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Biochemistry

Interferon action: Induction of specific proteins in mouse and human cells by homologous interferons

(mechanism of interferon action/double-stranded RNA/interferon-induced enzymes)

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ABSTRACT **Treatment of mouse (Ehrlich ascites tumor and L929) and human (FS4, GM258, etc.) cells with homologous interferons results in the induction of several proteins. Extracts obtained from cells labeled with [^{35}S]methionine in the absence or presence of interferon were fractionated on poly(I)·poly(C)-agarose columns. The proteins retained on the columns revealed, upon sodium dodecyl sulfate/polyacrylamide gel electrophoresis, three protein bands in mouse cells (M_r 120,000; 80,000; and 67,000) and two in human cells (M_r 120,000 and 80,000) which were detected in the extracts of interferon-treated but not of untreated cells. These proteins were retained on double-stranded RNA [poly(I)·poly(C)-agarose] columns but very poorly, if at all, on single-stranded RNA [poly(I)- or poly(C)-agarose] columns, suggesting that they have an affinity for double-stranded RNA. In addition, interferon treatment of human fibroblasts greatly increased the labeling of three other protein bands (M_r 88,000; 67,000; and 56,000) which were detected in whole extracts but were not appreciably retained on poly(I)·poly(C)-agarose columns. The appearance of the induced proteins was blocked by actinomycin D if added together with interferon, indicating that transcription of certain genetic information is required. The possible correlation between the induced proteins described here and the elevated levels of certain enzymes in interferon-treated cells (a protein kinase and 2′-5′-oligoadenylate synthetase) is at present unclear.**

Interferons are known for their potent antiviral activity. The replication of a wide variety of RNA and DNA viruses is blocked in cells pretreated with interferon (1, 2). Interferon treatment also results in various other effects, such as: alterations at the cell surface, inhibition of cell multiplication, antitumor effects in experimental animals and in man, effects on immune functions, etc. (3, 4). The mechanism(s) whereby interferon treatment results in various biological effects remains to be elucidated.

The establishment of the antiviral effect of interferon is blocked by actinomycin D (5–7) and by inhibitors of protein synthesis (6–8), suggesting that the expression of certain cellular genetic information is required. This is supported by experiments showing that enucleated cells fail to develop an antiviral effect upon interferon treatment (9, 10). Studies from various laboratories have revealed that the level of at least two enzymes is greatly elevated in various cell types upon interferon treatment. These are: (*i*) a protein kinase(s) which, in the presence of ATP and double-stranded (ds) RNA, phosphorylates at least two proteins of M_rs 64,000–67,000 and 35,000–38,000, and histones (11–14). The M_r 35,000–38,000 protein appears to be the small subunit of the initiation factor eIF-2 required for protein synthesis (15, 16). (*ii*) The other is an enzyme (2′-5′-oligoadenylate synthetase) which synthesizes 2′-5′-linked oligoadenylates with the structure pppA(2′p5′A)$_n$ from ATP in the presence of ds RNA (17, 18). These oligonucleotides act as potent inhibitors of mRNA translation in cell-free systems (12, 15–19) and serve as activators of an endonuclease that is present in both interferon-treated and untreated cells (20–23). These enzymes have been partially purified and characterized (16, 20, 23, 24). However, the role of these enzymes in the antiviral mechanism, whether these enzymes are the products of the genetic information that is induced by interferon treatment and whether any proteins other than these enzymes are induced, remains unclear. In this paper, we report the identification of several proteins that are induced in mouse and human cells upon treatment with homologous interferons. The induction of these proteins by interferon treatment is blocked by actinomycin D. Part of this work was presented at the Second International Workshop on Interferons held at the Memorial Sloan-Kettering Cancer Center, New York (April 22–24, 1979).

