Inhibition of Viral mRNA Translation in Interferon-Treated L Cells Infected with Reovirus

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Murine L cells were treated with interferon (IFN) concentrations which reduced by 75 to 80% the synthesis of viral mRNA after infection with reovirus. Protein synthesis was not inhibited in these cells up to 6 h after infection, but a large fraction of the viral mRNA was not associated with polyribosomes and sedimented at about 50S. In contrast, most of the reovirus mRNA was associated with polyribosomes in control infected cells. This mRNA was of similar size to non-polyribosomal mRNA from IFN-treated cells when analyzed by Northern blot hybridization with a cloned cDNA for the *s2* reovirus mRNA, indicating that the non-polyribosomal mRNA was not appreciably degraded. Viral mRNA was labeled with [³H]uridine and the non-polyribosomal mRNA was isolated from IFN-treated cells. This mRNA could quantitatively bind to 80S initiation complexes when incubated in a rabbit reticulocyte cell-free system. These findings indicated that the non-polyribosomal RNA was translatable, but that its binding to functional initiation complexes was inhibited in IFN-treated cells by a discriminatory mechanism, which did not affect translation of cellular mRNA. Previous experiments showed that mRNA is blocked in 48S complexes when the α subunit of initiation factor eIF-2 is phosphorylated by the double-stranded RNA-dependent protein kinase induced by IFN. A localized activation of this kinase could explain the block of viral mRNA in 48S complexes. By labeling the phosphoproteins of IFN-treated cells with ³²P, eIF-2 (α P) was shown to cosediment with non-polyribosomal mRNA, presumably in 48S complexes.

Interferons (IFN) induce the synthesis of antiviral proteins, which are thought to inhibit the replication of infecting viruses by different mechanisms (7). Only two antiviral proteins have thus far been characterized: 2', 5'-oligoadenylate (2-5A) synthetase and a protein kinase which phosphorylates the α subunit of initiation factor eIF-2 (1). These enzymes are activated *in vitro* by RNAs containing double-stranded regions with at least 50 base pairs (9) and are thought to be activated in intact cells by transcriptional complexes of RNA viruses (1). The 2-5A activates an endoribonuclease (RNase L) which cleaves indiscriminately both viral and cellular RNAs (1). 2-5A is present in IFN-treated cells infected with encephalomyocarditis and reovirus (12, 15), and it may inhibit their replication by specifically promoting cleavage of viral RNA by RNase L (12). Evidence for this discriminatory antiviral mechanism in intact cells is provided by the observation that, even when 2-5A is present in IFN-treated cells infected with reovirus at low multiplicity, cleavage of cellular mRNA and rRNA is undetectable (12).

The role of the double-stranded RNA-activated protein kinase (PK_{ds}) in inhibiting virus replication in IFN-treated cells is presently unclear. Inhibition of protein synthesis and eIF-2 α phosphorylation is observed after infection with reovirus in HeLa cells with high levels of IFN-induced PK_{ds}, but not in cells with low levels of this kinase (11). The inhibition of protein synthesis is greater in cells infected with reovirus at high multiplicity than in cells infected at low multiplicity (11). In agreement with these observations, a three- to sixfold increase in eIF-2 α phosphorylation has been subsequently reported in IFN-treated murine L cells infected with reovirus at a multiplicity of infection (MOI) of 50 (13).

A cell-free system has recently been developed to couple mRNA transcription by isolated reovirions to translation by

reticulocyte lysate (6), which contains PK_{ds} levels comparable to those of IFN-treated cell extracts (6). In this cell-free system, a localized activation of PK_{ds} specifically inhibits binding of nascent reovirus mRNA to 80S initiation complexes, since other mRNAs bind to these complexes (6). This finding suggests that a discriminatory mechanism may specifically inhibit translation of viral mRNA. The present experiments were carried out to establish whether translation of viral mRNA in intact IFN-treated L cells infected with reovirus was inhibited by a similar mechanism. A large fraction of reovirus mRNA was apparently associated in these cells with 48S particles containing eIF-2(α P); this mRNA was functional since it formed 80S initiation complexes when incubated with reticulocyte lysate. These findings may provide an explanation for the observation that translation of early reovirus mRNA is inhibited in cells treated with IFN (15).

