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Cell Biology

Fibroblast interferon induces synthesis of four proteins in human fibroblast cells

(interferon action/protein synthesis/two-dimensional electrophoresis)

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ABSTRACT **Treatment of human diploid fibroblasts with fibroblast interferon for 8 hr inhibited replication of vesicular stomatitis virus. When the total cell protein of cells treated with interferon for 8 hr was compared to the total cell protein of untreated cells by two-dimensional gel electrophoresis, the interferon-treated cells were found to contain four proteins not found in untreated cells. Addition of actinomycin D to the cells concurrently with interferon inhibited the synthesis of the four proteins. We conclude that these proteins are induced by interferon and that they may be involved in the inhibition of virus replication.**

For interferon to induce an antiviral state in cells, the cells must be exposed to interferon for a period of time prior to infection. Soon after the discovery of interferon it was concluded that some metabolic activity must occur for the development of the antiviral state (1). Later it was discovered that inhibitors of either RNA or protein synthesis prevented the cellular antiviral response to interferon (2, 3). From these experiments and others it has been concluded that the synthesis of new mRNA(s) and new protein(s) is a prerequisite for the establishment of the antiviral state. This very plausible idea, however, has had little support from data confirming the existence of new proteins in interferon-treated cells.

With Ehrlich ascites cells it has been reported that washes of ribosomes from interferon-treated cells contain an additional protein of estimated molecular weight of 48,000 that is not found in ribosomal washes of untreated cells (4). Whether this additional protein is synthesized *de novo* in response to interferon is not known.

Two-dimensional gel electrophoresis is a powerful tool in analyzing complex mixtures containing many proteins (5). We have used this technique to analyze the proteins in human fibroblast cells that had been treated with interferon. Our data indicate that at least four proteins synthesized in these cells during an 8-hr exposure to interferon are not found in control cells.

MATERIALS AND METHODS

Cells and Viruses. Human diploid fibroblast cells were grown in monolayers at 37°C in a CO_2 incubator in Eagle's minimal essential medium (GIBCO) supplemented with 7% fetal calf serum (GIBCO), gentamicin (10 μg/ml), Hepes buffer (12 mM), and *N*-[tris(hydroxymethyl)methyl]glycine (Tricine) buffer (6 mM); final pH 7.4. The cells were grown in wells of Micro Test II tissue culture plates (Falcon, no. 3040). Cells were seeded at 7×10^3 per well and used for experiments either 4 or 5 days later when they were confluent and had reached 3×10^4 cells. Vesicular stomatitis virus was grown in HeLa cells.

Interferon and Interferon Assays. Human fibroblast interferon was purified as described (6). Homogeneous interferon was used in all experiments. Interferon was assayed by a microassay technique (7). Units are given in National Institutes of Health standard leukocyte interferon units, G023-901-527.

^{14}C-Labeled Amino Acid Incorporation and Preparation of Cell Extracts. The medium for ^{14}C-labeled amino acid incorporation was composed as follows: minimal essential medium containing glutamine (2 mM) but without other amino acids, 5% fetal calf serum, Hepes buffer (12 mM), Tricine buffer (6 mM) and 15 ^{14}C-labeled amino acids (New England Nuclear, mixture no. 445), at either 10 or 20 μCi/ml (1 Ci = 3.7×10^{10} becquerels); final pH 7.4. The growth medium was removed from the cell monolayers, and the cells were washed once with 0.2 ml of phosphate-buffered saline, then 0.2 ml of the ^{14}C-labeled amino acid medium was added to each well. Those cells to be exposed to interferon received the same medium but containing interferon at 50 units/ml. Incorporations were for 8 hr; the medium was removed and the cells were washed two times with 0.2 ml each of phosphate-buffered saline. Cells were lysed in a solution of 8 M urea, 0.5% Nonidet P-40, 0.2% sodium dodecyl sulfate, 1.6% pH 5–8 Ampholines, and 0.4% pH 3.5–10 Ampholines (LKB). The cells in three wells were lysed in a total of 0.1 ml of the buffer. After 8 hr of ^{14}C-labeled amino acid incorporation an extract from 9×10^4 cells (three wells) contained a total of 5×10^5 cpm incorporated into protein. Cell extracts were loaded onto the first dimension gel immediately after preparation. When the proteins secreted into the medium were to be analyzed, the incorporation medium contained 0.5% fetal calf serum. The medium was concentrated by vacuum centrifugation prior to loading onto the first dimension gel.

