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Interferon-Induced Proteins in Human Fibroblasts and Development of the Antiviral State

BERISH Y. RUBIN AND SOHAN L. GUPTA*
Interferon Laboratories, Memorial Sloan-Kettering Cancer Center, New York, New York 10021

Treatment of human fibroblasts with interferon induces the synthesis of several proteins, as detected by incorporation of [^{35}S]methionine followed by analysis of cell extracts by polyacrylamide gel electrophoresis. The induction of these proteins had features in common with the development of the antiviral effect of interferon, such as (i) sensitivity to actinomycin D and cycloheximide when these compounds were added together with interferon, (ii) insensitivity to actinomycin D if the actinomycin D was added 2 h after the addition of interferon, (iii) similar dependence on interferon concentration, and (iv) species specificity for interferon. When interferon treatment was given in the presence of cycloheximide and actinomycin D was added before the removal of cycloheximide, all four proteins were induced, thus suggesting that their inductions are coordinated. Labeling for 2-h periods at varying time intervals after the addition of interferon revealed that the synthesis of these proteins was induced within a few hours, peaked at different time intervals, and was soon followed by a marked decline, suggesting that the mRNA's for these proteins have short half-lives. Moreover, this decline occurred despite the fact that the cells were continuously exposed to interferon, and there was no measurable loss of interferon activity in the medium. This suggests that the induction of these proteins is transient and is apparently subject to further control.

The replication of a wide variety of animal viruses is strongly inhibited in cells pretreated with interferon (31). It is believed that the development of this antiviral effect of interferon involves an induction of certain cellular gene(s), as it is blocked by inhibitors of transcription and translation (14, 24, 33) and by enucleation of cells (25, 34). In various cell types, interferon treatment results in greatly enhanced levels of at least two enzymatic activities; these are (i) a protein kinase(s) which, in the presence of double-stranded RNA and ATP, phosphorylates at least two polypeptides having molecular weights about 67,000 and 38,000 (15, 22, 23, 27, 29, 35) (the latter is believed to be the small subunit of the initiation factor eIF-2 required for protein synthesis [12, 28, 36]), and (ii) a 2′,5′-oligoadenylate synthetase which synthesizes 2′,5′ linked oligoadenylates [pppA(2′p5′A)n] from ATP in the presence of double-stranded RNA (1, 4, 17, 18). These 2′,5′-oligoadenylates activate an endonuclease which is present in both untreated and interferon-treated cell extracts and degrades mRNA (2, 5, 9, 26). These enzymes apparently constitute two pathways for the inhibition of mRNA translation in cell extracts (8, 12, 23) and are considered as likely candidates for a role in the antiviral effect in vivo. Whether these enzymes are the products of the genetic information that is induced by interferon is not entirely clear. Ball (3) reported that in chick cells, interferon treatment induces the synthesis of a 56,000-molecular-weight protein which appears to be related to the 2′,5′-oligoadenylate synthetase.

The binding of interferon to cells is apparently the first step required for a cellular response to interferon (6, 13, 32). Studies on the process whereby interferon treatment results in the induction of specific genes, leading to the establishment of the antiviral state, require an identification of the gene products induced by interferon. Our recent studies have revealed that a number of proteins are induced in mouse and human cells by homologous interferons (16). In mouse cells, three proteins (apparent molecular weights, 120,000, 80,000, and 67,000) were found to be induced and were identified by their retention on double-stranded RNA (polyriboinosinic·polyribocytidylic acid-agarose) columns, followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. There are other reports of interferon-induced proteins in mouse cells (10, 11, 30). In human fibroblasts, the syntheses of at least five proteins (apparent molecular weights, 120,000, 88,000, 80,000, 67,000, and 56,000; referred to as $P120_H$, $P88_H$, $P80_H$, $P67_H$, and $P56_H$, respectively) were found to be induced (16). (We

are assuming that the 80,000-molecular-weight induced protein or proteins detected by gel electrophoresis of whole extracts and after fractionation on polyriboinosinic·polyribocytidylic acid-agarose columns are identical.) At least two of these proteins ($P120_H$ and $P80_H$) are retained on polyriboinosinic·polyribocytidylic acid-agarose columns. In agreement with the species specificity, human leukocyte and human fibroblast interferons are effective in inducing these proteins, but mouse interferon is not. The appearance of the induced proteins in both mouse and human cells is blocked by actinomycin D (16). Knight and Korant (19) also detected four new proteins, which were synthesized in human fibroblasts after treatment with interferon.