MATERIALS AND METHODS

Reovirus RNA synthesis. Murine L929 cells were grown as spinner cultures in minimum essential medium with 10% heat-inactivated calf serum. Cells at 3×10^5 /ml were treated for 18 h with murine IFN- β (5.6×10^7 IU/mg; Lee Biomolecular). Cells grown to $<7 \times 10^5$ /ml were infected with reovirus (Dearing III) at an MOI of 30. The cells were collected by centrifugation and suspended at 10^7 /ml in medium minus serum supplemented with 2 mM glutamine and 14 mM HEPES (*N*,2-hydroxyethylpiperazine-*N'*,2-ethanesulfonic acid) buffer, pH 7.2. After 1 h, 19 volumes of complete medium supplemented with glutamine and HEPES buffer were added. Viral RNA was labeled by incubating culture samples with 10 μ Ci of [³H]uridine per ml 15 min after the addition of 5 μ g of actinomycin D per ml.

Protein synthesis. Protein was labeled with 20 μ Ci of [³H]lysine per ml in incubations which were stopped by the addition of 3 volumes of ice-cold saline. The cells were collected by centrifugation, suspended in 1% sodium dode-

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cyl sulfate, and precipitated with 10% trichloroacetic acid for counting.

Polyribosome analysis. Pellets containing 2.5×10^7 cells were suspended in 0.5 ml of lysis buffer [10 mM NaCl, 1.5 mM $\text{Mg}(\text{OAc})_2$, 50 μg of dextran sulfate per ml, 10 mM Tris-hydrochloride, pH 7.4]. After 10 min on ice, Brij-58 and sodium deoxycholate were added to 0.3%. The supernatant obtained by 10-min centrifugation at $30,000 \times g$ was centrifuged for 2.5 h at 39,000 rpm through 15 to 40% (wt/wt) sucrose gradients in lysis buffer minus dextran sulfate to fractionate polyribosomes. Alternatively, the samples were centrifuged for 5 h at 38,000 rpm through 15 to 30% (wt/vol) sucrose gradients in 0.5 M NaCl–30 mM $\text{Mg}(\text{OAc})_2$ –20 mM Tris-hydrochloride, pH 7.5, to pellet polyribosomes and fractionate ribosomal subunits (4).

Blot hybridization of RNA. Gradient fractions were analyzed by dot hybridization according to White and Bancroft (14). A 30- μl amount of $20\times$ SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) and 20 μl of formaldehyde were added to 50- μl samples of gradient fractions. After being heated for 15 min at 60°C , the samples were filtered through nitrocellulose filters which had been soaked in $20\times$ SSC; the filters were baked for 2 h at 80°C , prehybridized for 18 h at 65°C with 0.1 mg of salmon sperm DNA per ml in $2\times$ SSPE– $5\times$ Denhardt solution (8), and hybridized for 24 h at 65°C with 5×10^5 cpm of alkali-denatured probe. The filters were washed with $0.5\times$ SSC–0.25% sodium dodecyl sulfate (8). Cloned cDNA for the *s2* mRNA of reovirus (3) was obtained from Wolfgang K. Joklik, Duke University Medical School, and cDNA for β -actin mRNA was a gift of Timothy W. Nilsen, Case Western Reserve University Medical School. The cDNAs were labeled to 10^8 cpm/ μg as described previously (8). The RNA in fractions of polyribosome gradients was precipitated by adding NaAc to 0.3 M and 2 volumes of ethanol. The RNA was phenol extracted and analyzed by Northern blot hybridization (8).

mRNA binding to initiation complexes. The RNA in fractions of ribosomal subunit gradients was precipitated with 2 volumes of ethanol, extracted with phenol, reprecipitated, and assayed for binding to 80S initiation complexes in incubations with reticulocyte lysate, as previously described (4). The binding assays were fractionated by centrifugation on sucrose gradients as described before (4).