Two-Dimensional Gel Electrophoresis. The method that we have used is a modification of that described by O'Farrell (5). The first-dimension separation consisted of isoelectric focusing in slab gels. Gels 14.5 cm wide, 10.5 cm long, and 0.75 mm thick were prepared of 5.5% acrylamide containing 8 M urea, 1.6% pH 5–8 Ampholines, and 0.4% pH 3.5–10 Ampholines. Gels were polymerized by the addition of 2.5 ml of a riboflavin solution (40 μg/ml) to 17.5 ml of acrylamide mixture then illumination with two 15-W fluorescent lamps 6 cm from the mixture. Polymerization was complete in one hour. Sample wells (0.05 ml) were formed with a Teflon comb placed between the glass plates at the top of the gel. The comb was inserted between the plates prior to the addition of the acrylamide mixture. Samples of 0.04 ml in each well were overlaid with 0.01 ml of a solution of 4 M urea, 1.6% pH 5–8 Ampholines, 0.4% pH 3.5–10 Ampholines. The anode solution was 0.01 M phosphoric acid and the cathode solution was 0.02 M sodium hydroxide. Focusing was at 4°C at constant voltage. The voltage was maintained at 300 V for 16 hr, then 400 V for 1 hr.

For separation in the second dimension a slab gel with a linear acrylamide gradient (8–16%) was prepared. A discontinuous

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buffer system (8) was used and the acrylamide was polymerized with ammonium persulfate. The slab gels were 12 cm wide, 1 mm thick, and either 9.5 cm or 17 cm long. A stacking gel (2 cm in length) of 5% acrylamide was poured on top of the running gel. The first-dimension gel was cut into strips 1 cm wide extending from the bottom of the sample well to the bottom of the gel. Each strip was inserted lengthwise onto the stacking gel of the second-dimension gel. The gel strip was covered with a warm 1% agarose solution in stacking gel buffer. For the 17-cm gels, electrophoresis was at 4 mA/gel, constant current, for 16 hours; for the 9.5-cm gels, electrophoresis was at 20 mA/gel, constant current, for 4–5 hr. Proteins were fixed and stained in a solution of 40% methanol, 10% acetic acid, 50% water (vol/vol) containing 1.25 mg of Coomassie blue R-250 per ml. Gels were dried and exposed to Du Pont Cronex 4 medical x-ray film.

RESULTS

The proteins synthesized in human fibroblast cells in an 8-hr period with or without interferon in the medium are displayed in the autoradiograms in Fig. 1. Four new polypeptides are synthesized after interaction of the interferon with the cells; they are indicated by the arrows on the second-dimension gel (Fig. 1*A*). These polypeptides are not detected in control cells during an 8-hr exposure to ^{14}C-labeled amino acids (Fig. 1*B*). The four polypeptides have estimated molecular weights from 44,000 to >68,000. It is concluded that the four polypeptides have been synthesized as a result of interferon induction because only homogeneous human fibroblast interferon was used in these experiments. The cells are markedly resistant to replication of vesicular stomatitis virus after only 8 hr of interferon treatment (50 units/ml): virus yields were reduced from 1×10^8 to 5×10^5 plaque-forming units per ml.

If the four polypeptides indicated in Fig. 1*A* are synthesized from new mRNAs whose transcription has been induced by interferon, inhibitors of RNA synthesis such as actinomycin D should also inhibit the polypeptides' synthesis. This was tested by adding actinomycin D to the cells simultaneously with the interferon, allowing the cells to interact with the interferon for 8 hr, then comparing the polypeptides synthesized in the presence of interferon and actinomycin D to those synthesized in the presence of interferon only. Fig. 2*A* shows the four polypeptides whose synthesis is induced by interferon. Their synthesis is inhibited by actinomycin D, because they are absent in the autoradiogram shown in Fig. 2*B* (interferon plus actinomycin D). Moreover, the profiles of polypeptides in Fig. 2 *A* and *B* are very similar with the exception of the interferon-induced polypeptides, indicating no extensive breakdown of mRNA in the presence of actinomycin D.

