rescue of the interferon-treated cell.

MATERIALS AND METHODS

Reagents

[^{35}S]Methionine (1000 Ci/mmol and [^{3}H]uridine (29 Ci/mmol) were supplied by the Radiochemical Centre (Amersham, England). [^{3}H]Polyuridylic acid ([^{3}H]poly(U)) was a generous gift from Dr. Caput (Pasteur Institute, Paris); it was synthesized as described by Bishop et al. [4]. Mouse L929 cells were grown in monolayer culture in Eagle's minimum essential medium, containing 6% newborn calf serum (Flow Laboratories, Irvine, Scotland) at 37°C in a humidified, 5% CO_2 atmosphere. Purified Mengo virus was obtained by precipitation of virus stocks with polyethylene glycol [26] followed by cesium chloride centrifugation. Extraction of RNA from purified virus was carried out as previously described [6]. Interferon was induced in mouse L929 cell monolayers infected with Newcastle disease virus, and purified as desrcibed previously [7]. The different preparations used had specific activities from 5×10^7 to 1×10^8 units/mg protein and contained the two molecular variants of mouse interferon. MuIFN-α and MuIFN-β.

Measurement of RNA and protein synthesis in intact cells

Mouse L929 cells were grown in monolayer culture. For the experiments described in Figs. 1 and 2, 6×10^5 cells were plated in 35 mm diam. tissue culture dishes (Nunc Plastics, Roskilde, Denmark) and incubated for 24 h. The cultures were then incubated with plain medium or medium containing interferon (250 units/ml, 2.5 ml) for 18 h. Subsequently, the cultures were mock-infected or infected with 25 p.f.u. of Mengo virus per cell in 0.2 ml of medium. This time was considered as time 0. After 1 h incubation at 37°C, cells were washed with phosphate-buffered saline (PBS) and replenished with 2.5 ml of medium containing the appropriate concentration of interferon.

To measure RNA synthesis at different times after infection, cells were pulse-labelled for 20 min with [^{3}H]uridine (10 μCi/ml) added to the culture medium (0.5 ml). To measure protein synthesis, cells were incubated in medium lacking methionine for 15 min and then labelled for 30 min in the same medium (0.5 ml) supplemented with [^{35}S]-methionine (5 μCi/ml). At the end of the assay, cells were washed with cold PBS, and 1 ml of 5% trichloroacetic acid (TCA) was added for 1 h at 4°C. Cells were washed twice with 5% TCA, digested with 1 ml of 0.1 M NaOH for 60 min at 37°C and neutralized with 0.1 ml of 1 M HCl. The radioactivity of an aliquot was measured in Biofluor (New England Nuclear, Boston, MA).

Isolation of cytoplasmic RNA

30×10^6 cells were plated in 800 ml flasks (Nunc Plastics, Roskilde, Denmark) and incubated for 24 h in 40 ml of culture medium. Treatment with interferon and subsequent infection were performed as described previously. At different times post infection, the cells were washed and lysed for 15 min with 2 volumes of hypotonic buffer [10 mM, 4(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), pH 7.5, 10 mM KCl, 1.5 mM Mg $(CH_3COO)_2$] and disrupted in a Dounce tissue grinder (50 strokes) in the absence or presence of 0.5% of the non-ionic detergent NP-40 (BDH Chemicals Ltd., Poole, England). Ionic conditions were adjusted to 20 mM Hepes, pH 7.5, 120 mM KCl, 5 mM Mg $(CH_3COO)_2$, 7 mM β-mercaptoethanol. Cell extracts were centrifuged for 10 min at $7000 \times g$. The total RNA was prepared from the supernatant by the phenol/chloroform extraction procedure according to Shore and Tata [35].

Titration of poly(A)-containing RNA by hybridization with [^{3}H]poly(U)

[^{3}H]poly(U) was titrated as described previously [16]. The amount of poly(A)-containing RNA was expressed in percent of total RNA, assuming an average content of 10% poly(A) in cytoplasmic poly(A)-containing RNA.