In this paper, we present further studies on the characteristics of this induction phenomenon, which indicate a correlation between the induction of these proteins and the development of the antiviral state. The kinetics of labeling of these proteins indicate that their syntheses can be detected within hours after interferon treatment and that their rates of accumulation decline several hours after induction despite the continued presence of interferon in the medium, thus suggesting that this induction is transient.

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MATERIALS AND METHODS

Materials. Human leukocyte interferon (specific activity, approximately 7×10^5 reference units per mg of protein) was kindly provided by V. Edy. [^{35}S]methionine (specific activity, 550 to 820 Ci/mmol) was purchased from New England Nuclear Corp.; cycloheximide and actinomycin D were from Calbiochem.

Preparation of [^{35}S]methionine-labeled extracts from interferon-treated and untreated cells and analysis by polyacrylamide gel electrophoresis. Human FS-4 fibroblasts (kindly provided by J. Vilcek) were grown in tissue culture dishes (60 or 100 mm; Falcon Plastics) in Eagle minimum essential medium supplemented with 10% heat-inactivated fetal calf serum (growth medium) at 37°C in an incubator flushed with 5% CO_2 in air. Confluent monolayers were labeled with [^{35}S]methionine (5 or 10 μCi/ml) in the presence or absence of interferon in this growth medium, except that in most experiments the concentration of methionine was 25% of the regular concentration (labeling medium). Other experimental details are specified in the figure legends. After labeling, the cells were washed with 35 mM Tris-hydrochloride (pH 7.5)-146 mM NaCl and lysed in the cold by adding 0.2 ml of a buffer containing 10 mM Tris-hydrochloride (pH 7.4), 50 mM KCl, 5 mM $MgCl_2$, and 0.2% Triton X-100. After 5 min, the lysed cells were scraped with a rubber policeman, the lysate was centrifuged at 1,500 × g for 10 min, and the resulting supernatant was centrifuged at 10,000 × g for 20 min. The supernatants from the centrifugation at 10,000 × g were further centrifuged at 200,000 × g (maximum) for 2 h at 2°C in either a 50Ti or a 75Ti rotor (Spinco), and the final supernatants (designated S-200) were collected. Hot trichloroacetic acid-insoluble counts were determined in 5 μl of S-200 in duplicate (7). S-200 samples containing approximately equal counts were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (15, 20), except that 12% polyacrylamide gels were used; after stacking, electrophoresis was carried out at 2.5 W (constant power) for about 20 h. The gels were processed for autoradiography (16) or fluorography (21).

The interferon-induced protein bands were quantitated by densitometric scanning of autoradiographs or fluorographs at 525 nm with a Quick Scan Jr. densitometer (Helena Laboratories). The densitometer was standardized against a band which did not show any apparent change after interferon treatment. The areas on the scans corresponding to various induced proteins were cut out and weighed. The values presented are expressed as percentages of the maximum obtained for an individual band in a particular experiment.

Infection with VSV and determination of virus yield. To determine the effect of different treatments on the development of the antiviral state in various experiments, parallel monolayers were challenged with vesicular stomatitis virus (VSV) at a multiplicity of infection of 50 PFU/cell. Virus adsorption was allowed for 1 h at 37°C, and the cells were then washed twice with growth medium and incubated in 3 ml of growth medium for 18 h at 37°C. After three cycles of freezing and thawing, the virus yield was determined by plaque assay on L929 cells.

RESULTS

Requirement for transcription and translation. We showed previously (16) that the appearance of interferon-induced protein bands is blocked by actinomycin D if this drug is added together with interferon, thus indicating that this induction requires transcription. Figure 1A shows an experiment in which actinomycin D was added at different times (1, 2, and 4 h) after the addition of interferon to determine the time interval required for this transcription event to occur. An induction of four protein bands ($P88_H$, $P80_H$, $P67_H$, and $P56_H$) by interferon treatment was clearly observed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the S-200 fractions of interferon-treated cells (Fig. 1, lane 2) as compared with untreated cells (Fig. 1, lane 1), as reported previously (16). The appearance of the induced proteins was inhibited if actinomycin D was added up to 1 h after the addition of interferon (Fig. 1, lane 3), but not if it was added 2 h after interferon (Fig. 1, lane 4) or later. These experiments suggest that under the conditions employed, the transcription event induced by interferon which leads to the appear-