Viral RNA fractionation. RNA precipitated from gradient fractions was applied to 5 to 15% (wt/vol) sucrose gradients and centrifuged for 17 h at 25,000 rpm, as previously described (2). Gradient fractions were precipitated with 5% trichloroacetic acid and counted.

Protein phosphorylation. Cells were infected or mock-infected as described above. At 4.5 h after infection, the cells were transferred to medium minus phosphate supplemented with glutamine and HEPES buffer. After 1 h the cells were concentrated by centrifugation and suspended at $3 \times 10^6/\text{ml}$; 1-ml samples were incubated for 1 h with 0.1 mCi of $^{32}\text{P}_i$. The cells were collected by centrifugation, washed with saline, and suspended in 2 volumes of lysis buffer containing 0.5% Triton X-100 and 10 mM NaF. The lysate was centrifuged at $30,000 \times g$, and the supernatant was used either for the isolation of 48S complexes, as described above, or for immunoprecipitation with an antiserum against purified reticulocyte eIF-2 (a kind gift of William C. Merrick, Case Western Reserve University Medical School). A 20- μl portion of cell extract was reacted with 2 μl of antiserum in a reaction containing 10 mM NaF to inhibit phosphatases. After 20 min on ice, 25 μl of SAC buffer (1% Triton X-100, 0.5% sodium dodecyl sulfate, 2.5 mM EDTA in phosphate-

FIG. 1. Protein (○ and △) and viral RNA (● and ▲) syntheses in IFN-treated (△, ▲) and untreated L929 cells (○, ●) infected with reovirus. The cells were treated for 18 h with 200 U of IFN- β per ml before infection with reovirus at an MOI of 30. Protein and viral RNA syntheses were measured as described in the text. Samples (1 ml) of cell cultures were labeled for 1 h for each point.

buffered saline) was added together with 15 μl of IgG-Sorb (Enzyme Center, Inc.). After 30 min on ice, 0.2 ml of SAC buffer was added and the samples were filtered through GVWP (polyvinylidene fluoride) membranes of a Multiwell Millititer filtration plate (Millipore Corp.). Each well was washed with 2 ml of SAC buffer, and the filters were then cut out and eluted by boiling in electrophoresis sample buffer. The eluted proteins were analyzed on 10% polyacrylamide gels.

RESULTS

Presence of non-polyribosomal viral RNA in IFN-treated cells. To follow the fate of reovirus mRNA in IFN-treated cells, it was necessary to treat L cells with IFN concentrations which did not completely inhibit synthesis of viral mRNA. Treatment with 200 U of murine IFN- β per ml reduced by 75 to 80% the synthesis of reovirus mRNA (Fig. 1). The time course of reovirus mRNA synthesis was determined in IFN-treated and control L cells and significant synthesis of viral mRNA was observed 4 to 6 h after infection (Fig. 1). The rate of protein synthesis was measured in parallel experiments and no inhibition was detected in either control or IFN-treated cells (Fig. 1). This finding was confirmed by examining the polyribosome profile of infected cells (Fig. 2). No significant difference between control and IFN-treated cells was detected by this analysis. We concluded that, under the conditions selected for this study, with L cells infected at an MOI of 30, there was no detectable inhibition of protein synthesis in IFN-treated cells.