In vitro translation of RNA extracted from cells

The micrococcal nuclease-treated mRNA-dependent reticulocyte lysates were prepared according to the procedure of Pelham and Jackson [28] as already described [5]. Cell-free systems (12.5 μl) were incubated at 30°C under optimal conditions for protein synthesis (50% v/v lysate with additional 2 mM Mg $(CH_3COO)_2$ and 75 mM KCl) in the presence of [^{35}S] methionine (2 μCi) as described by Vaquero and Clemens [37]. After 1 h incubation, the radioactive products were analysed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis [8]. Molecular weight was determined by comparison with marker proteins from BDH Chemical Ltd., (Poole, England, Cat. No. 44223.2u).

RESULTS

To study the role of interferon on the virus-induced shut-off and the cell recovery, the following optimal experimental conditions were established: monolayers of L929 cells were treated with interferon (2.50 units/ml) for 18 h, then mock-infected or infected with Mengo virus (25 p.f.u./cell). After infection, interferon was maintained in the culture medium.

When the multiplicity of infection was lower than 25 p.f.u./cell, the shut-off was delayed and was lesser in extent [3,9]. With low doses of interferon, although the virus replication was reduced, the cells did not recover from virus infection [10]. Moreover, interferon had to be maintained in the culture medium to allow recovery of cellular synthetic activity as well as ultimate survival. When interferon was washed off at the time of virus inoculation, shut-off and recovery did occur later (24–48 h), but the cells died from viral infection.

Kinetics of RNA and protein synthesis in intact cells

The pattern of inhibition of protein synthesis in interferon-treated, Mengo virus-infected cells has already been described [9]. There was an inhibition of the rate of [^{35}S]methionine incorporation into cellular proteins in addition to inhibition of viral protein synthesis. However, 8 h post infection, cellular protein synthesis resumed and at 24 h reached a level as high as that in interferon-treated uninfected cells.

Fig. 1 shows the rate of RNA synthesis at various times after infection as measured by pulse-labelling with [^{3}H]uridine. RNA synthesis decreased 1 h post infection whether or not the cells had been terated with interferon. Without interferon treatment [^{3}H]-uridine incorporation increased again at about 4 h post infection, reflecting the formation of newly synthesized viral RNA. Such resumption of [^{3}H]uridine incorporation did not occur in interferon-treated, infected cells. In these cells, the apparent shut-off of cellular RNA synthesis remained unchanged for 8 h. Later, RNA synthesis did increase: after 15 h it reached the rate of synthesis observed in control cells. Moreover, recovery of RNA synthesis preceded recovery of protein synthesis, as shown by the experiments performed in the absence of actinomycin D (Fig. 2).

In the presence of actinomycin D, [^{3}H]uridine incorporation was abolished, strengthening the interpretation that hardly any viral RNA was synthesized in interferon-treated cells. The shut-off of protein synthesis occurred as in the absence of actinomycin D, but after 8 h almost no increase in the protein synthetic activity was observed, suggesting that the recovery of cellular protein synthesis was a consequence of recovery of cellular RNA synthesis.