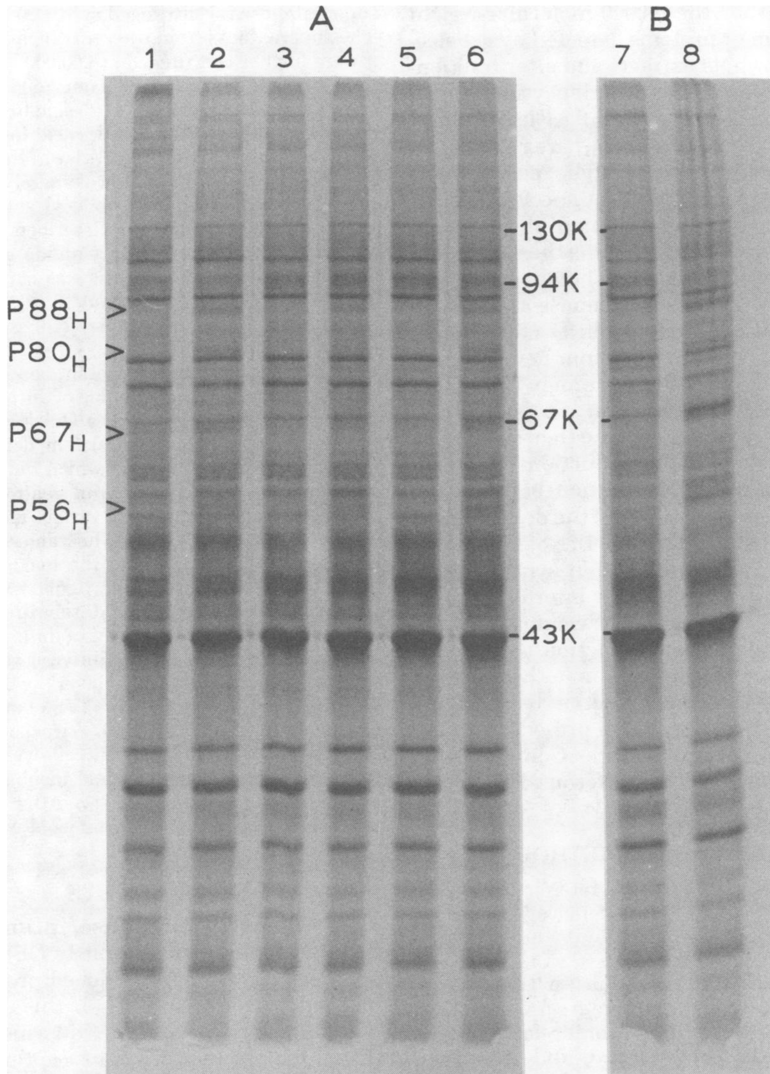

FIG. 1. *(A) Effect of actinomycin D on the appearance of the interferon-induced proteins. FS-4 cells in monolayers (100-mm plates) were treated with human leukocyte interferon (400 reference units per ml), and at varying times (1, 2, and 4 h), actinomycin D (2 μg/ml) was added. At 1 h after the addition of actinomycin D, the cells were washed with growth medium and then incubated in labeling medium (8 ml) containing [^{35}S]methionine (5 μCi/ml) and actinomycin D (0.5 μg/ml) for 18 h at 37°C. S-200 extracts were prepared, and samples containing 1.5 × 10^5 cpm were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described in the text. An autoradiograph of the dried gel is shown. Lane 1, control cells; lane 5, control cells treated with actinomycin D; lanes 2, 3, 4, and 6, cells treated with interferon and either no actinomycin D added (lane 2), or actinomycin D added at 1 h (lane 3), 2 h (lane 4), or 4 h (lane 6) after interferon. The induced proteins are indicated by arrowheads. (B) Induction of various proteins appears to be coordinated. Two monolayers of FS-4 cells were incubated for 4 h at 37°C in labeling medium containing cycloheximide (50 μg/ml), one without and the other with human leukocyte interferon (400 U/ml). Actinomycin D (2 μg/ml) was then added, and after 1 h the cells were washed and incubated in labeling medium containing [^{35}S]methionine (5 μCi/ml) and actinomycin D (0.5 μg/ml) for 18 h at 37°C. Cell extracts (S-200) were prepared, and samples containing 1.5 × 10^5 cpm were fractionated on slab gels as described in the text. An autoradiograph of the dried gel is shown. Lane 7, Cells not treated with interferon; lane 8, cells treated with interferon. The numbers between the gels indicate the positions of molecular weight markers (130K = 130,000 daltons).*

ance of these induced proteins occurs within 2 h after the addition of interferon. In a separate experiment, we determined that actinomycin D added 2 h after the addition of interferon did not affect the establishment of the antiviral effect (data not shown). In these experiments, the medium was changed after actinomycin D treatment; therefore, these results also indicate that once this actinomycin D-sensitive (transcription) event has been induced, the presence of interferon in the medium is no longer required for either the development of the antiviral state or the appearance of the induced proteins.