We have so far scored only the reproducible qualitative changes in the polypeptides of control and interferon-treated cells. There are, however, quantitative changes in various

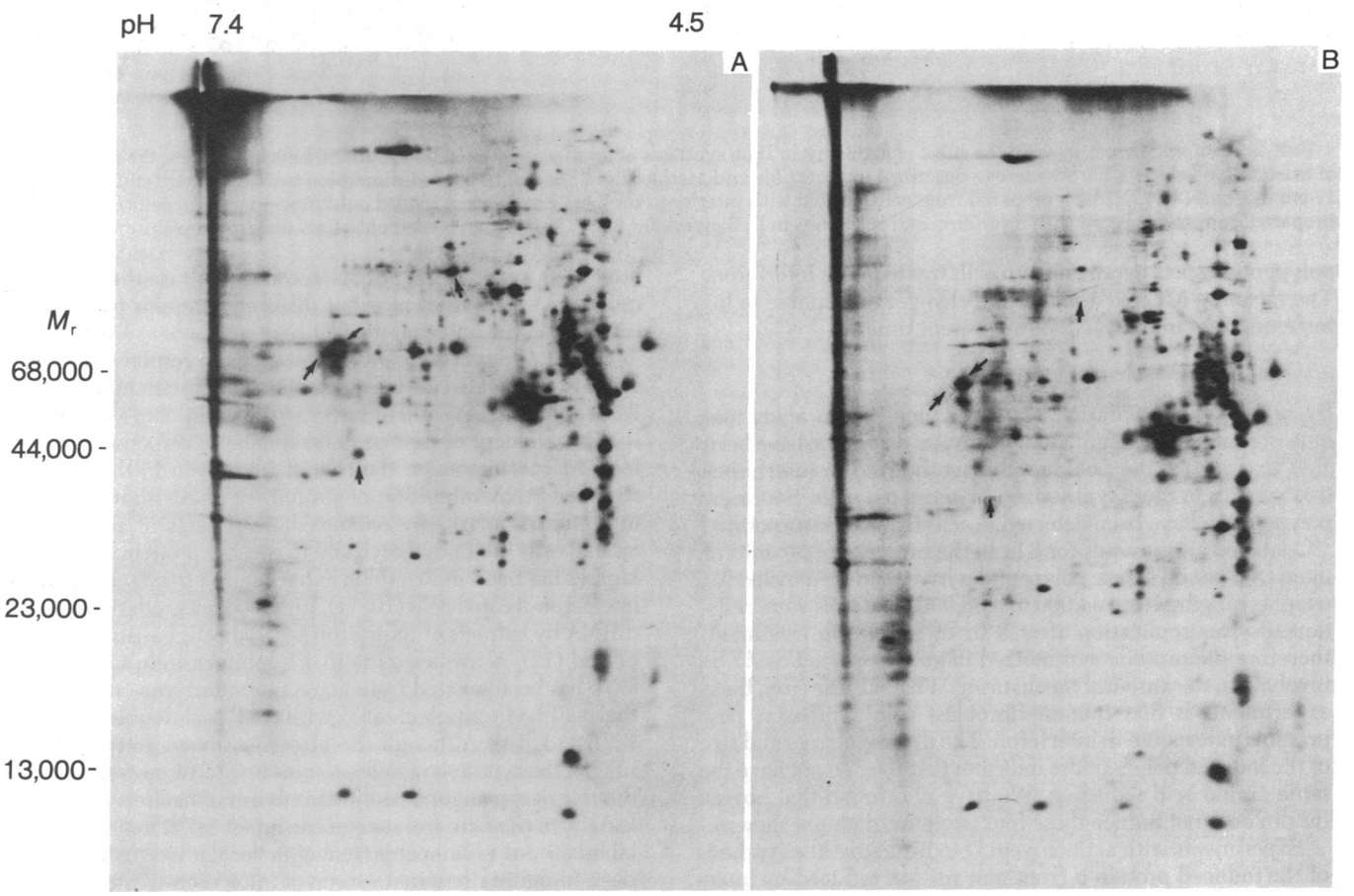

FIG. 1. Autoradiograms of polypeptides from cells labeled with ^{14}C-labeled amino acids for 8 hr in the presence of interferon. Interferon and ^{14}C-labeled amino acids were added simultaneously, and the cells were allowed to incorporate the amino acids for 8 hr. An identical culture of cells was labeled for 8 hr, but the medium contained no interferon (untreated cells). The second-dimension sodium dodecyl sulfate gel was 17 cm in length. (*A*) Extracts prepared from cells treated with interferon for 8 hr; 200,000 cpm loaded onto the first-dimension gel. (*B*) Extracts prepared from untreated cells; 200,000 cpm loaded onto the first-dimension gel. Arrows show the positions of the additional polypeptides that are not present in untreated cells.