MATERIALS AND METHODS

Materials. Mouse interferon partially purified (25) up to the CM-Sephadex step (specific activity $\approx 10^7$ reference units/mg of protein) was a generous gift from P. Lengyel (Yale University). Partially purified human leukocyte interferon (specific activity $\approx 7 \times 10^5$ reference units/mg of protein) and human fibroblast interferon (specific activity $\approx 10^5$ reference units/mg of protein) were kindly provided by V. Edy and W. E. Stewart II, respectively. Poly(I)-, poly(C)-, and poly(I)·poly(C) bound to agarose were purchased from P-L Biochemicals, and [^{35}S]-methionine was from New England Nuclear. Human fibroblasts (FS4) were obtained from J. Vilcek, and GM258 and GM2767 were from the Institute for Medical Research (Camden, NJ).

Preparation of [^{35}S]Methionine-Labeled Extracts from Interferon-Treated and Untreated Cells. Mouse Ehrlich ascites tumor (EAT) cells grown in spinner culture (26) or human fibroblasts grown in monolayers in 150-mm tissue culture dishes (Falcon) in Eagle's minimum essential medium containing 10% fetal calf serum were divided into two groups; one group received homologous interferon (400–500 reference units/ml) and the other served as a control. Both cultures were labeled with [^{35}S]methionine (5 μCi/ml, specific activity 550–700 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) for 16–20 hr. The cells were then washed and lysed and ribosomal wash and supernatant (S-200) fractions were prepared as described in the figure legends.

Fractionation on Poly(I)·Poly(C)-Agarose Columns and Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. Aliquots of [^{35}S]methionine-labeled S-200 and ribosomal wash fractions from interferon-treated and untreated cells were applied to poly(I)·poly(C)-agarose columns (ap-

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Abbreviations: poly(I), poly(inosinic acid); poly(C), poly(cytidylic acid); ds, double-stranded; $NaDodSO_4$, sodium dodecyl sulfate; S-200, final supernatant of lysed cells; EAT, Ehrlich ascites tumor.

proximately 0.1 ml) equilibrated with buffer I (10 mM Hepes·KOH, pH 7.5/90 mM KCl/1.5 mM magnesium acetate/7 mM 2-mercaptoethanol/20% glycerol). The samples were mixed gently with the column bed and incubated in the cold for 30 min and then at room temperature for 20 min with occasional mixing. The columns were extensively washed with buffer I and the proteins retained on the columns (approximately 2% in the case of S-200 and 5% in the case of ribosomal wash) were eluted with 150 μl of sample buffer (27) containing 2.5% $NaDodSO_4$ at 95°C for 10 min. The extracts were collected and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis in slab gels (27) as described (14). In several experiments, the eluted proteins were precipitated by adding trichloroacetic acid to 10% (wt/vol) final concentration after adding 10 μl of a mixture of proteins [bovine serum albumin/catalase/gamma globulin/aldolase/ribonuclease (100 μg/ml of each)] as carrier. The precipitates were collected by centrifugation for 10 min in a Microfuge (Beckman), washed once with 10% trichloroacetic acid and twice with acetone, dissolved in sample buffer, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The gels were stained with Coomassie blue, destained, and prepared for autoradiography (14) or fluorography (28) with Kodak XR-5 film.

RESULTS

Fractionation of [^{35}S]Methionine-Labeled Extracts from Interferon-Treated and Untreated EAT Cells. Addition of ds RNA to extracts from interferon-treated cells results in the activation of two enzymes, a protein kinase(s) and a 2′-5′-oligoadenylate synthetase (11–19, 24). We tested whether the factors induced by interferon treatment can be identified by virtue of a possible interaction with ds RNA. [^{35}S]Methionine-labeled ribosomal wash and S-200 preparations from interferon-treated and untreated cells were fractionated on poly(I)·poly(C)-agarose columns, and the material retained on the columns was extracted and analyzed by $NaDodSO_4$/poly-