Subsequent experiments established that a large fraction of reovirus mRNA synthesized in IFN-treated cells was not associated with polyribosomes. This was shown by dot hybridization of gradient fractions with a cloned cDNA probe for the *s2* genomic RNA of reovirus. The pattern of

FIG. 2. Polyribosome gradients of control (A) and interferon-treated cells (B) infected with reovirus as described in the legend to Fig. 1. Underneath the A_{260} profile are shown dot hybridizations of gradient fractions carried out as described in the text. The probes for reovirus s2 mRNA and actin mRNA were used for hybridization of separate blots, as indicated. The position of the 40S ribosomal subunit is shown by the arrow.

hybridization is shown in Fig. 2 underneath the polyribosome profile. In untreated cells the RNA hybridizing with the s2 probe was mostly associated with polyribosomes, whereas in IFN-treated cells a large part of this RNA sedimented at about 50S and was therefore not associated with polyribosomes. The gradient fractions were also probed for a cellular mRNA with cloned β -actin cDNA. The RNA hybridizing with this probe was found associated with polyribosomes in both IFN-treated and control cells (Fig. 2).

Gradient fractions of control and IFN-treated cells containing reovirus RNA were combined. The RNA was ex-

tracted and analyzed by Northern blot hybridization (Fig. 3). A single major band was detected by this analysis in both polyribosome-associated and non-polyribosomal RNA, indicating that the RNA sedimenting at about 50S in gradients of IFN-treated cells was not significantly degraded. Subsequent experiments indicated that this RNA could bind to initiation complexes when incubated in a rabbit reticulocyte cell-free system.

Binding of non-polyribosomal reovirus mRNA to initiation complexes. We have previously reported that activation of the IFN-induced eIF-2 α kinase by double-stranded RNA results in the formation of 48S complexes, which contain mRNA and Met-tRNA_f bound to a 40S ribosomal subunit (4). These 48S complexes were isolated by centrifugation on sucrose gradients prepared in high-ionic-strength buffer. Under these conditions, mRNA bound to 48S complexes was separated from unbound free mRNA (4). We used a similar fractionation procedure to isolate non-polyribosomal RNA present in IFN-treated cells infected with reovirus. Viral RNA was labeled by incubating infected cells with [³H]uridine, and cell extracts were fractionated by gradient centrifugation (Fig. 4). A peak of viral RNA sedimenting at about 50S was detected in the gradient of IFN-treated cells, whereas a smaller peak was present in a corresponding position in the gradient of control cells. The RNA sedimenting with polyribosomes in the pellet of these gradients and that present in the non-polyribosomal fractions were measured in several experiments. Greater than 80% of the RNA labeled in control cells was recovered in the polyribosome pellet, whereas <50% of the RNA labeled in IFN-treated cells was recovered in this pellet.

FIG. 3. Northern blot analysis of RNA isolated (A) from polyribosomal fractions (4 to 11) of the gradient shown in Fig. 2A and (B) from the non-polyribosomal fractions (16 to 19) of the gradient shown in Fig. 2B. The RNA was fractionated by electrophoresis and hybridized to the s2 probe as described in the text.

Fractions containing non-polyribosomal RNA of IFN-treated cells were pooled. The RNA was phenol extracted and assayed for its ability to form initiation complexes in a rabbit reticulocyte cell-free system (Fig. 5). The viral RNA could bind efficiently to reticulocyte ribosomes. This finding indicated that the non-polyribosomal RNA was functional viral mRNA which was apparently prevented from binding to ribosomes in IFN-treated cells. All three size classes of reovirus mRNA were present in the non-polyribosomal RNA, as shown by gradient centrifugation of this RNA (Fig. 6).

Phosphorylation of eIF-2 α in IFN-treated cells infected with reovirus. Protein synthesis was not inhibited in IFN-treated cells infected with reovirus at an MOI of 30 (Fig. 1). It could not be excluded, however, that eIF-2 α was phosphorylated in these cells and that its phosphorylation could account for the association of a fraction of reovirus mRNA with 48S complexes. To measure eIF-2 α phosphorylation, the cells were labeled with 32 P between 5.5 and 6.5 h after infection with reovirus at MOIs ranging from 3 to 100. The immunoprecipitates obtained with an antiserum prepared against purified eIF-2 were analyzed by gel electrophoresis (Fig. 7). The level of eIF-2(α P) was not appreciably increased in either control or IFN-treated cells infected at an MOI of 30, but was significantly elevated in IFN-treated cells infected at an MOI of 100. This finding indicated that an overall increase in the eIF-2(α P) level is not responsible for the failure of a large fraction of reovirus mRNA to bind to polyribosomes.

