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Biochemistry

Accumulation of newly synthesized mRNAs in response to human fibroblast (β) interferon

(mRNA isolation/cell-free translation/interferon-induced proteins/antiviral state)

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ABSTRACT **Treatment of human fibroblast cells with human fibroblast (β) interferon for up to 8 hr resulted in the accumulation of at least four mRNAs. The mRNAs were isolated from cellular polysomes and characterized by stimulation of translation in a wheat germ cell-free protein synthesis system. The mRNAs appear as early as 2 hr after exposure to interferon and can be translated *in vitro* into proteins having molecular weights of 61,000, 62,000, 64,000, and 68,000. The response is not elicited by mouse interferon or insulin and does not occur in the presence of actinomycin D. Chase experiments indicated that the induced mRNAs remain ribosome-associated for at least 3 hr after their synthesis. The appearance of the induced mRNAs correlated directly with the onset of an antiviral state. Velocity sedimentation of the induced mRNAs on sucrose gradients demonstrated that each of the four induced proteins are encoded by different-sized mRNAs.**

Interferons are proteins that are synthesized in minute quantities by various vertebrate cells in response to either virus infection or treatment with double-stranded RNA. The secreted interferon protein interacts with other cells and causes them to develop an antiviral state (1). Although the precise mechanism for development of an antiviral state is not known, transcription and translation are postulated prerequisites for the establishment of the antiviral state because actinomycin D and inhibitors of protein synthesis prevent or delay the effect (2, 3). Several laboratories have reported increased amounts of a protein kinase, 2′,5′-oligo(A) synthetase, a phosphodiesterase, and the presence of a methylation inhibitor in interferon-induced mouse (4–7), HeLa (8, 9), and chicken embryo (10) cells. All four activities have been proposed in schemes explaining inhibition of viral protein synthesis (11, 12). In addition, interferon appears to induce several proteins whose functions are not yet known. Three proteins with molecular weights of 120,000, 80,000, and 67,000 are induced in mouse cells, and as many as nine proteins with molecular weights of 120,000, 88,000, 67,000, and 56,000 and four proteins in the 44,000–68,000 molecular weight range are induced in human fibroblast cells (13–15).

Characterization of interferon-induced events by protein analysis has at least two major drawbacks. First, the vast number of background cellular proteins tend to camouflage the very small number of induced proteins, necessitating analysis on two-dimensional gels; second, the identified proteins cannot be used as genetic probes to study gene structure and expression. Characterization of interferon-induced mRNAs appears to overcome both of these problems. Farrell *et al.* (16) have used this approach in isolating mRNA from control and interferon-induced mouse ascites tumor cells. Analysis of the translation products of these mRNAs has revealed a 14,500 molecular weight protein not coded for by control mRNA preparations. Using a similar approach, I isolated polysomal mRNA from interferon-treated human diploid fibroblast cells and characterized these mRNAs with a wheat germ translation system as a first step to obtaining pure cellular DNA that encodes for the interferon-responsive genes.

MATERIALS AND METHODS

Human diploid fibroblast cells isolated from human foreskin were grown in 850 cm^2 roller bottles in GIBCO minimum essential medium containing 7% (vol/vol) fetal calf serum. Human fibroblast interferon (10^8 units/mg) was purified to homogeneity as described (17). Seven-day-old monolayers ($3–4 \times 10^8$ cells) were treated (see figure legends) in 25 ml of serum-free medium per bottle at 37°C. After 4 hr, cells were rinsed with cold phosphate-buffered saline (137 mM NaCl/2.7 mM KCl/8.1 mM Na_2PO_4, pH 7.2) and recovered by scraping. The cells were pelleted and resuspended in a hypotonic medium (10 mM NaCl), then disrupted by Dounce homogenization, and the nuclei were removed. The cytoplasmic extract was treated with nonionic detergents, and the polysomes were pelleted. Polysomal pellets were disrupted in 10 mM Tris·HCl/1 mM EDTA/0.3% lithium dodecyl sulfate, pH 7.5, and digested with proteinase K (0.5 mg/ml) for 15 min at 37°C. NaCl was added to 0.5 M, and the digest was chromatographed on oligo(dT)-cellulose as described (18). RNA that bound to the column was precipitated with ethanol and used at a concentration of 40 μg/ml to direct translation in a wheat germ cell-free system containing 0.4 mCi (1 Ci = 3.7×10^{10} becquerels) of [^{35}S]methionine per ml (19). Reaction mixtures (25 μl) were incubated at 24°C for 2 hr and stopped by the addition of $NaDodSO_4$ to 1%. Samples were analyzed on a 5.5–14% (wt/vol) gradient $NaDodSO_4$/polyacrylamide gel (20) and visualized by fluorography with EN^{3}HANCE (New England Nuclear).