Kinetic study of the occurrence of poly(A)-containing RNA

In order to appreciate the amount of mRNA, total cytoplasmic RNA was extracted

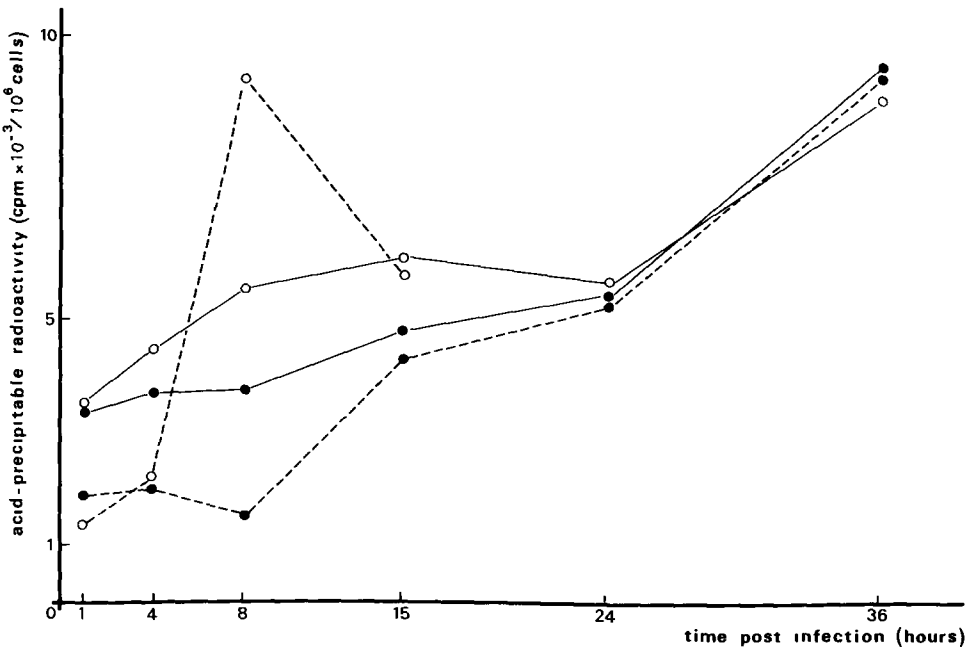

Fig. 1 Effect of interferon pretreatment on RNA synthesis in Mengo virus-infected cells. Monolayer cultures of L929 cells were incubated for 18 h with 250 units/ml of interferon, and subsequently infected with Mengo virus (25 p.f.u./cell). At different time intervals, the cultures were washed and pulse-labelled with [^{3}H]uridine (see Materials and Methods). Incorporated uridine was measured as acid-precipitable radioactivity. ○, Not treated with interferon. ●, Treated with interferon. ——, Not infected. - - -, Infected.

from control and interferon-treated cells with or without infection, and the content of poly(A)-containing RNA was determined by hybridization with [^{3}H]poly(U) (Fig. 3). The amount of poly(A)-containing RNA in interferon-treated cells was similar to that in control cells. The higher content of poly(A)-containing RNA in infected cells, not treated with interferon, reflected the presence of viral RNA. We have characterized it as viral RNA by its translation in a micrococcal nuclease-treated reticulocyte lysate (see next section).

In interferon-treated, infected cells the level of poly(A)-containing RNA was reduced within the first hours post infection to 70% of the control and remained constant up to the 8th hour, suggesting that severe degradation did not occur. Later in infection poly(A)-containing RNA increased and reached the level seen in appropriate controls.

The moderate reduction of poly(A)-containing RNA content observed early after infection does not seem sufficient to explain the 90% inhibition of cellular protein synthesis. It might be due partly to shut-off of cellular RNA synthesis (Fig. 1) as was found to be the case in Mengo virus-infected cells [3]. Our data also allow the exclusion of an