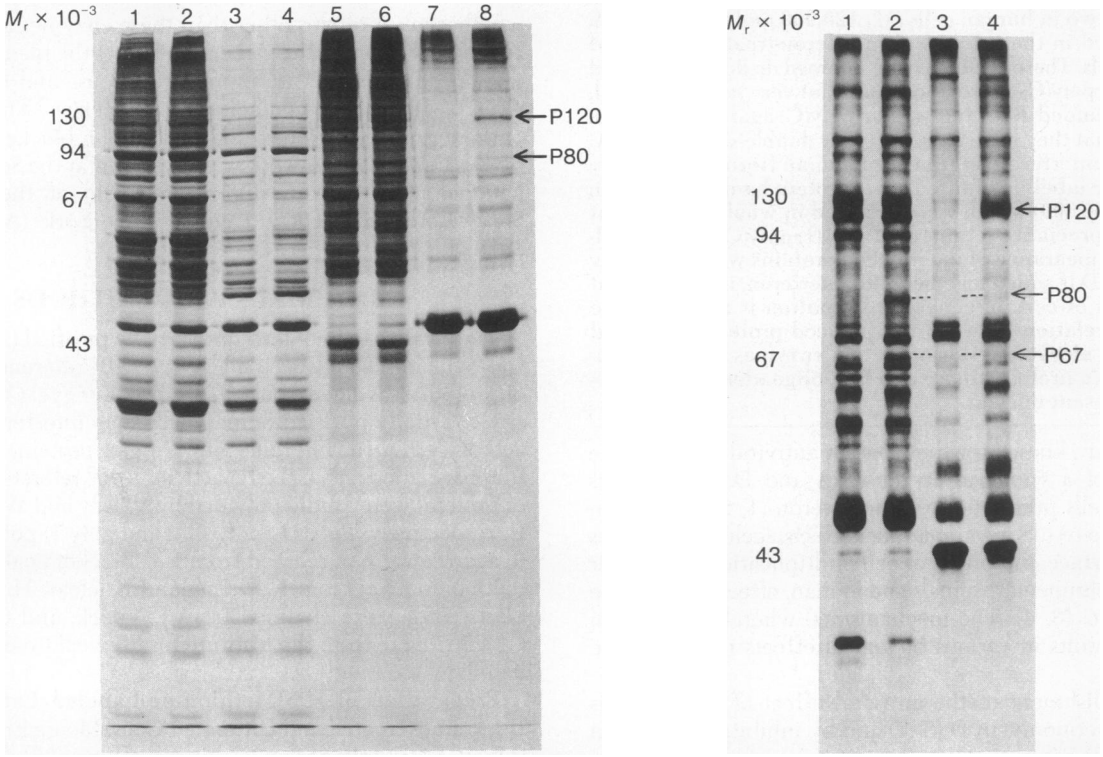

FIG. 1. (*Left*) $NaDodSO_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled ribosomal wash (2 μl containing ≈2.5 × 10^5 cpm; lanes 1 and 2) and S-200 (3 μl containing ≈1 × 10^5 cpm; lanes 3 and 4) fractions from interferon-treated and untreated EAT cells, and of the material retained on poly(I)·poly(C)-agarose columns from the ribosomal wash (lanes 5 and 6) and S-200 (lanes 7 and 8) fractions. Lanes 2, 4, 6, and 8 represent samples from interferon-treated cells, and lanes 1, 3, 5, and 7 represent samples from untreated cells. EAT cells were labeled with [^{35}S]methionine in the absence or presence of mouse interferon (500 reference units/ml) for 16 hr. The cells were washed, their lysates were prepared as described (26) and centrifuged at 1500 × *g* for 10 min, and the supernatants were centrifuged at 10,000 × *g* for 20 min in the cold. The 10,000 × *g* supernatants were centrifuged in a 75 Ti rotor (Spinco) at 200,000 × *g* for 2 hr at 2°C and the final supernatants (S-200) were collected. The ribosomal pellets were extracted in 25 mM Tris·HCl, pH 7.8/500 mM KCl/5 mM magnesium acetate/1 mM EDTA/1 mM ATP/30 mM 2-mercaptoethanol (using 250 μl of buffer per ml of S-200) by stirring for 1 hr in the cold, and the ribosomes were pelleted by centrifugation at 200,000 × *g* for 2 hr. The supernatants (ribosomal wash) were dialyzed overnight against 300 ml of buffer (10 mM Tris·HCl, pH 7.8/120 mM KCl/5 mM magnesium acetate/30 mM 2-mercaptoethanol/1 mM EDTA/10% glycerol) with one buffer change. The S-200 and ribosomal wash fractions were stored in aliquots in liquid nitrogen. A typical preparation of S-200 and ribosomal wash contained 8–10 mg of protein per ml. Ribosomal wash (30 μl containing ≈4 × 10^6 cpm) or S-200 (120 μl containing ≈3.6 × 10^6 cpm) was fractionated on poly(I)·poly(C)-agarose columns. The material retained on the columns was extracted with $NaDodSO_4$, precipitated with 10% trichloroacetic acid, washed, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. The following proteins were run in parallel as M_r markers: myosin (200,000), β-galactosidase (130,000), phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), and carbonic anhydrase (30,000). (*Right*) [^{35}S]methionine-labeled ribosomal wash (40 μl) and S-200 (160 μl) from interferon-treated and untreated cells were fractionated on poly(I)·poly(C)-agarose columns. The proteins retained on the columns were eluted, precipitated with trichloroacetic acid, washed, and applied to a $NaDodSO_4$/polyacrylamide slab gel as described in *Left*. Electrophoresis was carried out at 18 mA for 1 hr, then at 22 mA