Velocity sedimentation in sucrose gradients and vesicular stomatitis virus (VSV) plaque assay were as described (21, 22).

RESULTS

Polysomal messenger RNA was prepared from control cells and from human diploid fibroblast cells incubated with 100 units/ml of homogeneous human fibroblast interferon for 4 hr. The two mRNA preparations were translated in a wheat germ cell-free translation system, and [^{35}S]methionine-labeled translation products were analyzed by $NaDodSO_4$/gel electrophoresis. In Fig. 1, lanes 3 and 4 contain the translation products of control and interferon-induced mRNAs, respectively. Four proteins with molecular weights of 61,000, 62,000, 64,000, and 68,000 appeared to accumulate upon translation of interferon-induced mRNAs (Fig. 1, arrows) but were either absent (61,000, 62,000,

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Abbreviation: VSV, vesicular stomatitis virus.

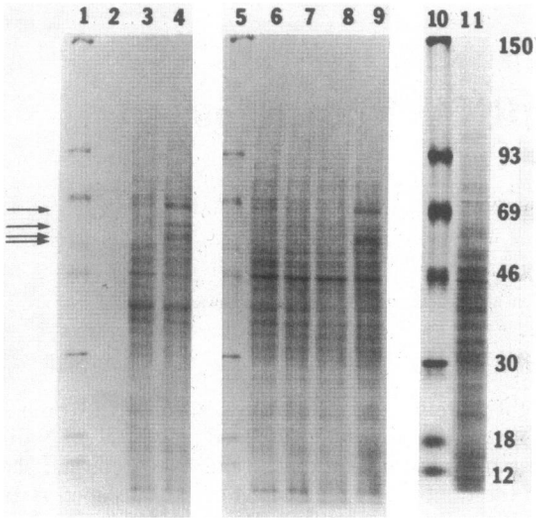

FIG. 1. Induction of human fibroblast mRNAs after interferon treatment. Polysomal mRNA was isolated from human fibroblast cells after the various treatments cited here and translated in a wheat germ translation system. The ^{35}S-labeled translation products were then analyzed on a 5.5–14% $NaDodSO_4$/polyacrylamide gel and visualized by fluorography. Lanes: 1, 5, and 10, ^{14}C-labeled protein markers (New England Nuclear) shown $\times 10^{-3}$; 2, translation products when no RNA is added; 3 and 6, translation of control mRNA; 4 and 9, translation of RNA from fibroblast interferon-treated (100 units/ml) cells; 7, RNA from mouse interferon-treated (100 units/ml) fibroblast cells; 8, RNA from insulin-treated (10 μM) fibroblast cells; 11, translation products of RNA when cells are treated with fibroblast interferon (100 units/ml) in the presence of actinomycin D (5 μg/ml).