It is not surprising that the addition of cycloheximide (50 μg/ml), which inhibited the incorporation of [^{35}S]methionine into total protein by 97%, also blocked the appearance of ^{35}S-labeled induced proteins (data not shown). This indicates that the induction of these proteins requires de novo protein synthesis. The possibility remains, however, that these proteins might be synthesized normally in human fibroblasts, but in a different form, and that they might be modified after interferon treatment to give rise to new (induced) bands (for example, due to the synthesis of a certain modifying factor[s] induced by interferon). To test this possibility, cells were prelabeled with [^{35}S]methionine for 18 h in order to label cellular proteins. The cells were then washed and incubated in growth medium for 6 h. The cells were washed again and incubated in the presence of interferon (400 U/ml) for 18 h to test whether any ^{35}S-labeled proteins synthesized in the absence of interferon could be subsequently chased into the induced bands upon interferon treatment. No ^{35}S-labeled induced bands appeared (data not shown). Parallel controls were run and showed that the cells which were treated similarly during prelabeling (except minus the label) retained the capacity to incorporate [^{35}S]methionine into induced proteins when they were treated with interferon (data not shown). Thus, the results indicate that the induced proteins detected are synthesized de novo and that their syntheses require de novo transcription.

Induction of these proteins appears to be coordinated. The fact that the syntheses of several proteins were induced by interferon treatment raises the possibility that as a primary event, interferon may induce the synthesis of only one of them (and for that matter of yet some other totally different protein not included among those which we have detected) as a key component and that the induction of the other proteins may be a secondary event. The following experiment indicates that such a possibility is less likely. FS-4 cells were treated with interferon in the presence of cycloheximide (50 μg/ml) for 4 h. Under these conditions, the interferon treatment would be expected to activate the genetic information which is induced as a primary event; however, protein synthesis would be blocked, and therefore no secondary induction should occur. Actinomycin D was then added to block any subsequent genetic transcription; 1 h later the medium was sucked off, and the cells were washed and incubated in [^{35}S]-methionine-containing medium for 18 h. The analysis of the cell extracts by gel electrophoresis revealed that all four induced proteins were synthesized upon removal of cycloheximide (Fig. 1B, lane 8). Under these conditions, it is less likely that a secondary induction would have occurred. This experiment indicates that the genetic expression of all four proteins appears to be induced in a coordinated manner.

Dependence on interferon concentration and the kinetics of accumulation of the induced proteins. Figure 2 shows an experiment in which two groups of FS-4 monolayers were treated with increasing concentrations of interferon. We determined the relative levels of the induced proteins accumulated in one group (as measured by labeling with [^{35}S]methionine followed by analysis of the cell extracts) and the degree of the antiviral state, as established by VSV yield reduction, in the other group. The results indicate that the accumulation of the induced proteins and the inhibition of VSV yield showed a similar dependence on interferon concentration. The antiviral effect tapered off at 100 U of interferon per ml, and at least three of the induced proteins ($P88_H$, $P80_H$, and $P67_H$) also tended to level off at 100 U/ml.

Figure 3 shows the results of an experiment carried out to study the rate of accumulation of various induced proteins after treatment with interferon. Monolayers of FS-4 cells were treated with interferon and then labeled with [^{35}S]methionine for 2-h periods at varying time intervals; their extracts were prepared and analyzed. Figure 3A shows the gel pattern, and Fig. 3B shows a graphic representation of the relative intensities of various induced bands (as measured by densitometric scanning and expressed as percentages of maximum values). The results show that the synthesis of the induced proteins can be detected within a few hours after the addition of interferon. The induction of $P67_H$ was clearly detected within 1 to 3 h after interferon treatment, peaked at 3 to 5 h, and then declined quite sharply. The inductions of $P56_H$, $P80_H$, and $P88_H$ were detected more clearly within 3 to 5 h, peaked at varying times, and then declined. The accumulations of $P67_H$ and $P56_H$ declined to