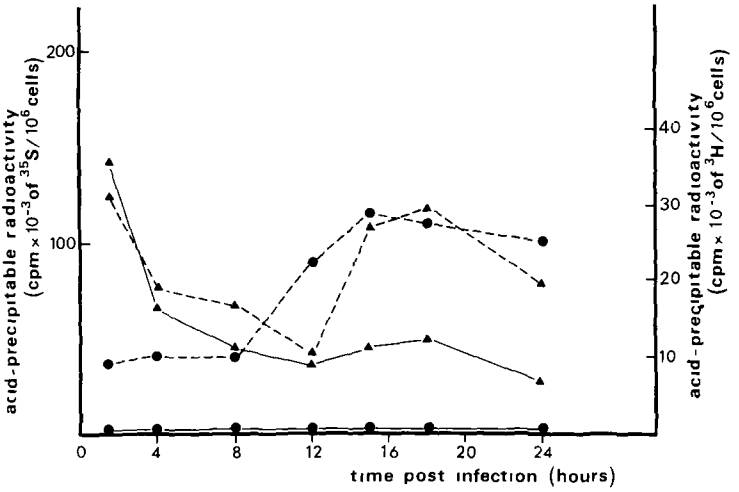

Fig. 2 Effect of actinomycin D on interferon-mediated rescue of RNA and protein synthesis in Mengo virus-infected cells. Monolayer cultures of L929 cells were incubated for 18 h with 250 units/ml of interferon and then inoculated with Mengo virus (25 p.f.u./cell). Actinomycin D (1 μg/ml) was added to the cultures 1 h post infection At different times post infection, some of the cultures were washed and pulse-labelled with either [^{3}H]uridine or [^{35}S]methionine (see Materials and Methods) Incorporation of label into RNA or protein was measured as acid-precipitable radioactivity. ●, [^{3}H]uridine. ▲, [^{35}S]methionine - - -, Without actinomycin D. ——, With actinomycin D added.

extensive degradation of poly(A)-containing RNA, even in interferon-treated cells. A slight endonucleolytic attack induced by the 2′,5′A-system might have occurred, leaving poly(A) segments that were measurable by poly(U) hybridization but not by translational activity. The following experiments were done to examine this possibility.

Template activity of cytoplasmic RNA

Cytoplasmic RNA from control and interferon-treated cells, uninfected or infected with Mengo virus, was prepared by the phenol/chloroform extraction procedure, and the template activity of these preparations was assayed in a micrococcal nuclease-treated reticulocyte lysate. The cytoplasmic RNA was extracted 7 h post infection, when the shut-off of protein synthesis was established and when viral RNA synthesis had occurred in infected cells that had not been treated with interferon. Fig. 4 shows the [^{35}S]methionine-labelled proteins synthesized in reticulocyte lysates, programmed by the different cytoplasmic RNA preparations. RNA from infected cells, not treated with interferon, directed the synthesis of viral polypeptides (Fig. 4, lane 4) similar to those synthesized with a purified preparation of viral RNA (lane 2). The patterns of synthesized proteins obtained with the cytoplasmic RNA from control (lane 3), interferon-treated (lane 5), and from interferon-treated, infected cells (lane 6) were quite similar to each other and merely of cellular origin.

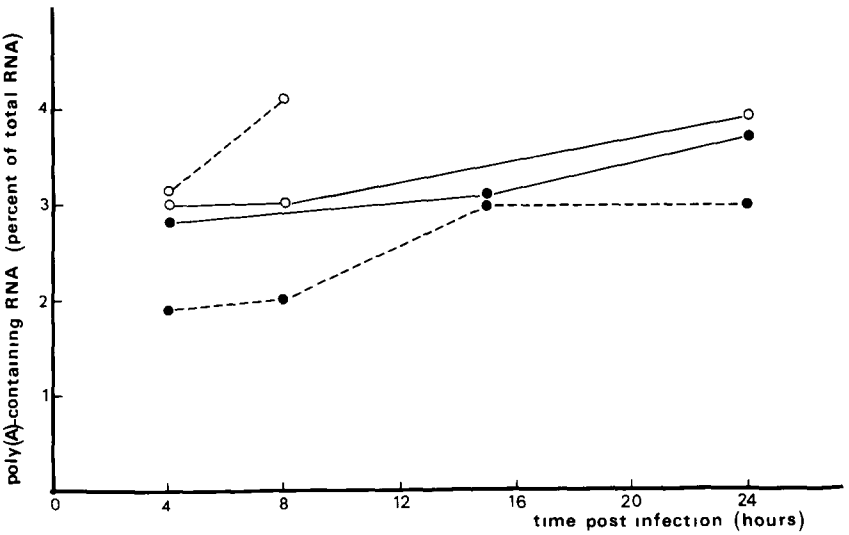

Fig. 3. Content of poly(A)-containing RNA in cytoplasma of interferon-treated and Mengo virus-infected cells as compared to appropriate controls. Monolayer cultures of L929 cells were incubated for 18 h with interferon (0 or 250 units/ml) and subsequently infected with Mengo virus (25 p.f.u./cells), or left uninfected. At the indicated time points, cytoplasmic RNA was extracted from the cell lysates and poly(A)-containing RNA was determined (see Materials and Methods). ○, Not treated with interferon. ●, Treated with interferon ——, Not infected - - -, Infected with Mengo virus.