at constant current until the current output increased to 2 W; then it was run at 2 W (constant power) for another 16 hr. The figure shows the gel pattern of proteins retained on the columns from the ribosomal wash (lanes 1 and 2) and from the S-200 (lanes 3 and 4) fractions from interferon-treated (lanes 2 and 4) and untreated (lanes 1 and 3) cells. Fluorography (28) was used to locate the bands.

acrylamide gel electrophoresis along with the S-200 and ribosomal wash fractions (Fig. 1 *left*). Under the fractionation conditions, the gel patterns of total proteins in S-200 or ribosomal wash fractions from interferon-treated and untreated cells revealed no detectable differences. However, the proteins that were retained on poly(I)·poly(C)-agarose columns revealed two bands that were present in samples derived from interferon-treated cells but were missing (or were present in considerably smaller amounts) in parallel samples obtained from untreated cells. This difference is most clearly seen in the case of proteins retained from the S-200 fractions (P120 and P80). P120 (M_r 120,000) was detected as a prominent band which has consistently been observed in samples derived from interferon-treated S-200. P80 (M_r 80,000) was usually detected as a faint band, which may be why it escapes detection in some experiments. A protein band similar to P120 was also detected by this approach in the S-200 fraction of interferon-treated L929 cells but not in untreated cells (data not shown). P120 was detected also when a 1:1 mixture of S-200 from interferon-treated and from untreated EAT cells was applied to poly(I)·poly(C)-agarose columns, thus suggesting that the lack of this protein band in samples from untreated cells is not due to inhibitors that may interfere with the retention of this protein on the column (data not shown).

The proteins that were retained on poly(I)·poly(C)-agarose columns from ribosomal wash fractions gave several bands in the high M_r region, thus making it difficult to conclude whether or not P120 and P80 are also present in the ribosomal wash from interferon-treated cells. However, upon prolonged electrophoresis of such samples, we detected a M_r 80,000 protein band that was induced by interferon treatment (Fig. 1 *right*, lane 2). In addition, these experiments revealed another protein band (P67, M_r 67,000) which showed increased labeling upon interferon-treatment. These results indicate that at least three proteins, which were detected in extracts of interferon-treated mouse EAT cells but not (or in considerably smaller amounts) in similar extracts of untreated cells, could be identified by this approach.