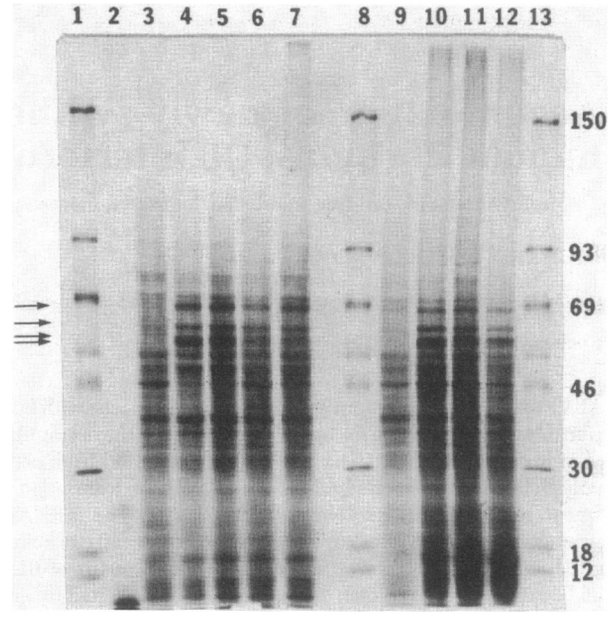

FIG. 2. Time course for the synthesis of interferon-induced mRNAs. Human diploid fibroblast cells were treated with human fibroblast interferon (100 units/ml), and mRNA was isolated and translated. Lanes: 1, 8, and 13, ^{14}C-labeled protein molecular weight markers shown $\times 10^{-3}$; 2, no mRNA; 3 and 9, translation products of control mRNA; 4 and 10, mRNA translations of 2-hr induced cells; 5–7, mRNA from 4-, 6- and 8-hr inductions, respectively; 11, mRNA after a 3-hr chase; 12, mRNA from a 3-hr chase in the presence of actinomycin D (5 μg/ml).

and 64,000) or present in reduced amounts (68,000) when mRNA from control cells was translated. Cell-free translation of the two mRNA preparations in a rabbit reticulocyte system gave identical results (data not shown). Fig. 1, lanes 6–9 show the translation products of RNA from fibroblast cells incubated with medium only, mouse interferon, insulin, and fibroblast interferon, respectively. Again the four induced mRNAs accumulated only when human interferon was present (lane 9), indicating that they result specifically from human interferon induction. When actinomycin D was present during the incubation period, the mRNAs for the induced proteins were not found (lane 11), suggesting that the induced mRNAs were newly synthesized.

To determine the length of time that fibroblast cells need after addition of interferon for the synthesis and transport of the newly synthesized mRNAs, fibroblast cells were incubated with interferon for various periods of time up to 8 hr, and the mRNAs were isolated as before. Electropherograms of the translation products (Fig. 2, lanes 3–7) of the mRNAs isolated at 0, 2, 4, 6, and 8 hr indicated that the mRNAs coding for the 61,000, 62,000, 64,000, and 68,000 molecular weight proteins appeared as early as 2 hr and were found on polysomes as late as 8 hr. Several other protein bands appeared to either increase or decrease over the time course chosen, but because of their nonreproducible nature, it was difficult to relate them to interferon induction.

Experiments were done to determine if the new mRNAs found throughout the 8-hr induction period required the continued presence of interferon or were stable on polysomes for hours after their synthesis. Fibroblast cells were treated for 2 hr with fibroblast interferon, washed with phosphate-buffered saline, and incubated in interferon-free medium for 3 hr at 37°C. Cell-free translation of the control and 2-hr induced mRNAs (Fig. 2, lanes 9–10) again demonstrated that the mRNAs were induced at 2 hr and absent in untreated cells. Three hours after interferon was removed, the induced mRNAs are still present on polyribosomes (Fig. 2, lane 11). Actinomycin D was added at the time of interferon removal (lane 12) to ascertain whether the mRNAs found were newly synthesized or preexisting molecules. The interferon-specific mRNAs were again found on polyribosomes, indicating that they are synthesized within 2 hr after exposure to interferon and can remain ribosome-associated for at least 3 hr when actinomycin D is present.