The translation products of RNA from interferon-treated, virus-infected cells contained at least one extra polypeptide (besides the cellular ones) which co-migrated with a high molecular weight viral protein (∿ 110,000). A similar result was obtained with cytoplasmic RNAs extracted from interferon-treated, infected cells up to 36 h post infection (data not shown). The synthesis of some viral proteins occurring in spite of interferon pretreatment could be due to the translation of some newly synthesized viral RNA escaped from degradation by the 2′,5′A-induced nuclease, or to translation of parental viral RNA. In fact, it is known that only few particles of the incoming virus do initiate infection [3]; some particles may remain uncoated or partially coated, and their RNA extracted with the cytoplasmic RNA might be responsible for the translation of the 110,000 mol. wt. protein. Another possibility, not excluded by our data is that this protein is a cellular gene product. Hence, its viral character remains to be ensured by immunoprecipitation with antiserum raised against Mengo virus.

DISCUSSION

We have presented evidence that in L929 cells, treated with interferon, Mengo virus infection triggers early intracellular events similar to those already described in infected cells, not treated with interferon [25]. First, there is a rapid shut-off of cellular RNA synthesis followed by gradual inhibition of protein synthesis shown respectively by

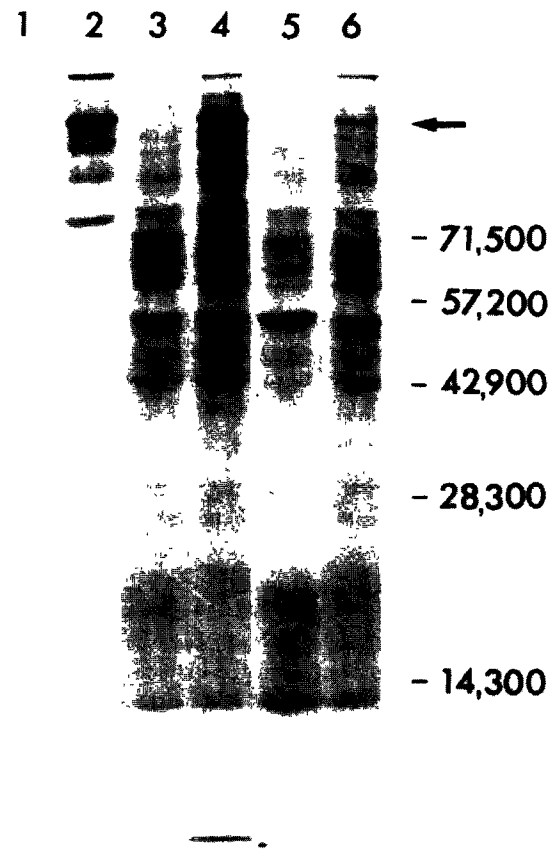

Fig. 4 SDS-polyacrylamide gel electrophoretic analysis of radioactive cell-free translation products obtained from interferon-treated, Mengo virus-infected cells as compared to appropriate controls. Monolayer cultures of L929 cells were incubated for 18 h with 250 units/ml of interferon and subsequently infected with Mengo virus (25 p.f.u./cell) 7 h post infection the cells were lysed and RNA extracts were prepared that were used as templates for translation in reticulocyte lysates (for details, see Materials and Methods). After 1 h of incubation, the total reaction mixture was subjected to SDS-gel electrophoresis. Lanes: 1) no RNA added; 2) purified Mengo virus RNA (5 μg/ml); 3) RNA from control cells (320 μg/ml), 4) RNA from cells infected with Mengo virus, but not pretreated with interferon (360 μg/ml); 5) RNA from uninfected, interferon-pretreated cells; 6) RNA from cells infected with Mengo virus, and pretreated with interferon (360 μg/ml). Arrow: location of 110,000 mol. wt. polypeptide. The location of bands obtained with molecular weight markers are shown on the right-hand side of the figure

the incorporation of [^{3}H]uridine and [^{35}S]methionine into acid-precipitable radioactivity. These results indicate that the early shut-off of cellular RNA synthesis could be partly responsible for the inhibition of cellular protein synthesis. Moreover, in interferon-treated cells, viral replication could not be detected by 20 min pulse-labelling experiments, even in the presence of actinomycin.