A relatively small increase in eIF-2(α P) could not be detected in the experiment shown in Fig. 7, since a fraction of eIF-2 α is normally phosphorylated even in untreated uninfected L cells (13). However, eIF-2(α P) specifically associated with 48S complexes could be detected by analyzing the phosphoproteins cosedimenting with these complexes. Extracts prepared from 32 P-labeled IFN-treated and control cells infected with reovirus at an MOI of 30 were

FIG. 4. Fractionation of ribosomal subunits from untreated (A) and IFN-treated (B) cells infected with reovirus. The cells were labeled with [3 H]uridine from 4 to 6 h after infection, as described in the legend to Fig. 1. Cell extracts were centrifuged for 5 h at 260,000 $\times$ g, as described in the text. Samples of each fraction were precipitated with 5% trichloroacetic acid and counted. The positions of 80S ribosomes and 40S ribosomal subunits are indicated.

FIG. 5. Binding of non-polyribosomal reovirus RNA from IFN-treated cells to reticulocyte initiation complexes. Fractions 12 to 14 of the gradient shown in Fig. 4B were combined and precipitated with ethanol. The RNA was extracted with phenol and assayed for binding in an incubation with reticulocyte lysate as described in the text. (A) Incubation kept at 0°C; (B) incubation for 4 min at 30°C. The binding assays were centrifuged on sucrose gradients and the fractions obtained were directly counted. The positions of 80S initiation complexes and 40S ribosomal subunits are indicated.

fractionated by gradient centrifugation. The fractions containing non-polyribosomal RNA (Fig. 4) were combined and analyzed by gel electrophoresis. A band corresponding to eIF-2(α P) was clearly increased in the sample obtained from IFN-treated cells (Fig. 8). This band was identified with eIF-2(α P) by immunoprecipitation as described above.

DISCUSSION

A large fraction of reovirus mRNA synthesized in IFN-treated L cells did not associate with polyribosomes. This was not caused by generalized inhibition of protein synthesis, since amino acid incorporation into proteins was not significantly decreased and the polyribosome profile was similar to that of control cells. The failure to bind to polyribosomes was specific for reovirus mRNA, since actin mRNA was found associated with polyribosomes. The non-polyribosomal viral mRNA had the same size as polyribosomal RNA when analyzed by electrophoresis and Northern blot hybridization with a cloned probe for the *s2* mRNA of reovirus. The isolated non-polyribosomal RNA was bound to 80S initiation complexes when incubated in a reticulocyte cell-free system, indicating that it was functional mRNA. Apparently, all reovirus mRNAs failed to some extent to bind to polyribosomes, as shown by the presence of the different size classes of viral mRNAs in the non-polyribosomal fraction. It could not be excluded, however, that specific reovirus mRNAs were prevented from binding to initiation complexes more effectively than other mRNAs.

The most likely explanation for the presence of reovirus mRNA not associated with polyribosomes is an activation of

FIG. 6. Gradient centrifugation analysis of non-polyribosomal reovirus RNA from IFN-treated cells. RNA from fractions 12 to 14 of the gradient shown in Fig. 4B was isolated as described in the legend to Fig. 5 and centrifuged on sucrose gradients as described in the text. The three size classes of reovirus mRNA are indicated.

PK_{ds} by viral transcriptional complexes. We have previously reported that in a coupled cell-free system nascent reovirus mRNA specifically fails to bind to 80S initiation complexes and is found associated with 48S complexes (6), which contain eIF-2(α P) (4). The PK_{ds} and eIF-2(α P) are both bound to viral particles after incubation with reticulocyte lysate, suggesting that localized activation of PK_{ds} and association of eIF-2(α P) with nascent viral mRNA are responsible for the selective inhibition of binding of this mRNA to 80S initiation complexes. This mechanism may account for the presence of non-polyribosomal reovirus mRNA in IFN-treated cells. We propose that PK_{ds} is bound to and activated by viral transcriptional complexes and that nascent reovirus mRNA becomes preferentially associated with eIF-2(α P).