When ^{35}S-labeled S-200 preparations were fractionated on poly(I)-agarose or poly(C)-agarose in place of poly(I)·poly(C)-agarose and the proteins that were retained on the columns were extracted and analyzed on slab gels, it was observed that P120 detected in S-200 from interferon-treated cells was strongly retained on polyI)·poly(C)-agarose but not, or very poorly, on poly(I)- or poly(C)-agarose columns (data not shown), thus suggesting an affinity for ds RNA.

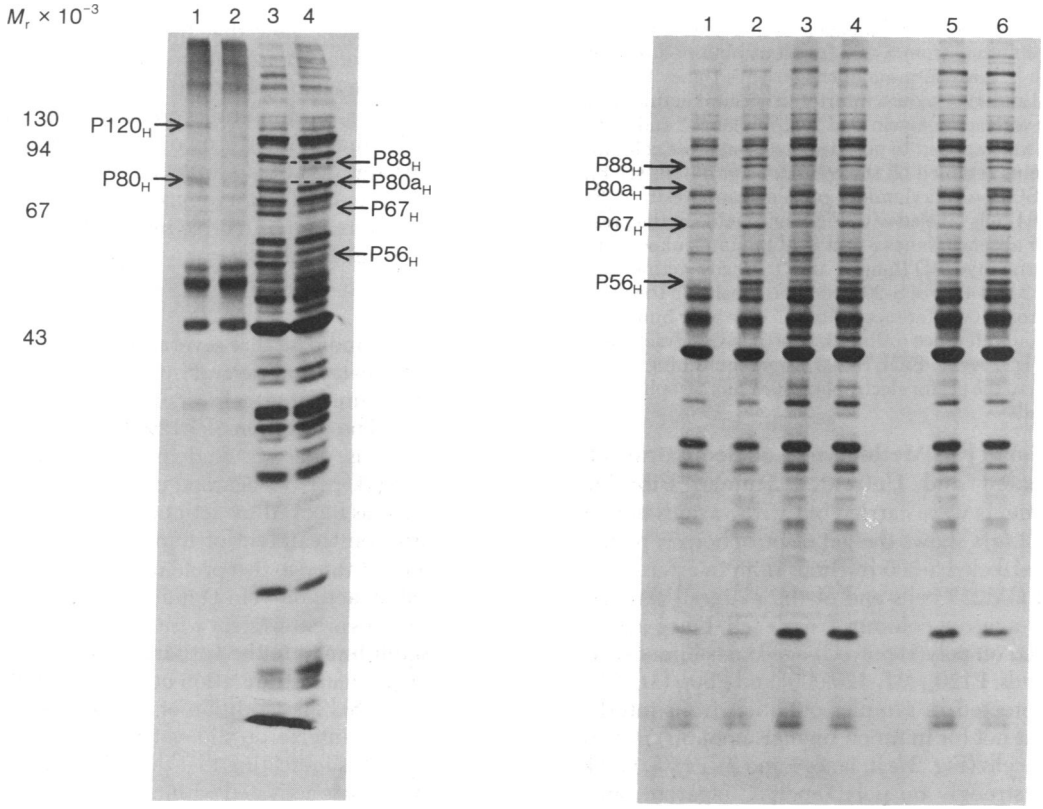

FIG. 2. $NaDodSO_4$/polyacrylamide gel electrophoresis of [^{35}S]methionine-labeled S-200 and of material retained on poly(I)·poly(C)-agarose columns from S-200 of interferon-treated and untreated human fibroblasts. (*Left*) GM258 cells in monolayers were labeled with [^{35}S]methionine in the absence or presence of human leukocyte interferon (400 reference units/ml) for 18 hr and their extracts were prepared as described (ref. 14, procedure *ii*), except that the 10,000 × *g* supernatants obtained were not dialyzed and were centrifuged further at 200,000 × *g* for 2 hr at 2°C, and the final supernatants (S-200) were collected. Each S-200 (70 μl containing 7.0–8.5 × 10^5 cpm) was applied to poly(I)·poly(C)-agarose columns to isolate proteins retained on the columns. Lanes: 1 and 2, proteins retained on poly(I)·poly(C)-agarose column from interferon-treated and untreated S-200, respectively; 3 and 4, S-200 (4 μl containing 4–4.8 × 10^4 cpm) from interferon-treated and untreated cells, respectively. (*Right*) Various human fibroblasts grown in monolayers were labeled with [^{35}S]methionine in the absence or presence of human leukocyte interferon (400 reference units/ml) for 16 hr. The cells were washed with 35 mM Tris·HCl, pH 7.5/147 mM NaCl and lysed in the cold by adding 0.5 ml of buffer (10 mM Tris·HCl, pH 