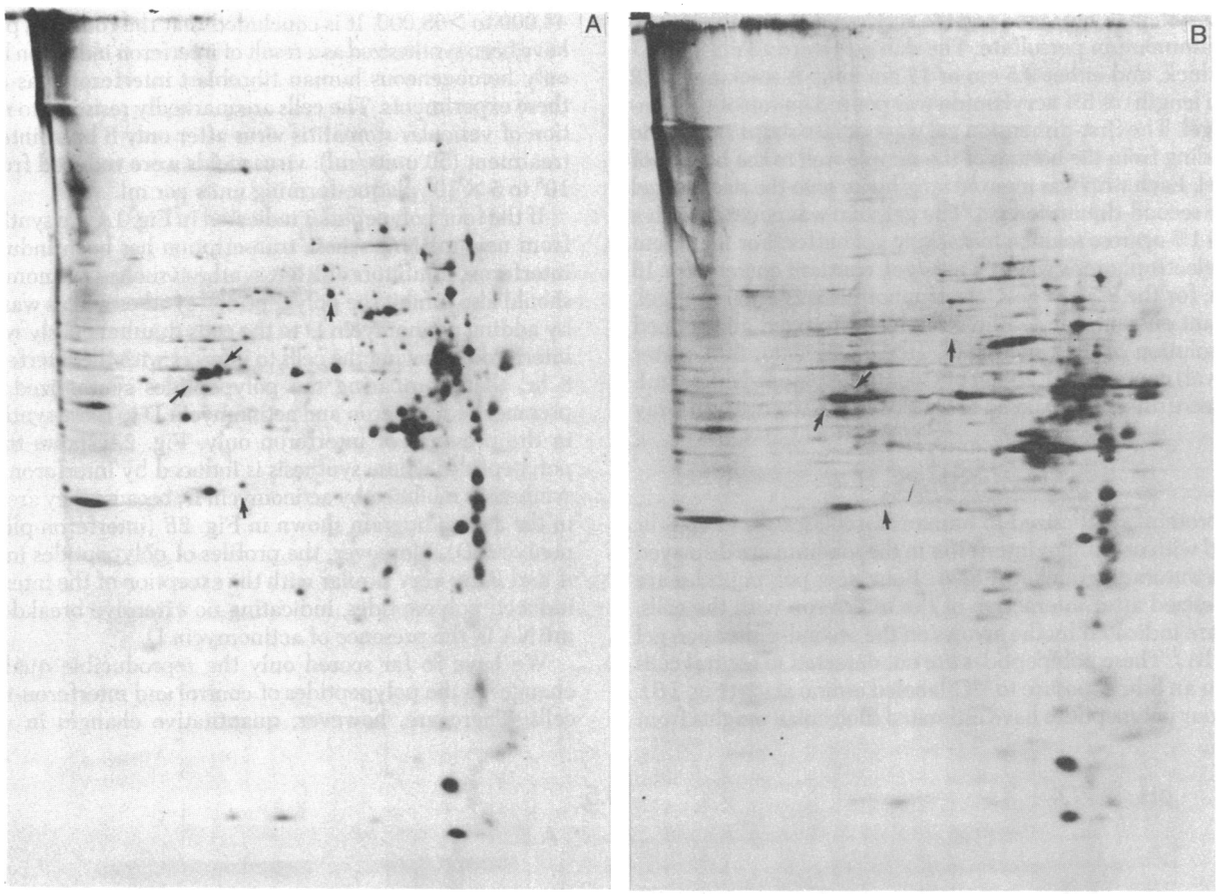

FIG. 2. Autoradiogram showing the effect of actinomycin D on synthesis of interferon-induced polypeptides. Labeling of cells, the preparation of extracts, and electrophoresis were as described in *Materials and Methods* and Fig. 1. The second-dimension sodium dodecyl sulfate gel was 17 cm in length. (*A*) Extracts prepared from cells treated with interferon for 8 hr; 130,000 cpm loaded onto first-dimension gel. (*B*) Extracts prepared from cells treated with interferon and actinomycin D, 5 μg/ml, for 8 hr; 130,000 cpm loaded onto first-dimension gel.

polypeptide spots obtained from cells treated with interferon. The meaning of these quantitative changes in response to interferon is not understood at the present time.

DISCUSSION

By utilizing incorporation of ^{14}C-labeled amino acids into human fibroblast cells grown in microtiter dishes, we have been able to compare the polypeptides synthesized in interferon-treated cells to those synthesized in untreated cells. Four new polypeptides have been detected in cells that had incorporated ^{14}C-labeled amino acids for 8 hr in the presence of 50 units of interferon per ml. These polypeptides have estimated molecular weights ranging from 44,000 to >68,000. The cells were resistant to virus replication after 8 hr of interferon treatment, therefore the proteins synthesized in an 8-hr period could be involved in the antiviral mechanism. What is clear from these experiments is that human fibroblast cells synthesize new proteins in response to interferon. The diverse charge and size of the induced polypeptides indicates that they do not have the same amino acid sequence. We have also found that bovine insulin does not induce these four proteins (data not shown).

Experiments with actinomycin D indicate that the synthesis of the induced protein is from new mRNA induced by interferon and is not from existing mRNA. We have no evidence at this time suggesting roles for these proteins in the antiviral activity that is induced by interferon. Because the antiviral mechanism induced by interferon is poorly understood we cannot speculate as to the number of proteins that may be involved in such a mechanism. Furthermore, interferon induces numerous nonantiviral effects in cells in culture that may require the synthesis of new but different proteins from those involved in the antiviral mechanism.