The size of the induced mRNAs was determined by velocity sedimentation of [^3H]uridine-labeled mRNAs on sucrose gradients. Both the induced and uninduced mRNA profiles were nearly identical with no evidence of degradation in either case (Fig. 3*A*). The induced mRNA profiles reproducibly contained an extra shoulder at 16.5 S and an extra peak at 21 S, which were missing in uninduced profiles. To determine the location of the four induced mRNAs, gradient fractions between 13–32 S were each precipitated with ethanol and translated in the wheat germ translation system. The mRNAs coding for the four induced proteins sedimented as three different-sized mRNAs (Fig. 3*B*). The 61,000, 62,000, 64,000, and 68,000 molecular weight proteins were coded for by mRNAs that sedimented at 21 S, 16.5 S, 19.5 S, and 21 S respectively. This result shows that the newly identified proteins are not the result of aberrant initiation or termination of a single mRNA in the cell-free translation system but instead result from the translation of at least three unique mRNA species.

It was of interest to determine if the early appearance of the induced mRNAs correlated with the onset of an antiviral state. Fibroblast cells were treated with homogeneous interferon as before, but instead of harvesting the RNA at a given time, the interferon-containing medium was removed, the cells were washed twice with phosphate-buffered saline, and interferon-

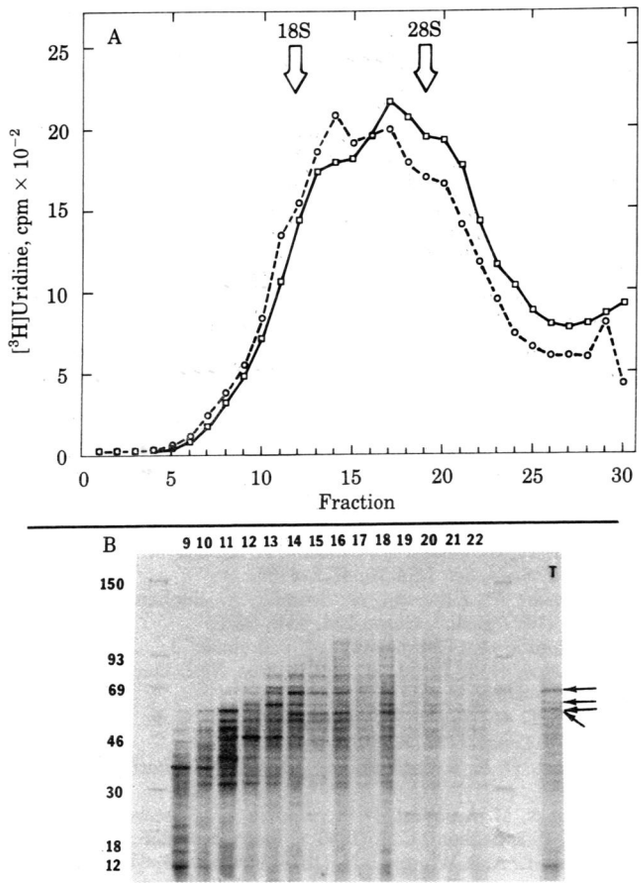

FIG. 3. Velocity sedimentation of control and induced mRNAs in sucrose gradients. Fibroblast cells were incubated for 2 hr at 37°C in the presence of [^{3}H]uridine (20 μCi/ml) with (○——○) or without (□——□) 100 units/ml of fibroblast interferon per ml. Polysomal mRNA was isolated from polysomes and analyzed by velocity sedimentation on a 15–30% (wt/vol) sucrose gradient containing 0.5% $NaDodSO_4$ (22). Sedimentation was for 16 hr at 26,000 rpm in a Beckman SW 40 rotor at 24°C. Gradients were fractionated (0.4 ml), aliquots (50 λ) were assayed for radioactivity (*A*). Fractions (9–22) expected to contain the induced mRNAs were each precipitated with ethanol and translated by using the wheat germ translation system as described (19). The polypeptides directed by the mRNA in each gradient fraction were analyzed on a 5.5–14% polyacrylamide gel (20) and visualized after fluorography (*B*). Lane T, translation of unfractionated induced mRNA; arrows, position of the four induced proteins. Protein molecular weight markers are shown $\times 10^{-3}$; numbers above gel lanes indicate the gradient fraction from which the mRNA was obtained.