7.4/50 mM KCl/5 mM $MgCl_2$) containing 0.2% Triton X-100 and spreading it uniformly. After 5 min, the lysed cells were scraped with a rubber policeman and the suspensions were centrifuged to prepare S-200 fractions as in Fig. 1. Each S-200 (20 μl containing 2–2.6 × 10^4 cpm) was applied to gels for electrophoresis. Lanes: 1 and 2, FS4 cells; 3 and 4, FR8 cells; 5 and 6, GM2767 cells. Lanes 2, 4, and 6 represent samples from interferon-treated cells and lanes 1, 3, and 5 represent samples from untreated cells. The bands were located by fluorography (28). FR8 is a human foreskin fibroblast cell line.

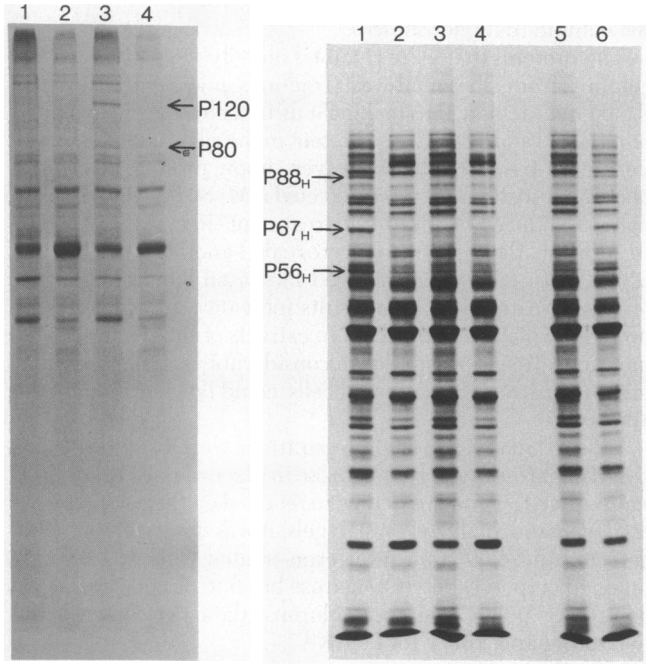

FIG. 3. Effect of actinomycin D. (*Left*) NaDodSO$_4$/polyacrylamide gel electrophoresis of ^{35}S-labeled proteins retained on poly(I·poly(C)-agarose columns from S-200 fractions obtained from EAT cells labeled with [^{35}S]methionine in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of mouse interferon without actinomycin D (lanes 1 and 3) or with actinomycin D at 2 μg/ml (lanes 2 and 4). S-200 samples (125 μl) were applied to poly(I)·poly(C)-agarose columns for isolation of proteins retained on the columns. For details see Fig. 1. (*Right*) NaDodSO$_4$/polyacrylamide gel electrophoresis of S-200 fractions from FS4 cells labeled with [^{35}S]methionine in the presence (lanes 1 and 2) or absence (lanes 3 and 4) of human leukocyte interferon without actinomycin D (lanes 1 and 3) or with actinomycin D at 2 μg/ml (lanes 2 and 4) or of S-200 fractions labeled in the presence of mouse interferon (400 reference units/ml, lane 5) or human fibroblast interferon (40 reference units/ml, lane 6). S-200 fractions were prepared as in Fig. 2 *right*. Each S-200 (20 μl containing 3–4 × 10^4 cpm) was applied to the gel for electrophoresis. Fluorography was used to locate the bands.