A large increase in eIF-2(α P) can be detected in IFN-treated L cells only after high-multiplicity infection with reovirus (Fig. 7). Under these conditions, the activation of PK_{ds} is apparently no longer localized and cellular protein synthesis may be inhibited, as previously reported for IFN-treated HeLa cells infected with reovirus at high multiplicity (11). The non-polyribosomal reovirus mRNA was detected in cells infected at a relatively low MOI, which did not result in a significant overall increase in eIF-2 phosphorylation. However, high levels of eIF-2(α P) were detected in the complexes containing non-polyribosomal reovirus mRNA (Fig. 8). Therefore, whereas the overall rate of eIF-2 phosphorylation may not be significantly increased in these cells, the eIF-2(α P) formed is apparently specifically associated with viral mRNA.

The mRNA in 48S complexes may be rescued and translated if the eIF-2(α P) is dephosphorylated by cellular phosphatases (4, 5, 5a). However, the kinetics of

dephosphorylation of eIF-2(α P) bound to 48S complexes is relatively slow, since unbound eIF-2(α P) is a better substrate for phosphatases (5a). It seems possible, therefore, that viral mRNA may be blocked in 48S complexes for a significant time when PK_{ds} continuously phosphorylates eIF-2 α and the cellular phosphatases preferentially dephosphorylate "free" eIF-2 α (5). It should be pointed out that this is a tentative explanation for the presence of untranslated reovirus mRNA in IFN-treated cells and that other mechanisms cannot be excluded at this time. This mechanism for the localized activation of PK_{ds} is in principle similar to that previously proposed for the localized activation of the 2-5A synthetase-RNase L system (10). The main difference between these inhibitory mechanisms is that the translational block due to eIF-2 α phosphorylation is potentially reversible, in contrast to RNA degradation by RNase L. However, it should be pointed out that the block of viral mRNA in 48S complexes may render it more susceptible to cleavage by RNase L, as suggested by recent observations (5a). The function of only PK_{ds} and 2-5A synthetase, among all the antiviral proteins induced by IFN, is known at this time. Consequently, there is a justifiable tendency to attribute all translational inhibitory phenomena to the activity of these enzymes. When the functions of other antiviral proteins are uncovered, it will become clear whether this assumption is correct.

FIG. 7. Phosphorylation of eIF-2 α in control and IFN-treated (200 U/ml) L cells infected with reovirus at different MOIs. The cells were infected or mock-infected for 4.5 h and labeled with ³²P. Cell lysates were immunoprecipitated with antiserum against eIF-2; the precipitates were analyzed by gel electrophoresis, as described in the text. The MOI is indicated at the top. Authentic eIF-2 phosphorylated by PK_{ds} (5a) was run in parallel; the positions of the three subunits of this protein were determined by staining the gel and are indicated.

FIG. 8. Presence of eIF-2(α P) in gradient fractions containing reovirus mRNA not bound to polyribosomes. Control and IFN-treated (200 U/ml) L cells were infected at an MOI of 30 and labeled with 32 P as described in the legend to Fig. 7. Cell extracts were fractionated by centrifugation as described in the legend to Fig. 4. The fractions containing non-polyribosomal mRNA were combined as described in the legend to Fig. 5 and centrifuged for 17 h at $200,000 \times g$. The pellets were suspended in lysis buffer without dextran sulfate and either directly analyzed by gel electrophoresis (left) or treated with anti-eIF-2 antiserum as described in the text (right). A sample of purified eIF-2 phosphorylated in the α subunit (5a) was run in parallel (track e). Treatment of cells with IFN and infection with reovirus are indicated at the bottom of the gel (left).

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