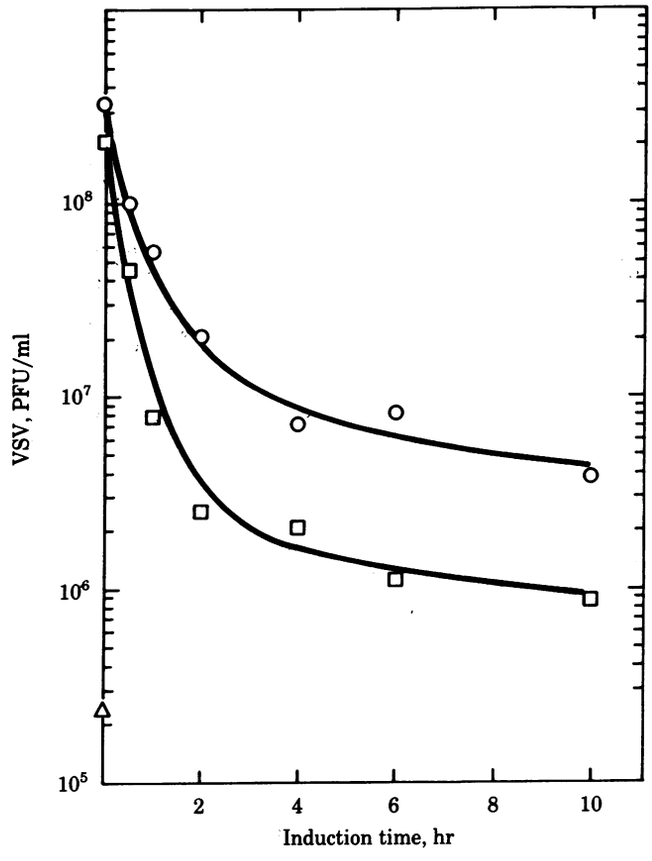

FIG. 4. Development of an antiviral state in fibroblast cells. Fibroblast cells were treated with fibroblast interferon (100 units/ml). At the indicated times, cells were washed twice with phosphate-buffered saline and infected with VSV (○——○) and VSV with actinomycin D (5 μg/ml) at a multiplicity of infection of 2 (□——□). Cells were incubated at 37°C for 20 hr, and VSV titers were determined by using an L-cell plaque assay (21). △, Titer of VSV after incubation in the absence of cells; PFU, plaque-forming unit(s).

free medium containing VSV was added (multiplicity of infection of 2). After incubation at 37°C for 24 hr, VSV in the medium was assayed on L-cell monolayers. A 30% drop in VSV titer occurred after only a 30-min exposure to interferon, with 90% inhibition within 2 hr of interferon exposure (Fig. 4). Interferon assays on the medium removed from cells after 2 hr of incubation indicated that only 10% of the interferon biological activity could be recovered (data not shown). This result suggests that interferon molecules are either irreversibly bound to cell membranes or degraded during the incubation period.

The question remained whether interferon required hours to cause an antiviral state to develop under the conditions employed (i.e., a race between host shutoff by VSV and development of an antiviral state). To answer this question, the previous experiment was repeated with actinomycin D added along with the VSV to prevent further synthesis of interferon-induced mRNAs and, thus, cellular proteins. From 30 min through 8 hr, the addition of actinomycin D did not inhibit the development of the antiviral state but instead paralleled the results obtained in the non-actinomycin D-treated cells (Fig. 4). Taken together with the results that interferon-induced mRNAs appear stable in the presence of actinomycin D (Fig. 2, lane 12), it appears that the expression of the antiviral gene(s) occurs within minutes and that stability of the mRNAs may increase if further transcription is prevented.