Fractionation of [^{35}S]Methionine-Labeled Extracts from Interferon-Treated and Untreated Human Fibroblasts. Similar experiments were carried out with various human fibroblasts. Fig. 2 *left* shows the gel electrophoresis pattern of [^{35}S]methionine-labeled S-200 fractions from interferon-treated and untreated GM258 cells and of the material retained on poly(I)·poly(C)-agarose columns from each. Once again, the proteins retained on poly(I)·poly(C)-agarose columns revealed two protein bands, P120$_H$ (M_r 120,000) and P80$_H$ (M_r 80,000), which were detected in samples obtained from interferon-treated cells but not (or in much smaller amounts) in samples from untreated cells (Fig. 2 *left*, lanes 1 and 2). P120$_H$ and P80$_H$ were retained strongly on poly(I)·poly(C)-agarose but very poorly on poly(C)-agarose (data not shown), again suggesting an affinity for ds RNA. Unexpectedly, the electrophoresis of S-200, without any prior fractionation, revealed two protein bands in S-200 from interferon-treated cells but not from untreated cells. One of these had a mobility similar to P80$_H$ and is therefore referred to as P80a$_H$ (P80$_H$ and P80a$_H$ may be identical), and the other had a mobility corresponding to M_r 88,000 (P88$_H$). In addition, two protein bands (P67 and P56) showed a quantitative increase upon interferon treatment. It is conceivable that these bands consist of several proteins, and that what appears as a quantitative change may in fact be due to a marked induction of specific proteins. A preliminary result from prolonged electrophoresis suggests that this may be true, at least for the M_r 67,000 protein band (data not shown). Further fractionation is necessary to evaluate this possibility. Interferon treatment of various other lines of human fibroblasts (FS4, FR8, and GM2767) revealed similar differences upon NaDodSO$_4$/polyacrylamide gel electrophoresis of their extracts (Fig. 2 *right*). Therefore, the differences observed after interferon treatment do not seem to depend on trisomy for chromosome 21, a characteristic of GM258 and GM2767 cells. The [^{35}S]methionine-labeled bands detected in the gels were found to be sensitive to Pronase (250 μg/ml, Calbiochem) but resistant to pancreatic RNase (100 μg/ml, Worthington) and DNase I (250 μg/ml, Worthington) at 37°C for 15 min (data not shown). Thus, three to five proteins that were induced in human fibroblasts by treatment with human interferon were detected.

Inhibition by Actinomycin D and Species Specificity. Fig. 3 *left* shows that, whereas P120 and P80 were detected in the S-200 fraction from interferon-treated EAT cells (lane 3), they were not detected if the interferon treatment was given in the presence of actinomycin D (track 4), indicating that this induction requires RNA synthesis. The appearance of proteins induced in human fibroblasts by interferon treatment was also blocked by actinomycin D if added together with interferon (Fig. 3 *right*, lanes 1 and 2) but not if added 4 hr after the addition of interferon (data not shown). These results are consistent with the present view that the antiviral effect of interferon requires cellular transcription and translation. Human fibroblasts are known to be insensitive to mouse interferon. Consistent with this, we found that treatment of human fibroblasts (FS4) with mouse interferon did not result in the appearance of the proteins that were induced by human interferon (Fig. 3 *right*, lane 5). Human leukocyte and human fibroblast interferons were both effective in inducing these proteins (Fig. 3 *right*, lanes 1 and 6).