It has been shown that the introduction of dsRNA in extracts from interferon-treated cells triggers two enzymatic activities which participate in the inhibition of translation via phosphorylation of the α-subunit of initiation factor eIF-2 and via activation of an endonuclease. The latter can degrade viral as well as cellular RNA in vitro [17, 24, 34].

Several lines of evidence indicate that dsRNA does occur in Mengo virus-infected cells, whether or not they have been treated with interferon. By the use of a complementary DNA probe, Jacquet et al. [16] could detect some newly synthesized single-stranded Mengo virus RNAs in interferon-treated cells, implying the existence of a replicative complex. Also, by indirect measurement [1], it was possible to demonstrate that, even if there was a marked reduction in the amount of viral replicative forms in interferon-treated cells, residual dsRNA was still sufficient to trigger the two enzymatic activities shown to be induced in the lysates from interferon-treated uninfected cells. Recently, the natural occurrence of 2′,5′A has been demonstrated in interferon-treated cells infected with encephalomyocarditis virus [38]. In lysates of interferon-treated, Mengo virus-infected cell lysates, we have also found 2′,5′A activity (unpublished results).

It is reasonable to assume that the viral replicative form could induce, in vivo, the activation of an endonuclease leading to degradation of RNA. The inhibition of viral protein synthesis observed in cells treated with interferon and infected with Mengo virus might be due to degradation of single-stranded viral RNA soon after its synthesis. Similarly, the virus-induced shut-off of host protein synthesis, which is evident in interferon-treated as well as untreated cells, might be due to the breakdown of host mRNA. However, our results suggest that the inhibition of host protein synthesis is not due to a marked degradation of the host mRNA, as already reported for infected cells not pretreated with interferon [21]. Determinations of poly(A)-containing RNA during shut-off showed that cellular mRNA was not drastically degraded. The lower content of poly(A)-containing RNA observed in these cells, as compared to the uninfected control cells, could be due either to moderate degradation induced by the 2′,5′A system or to virus-induced inhibition of cellular mRNA synthesis or to both types of phenomena simultaneously. Nevertheless, the cellular mRNAs already present before virus infection had preserved their template activity, as evident from their efficient translation in a micrococcal nuclease-treated reticulocyte lysate.

Thus, it seemed that the dsRNA-induced endonuclease shows some specificity towards viral RNA in intact cells, despite the lack of specificity observed in cell-free systems. This finding is in agreement with the presence of an enhanced nuclease activity in a sedimentable fraction containing the viral replication complex [1] and with the experiments of Nilson and Baglioni [27] on discrimination for the cleavage of cellular and viral RNA in extracts from interferon-treated cells.

Our results are consistent with a model in which viral replicative forms activate nucleases through the 2′,5′A system in interferon-treated cells, as described in experiments with cell-free systems. This may then lead to the destruction of viral RNA. The restricted location of the nuclease activity in the vicinity of the replication complex would explain the absence of overall degradation of cellular mRNA. At the same time, an alteration

of certain initiation factors might occur and this might be responsible for the shut-off of cellular protein synthesis, as also seen in infected cells not pretreated with interferon [11,12,31,36].

The shut-off of cellular RNA and protein synthesis is transient in the interferon-treated, infected cells. Although during shut-off, most of the cellular mRNA remains undegraded and active in protein synthesis, actinomycin D can prevent resumption of protein synthesis, suggesting that newly synthesized RNAs are needed. To explain this, we propose that the inhibition of RNA synthesis early during infection brings about a shortage of certain types of mRNA, especially those with a short half-life time. One might speculate that amongst the mRNAs that are depleted are those coding for initiation factors necessary for resumption of cellular protein synthesis.

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