DISCUSSION

The data in this paper clearly demonstrate that treatment of human diploid fibroblast cells with purified human fibroblast (β) interferon induces the synthesis of at least three and probably four mRNAs. These mRNAs are induced within 2 hr of interferon treatment (Fig. 2) and, in fact, have been detected as early as 1 hr after interferon treatment (data not shown). Similar results also have been obtained with interferon-induced proteins (14). It has not yet been determined whether continuous interaction with interferon is needed for mRNA induction because quantitative removal of interferon from treated cells has proved to be difficult, and mRNA concentrations have not been determined. Treatment of the fibroblast cells with fibroblast interferon in the presence of actinomycin D prevented the appearance of the induced mRNAs (Fig. 1, lane 11). This result demonstrates that the induced mRNAs are newly synthesized because there was virtually no effect on the normal distribution

of cellular mRNAs found on polysomes. Chase experiments (Fig. 2, lanes 10–12) also tend to support the finding that the induced mRNAs are newly synthesized shortly after treatment with interferon because addition of actinomycin D after a 2-hr exposure period does not affect their isolation from polysomes. These results seem to suggest that transcription, transport to the cytoplasm, and translation of these genes occurs within 1–2 hr of interferon treatment.

The induction of the newly described mRNAs appears to be specific for human interferon. Attempts to induce the mRNAs with mouse interferon or insulin proved to be unsuccessful. Interestingly, human leukocyte (α) interferon caused the identical mRNA response as that demonstrated by treatment with fibroblast interferon (unpublished results). Thus, the correlation exists that the only two proteins tested that caused the development of an antiviral state in fibroblast cells also induced the four mRNAs. This correlation is also supported by the results obtained in Fig. 4, in which there is a direct correlation between the development of an antiviral state and the appearance of the induced mRNAs. The fact that addition of actinomycin D at early times after exposure to interferon did not inhibit the development of an antiviral state further supports the assumption that the genetic information needed for development of an antiviral state is expressed within the first 2 hr of interferon addition. The enhancing effect of actinomycin D on the development of an antiviral state has been demonstrated (23) and may represent stabilization of the induced mRNAs.

Initial characterization of the induced mRNAs on sucrose gradients (Fig. 3) showed that the newly identified proteins synthesized in the wheat germ translation system are not the result of aberrant initiation or termination of a single mRNA. The fact that the new proteins are coded for by at least three different-sized mRNAs indicates that the induced proteins are the result of translation of at least three unique mRNAs. In addition, the size of the induced mRNAs correlated with their predicted sizes based on molecular weights of the proteins for which they code. The only exception is a 21S mRNA coding for the 61,000 molecular weight protein. Although the deviation from expected results is not that dramatic, it cannot be ruled out that a 21S mRNA could code for both the 61,000 and 68,000 molecular weight proteins. It is important to note that the protein ratios obtained by cell-free translation may not be an accurate measure of mRNA concentrations. The reasons for this are that mRNAs have different translation efficiencies, the use of [^{35}S]methionine as the sole radioactive precursor, and the fact that equal amounts of radioactivity rather than proteins are loaded into each gel lane.

Whether the interferon-induced mRNAs are translated into proteins that have the enzymatic activities found in interferon-treated cells or whether they code for as yet unidentified proteins remains unclear. None of the predicted enzymatic activities were found among cell-free translation products of these newly induced mRNAs, most likely because of their minute quantities (unpublished results). Two-dimensional gel electrophoresis of the cell-free translation products and interferon-induced cellular proteins indicates that one (65,000 molecular weight) of the proteins correlates with those found by Knight and Korant (ref. 13; B. Korant, personal communication). The fact that these mRNAs are newly induced, specific for human interferon, have fairly long half-lives, and parallel the onset of viral resistance strongly suggests that these mRNAs are involved in the establishment of an antiviral state.

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