DISCUSSION

The results indicate that several proteins were detected in interferon-treated mouse and human cells that were either not present in untreated cells or were present in much smaller amounts. The detection of P120, P80, and P67 in interferon-treated mouse cells was made possible by their retention on ds RNA [poly(I)·poly(C)-agarose] columns. The induction of these proteins was blocked by actinomycin D.

Interferon treatment of human fibroblasts resulted in the induction of three to five protein bands, and their induction was blocked by actinomycin D if added together with interferon. These results are consistent with the observations indicating that the establishment of the antiviral effect of interferon requires transcription and translation of certain cellular genetic information (5–8). The addition of actinomycin D 4 hr after the addition of interferon did not block the induction of these proteins, thus indicating that the genetic information induced by interferon is activated within 4 hr. In a separate experiment, we confirmed that the antiviral effect of interferon (as determined by infecting the cells with vesicular stomatitis virus and measuring the virus yield by plaque assay) was blocked if actinomycin D was added together with interferon but not if added 4 hr later (data not shown). In this respect, the appearance of the induced proteins correlates with the development of the antiviral state, thus suggesting that these proteins might be involved in the antiviral mechanism.

At least two proteins that were induced in mouse (P120 and P80) and human (P120$_H$ and P80$_H$) cells have similar characteristics. They are retained on poly(I)·poly(C)-agarose columns and have similar molecular weights. In various cell types, interferon treatment has been shown to result in elevated levels

of two enzyme activities, a protein kinase and a 2′-5′-oligoadenylate synthetase (11–19). It is at present unclear how the induced proteins described here are related to the above-mentioned enzymes. Proteins P120, P80, and P67 were detected by fractionation on poly(I)·poly(C)-agarose columns, thus indicating that these proteins have a strong affinity for ds RNA. It is conceivable that these proteins represent certain components of the interferon-induced protein kinase or 2′-5′-oligoadenylate synthetase enzyme(s), or they may be involved in the ds RNA-mediated activation of these enzymes. It is worth noting that, after fractionation on poly(I)·poly(C)-agarose columns, the P120 is detected upon $NaDodSO_4$/polyacrylamide gel electrophoresis not only in autoradiographs but also as a band stainable with Coomassie blue, thus indicating that it is induced in substantial amounts. From the staining intensity of this band in comparison to known amounts of bovine serum albumin run in parallel as standard, we estimated by making use of the Avogadro's number that, under the conditions employed, the interferon-treated EAT cells contain $>10^4$ molecules of this protein per cell.

The P67 detected in the ribosomal wash of interferon-treated EAT cells has certain characteristics in common with the M_r 64,000–67,000 protein that is phosphorylated by ds RNA-activated protein kinase (11–14). For example, both have similar size, both are retained on poly(I)·(poly(C)-agarose columns, and both are located predominantly in the ribosomal fraction. If the two proteins are identical, this would imply that the M_r 67,000 protein substrate for the kinase is also induced by interferon treatment. A protein of similar molecular weight showed increased labeling with [^{35}S]methionine in various human fibroblasts as well upon treatment with human interferon. Interferon treatment of chicken cells was recently reported to induce a protein of M_r 56,000 which appeared to correlate with the 2′-5′-oligoadenylate synthetase (29). We have detected a M_r 56,000 protein band which was greatly increased in human cells by interferon treatment. Whether this increase in the M_r 56,000 protein band is related to the 2′-5′-oligoadenylate synthetase is unclear.

Interferon treatment of human fibroblasts resulted in the induction of three protein bands ($P120_H$, $P88_H$, and $P80_H$) and in increased labeling of two bands ($P67_H$ and $P56_H$). It remains to be established whether all of the proteins that are induced by interferon treatment are related to the two enzymes (protein kinase and 2′-5′-oligoadenylate synthetase) that are known to be induced by interferon treatment or whether any of them are unrelated to these enzymes. The latter possibility may not be too surprising in view of the fact that interferon treatment results in various biological effects (3, 4).

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