The synthesis of new proteins by cells in response to interferon was indicated 14 years ago in the work with inhibitors of RNA and protein synthesis (2, 3). More recent data also suggest the involvement of new proteins in a cell's response to interferon. Experiments on the role of interferon in the double-stranded RNA inhibition of protein synthesis show that the inhibition is markedly enhanced in extracts of interferon-treated cells (9). Phosphorylation of specific proteins by protein kinases has been shown to be enhanced *in vitro* by extracts of interferon-treated cells (10, 11). Enhanced degradation of viral mRNA by extracts of interferon-treated cells has also been reported (12). A trinucleotide that inhibits protein synthesis *in vitro* has been isolated from extracts of interferon-treated cells that had been supplemented with ATP and double-stranded RNA (13, 14). Although the aforementioned activities may suggest the synthesis of new proteins in interferon-treated cells, the role of the oligonucleotide inhibitor(s), kinase(s), and nuclease(s) in the antiviral state of the intact cell remains to be established. An additional protein of molecular weight 48,000 has been found in ribosomal washes of interferon-treated Ehrlich ascites cells (4). Whether the synthesis of this protein (as opposed to recruitment) has been induced by interferon has not been determined.

Clearly, two-dimensional electrophoretic analysis provides a powerful tool for investigating the proteins in cells that have been treated by interferon. The effects of many variables on

the synthesis of the interferon-induced proteins can be examined by using this technique. Whether the four proteins synthesized during 8 hr of interferon treatment have roles in the antiviral mechanism or in the nonantiviral activities of interferon remains to be established. We conclude that under the conditions of our experiments human fibroblast cells synthesize at least four proteins in response to interferon.

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