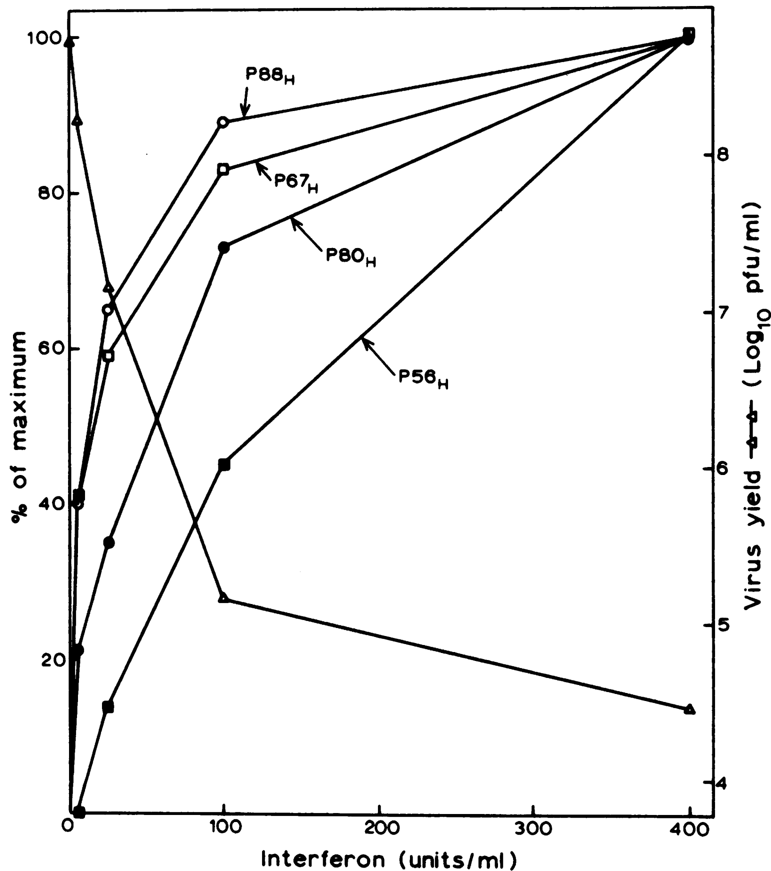

FIG. 2. *Effect of interferon concentration on accumulations of induced proteins and on VSV replication. Two groups of FS-4 monolayers were incubated in labeling medium with increasing concentrations of interferon at 37°C, group 1 in the absence of [35S]methionine and group 2 in the presence of 5 μCi of [35S]methionine per ml. After 18 h, the cells in group 1 were infected with VSV, and the virus yield was determined (Δ). The cells in group 2 were lysed and their S-200 extracts were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The bands of 35S-labeled induced proteins were identified by autoradiography and quantitated by densitometric scanning of the autoradiograph. The values are expressed as percentages of the maximum obtained for each band. For details, see text.*

undetectable levels at 10 and 18 h, respectively, and those of $P80_H$ and $P88_H$ were reduced to approximately 20 and 60%, respectively, of their maximum levels after 18 h of interferon treatment. On the other hand, the incorporation of [^{35}S]methionine into total cellular protein was unaffected. We determined that under these conditions there was no measurable loss of interferon titer in the medium. Thus, this decline occurred despite the fact that the cells were continuously exposed to interferon. This suggests that the induction of these proteins is transient and is apparently subject to further control.

In another experiment, FS-4 monolayers were labeled with [^{35}S]methionine in the presence of interferon for increasing time intervals to analyze the total accumulation of various induced proteins as a function of time. Monolayers in another group were treated similarly with interferon for varying time intervals, and the degree of antiviral state established was determined by infecting with VSV and measuring the virus yield. Figure 4 shows that the accumulation of $P67_H$ reached a maximum by 8 h and that the accumulation of $P56_H$ tapered off at 8 after interferon treatment, whereas the accumulations of $P80_H$ and $P88_H$ continued after 8 h. These results are consistent with the experiment shown in Fig. 3. The degree of antiviral state established also tended to level off at 8 h after interferon treatment (Fig. 4).

We wondered why there was little further decrease in VSV titer (Fig. 4), although the total

accumulation of $P88_H$ and $P80_H$ continued beyond 8 h after interferon treatment. A similar situation was observed in the experiment shown in Fig. 2, where the level of $P56_H$ increased further at 400 U/ml but the decline in virus titer was not much greater than at 100 U/ml. A simple calculation showed that whereas untreated cells produced >500 PFU/cell, in the case of cells treated with 100 U of interferon per ml, the virus titer amounted to <1 PFU/cell. Moreover, we determined the virus titers at varying times after infection of untreated and interferon-treated (400 U/ml) cells. We found that whereas in untreated cells the virus titer increased markedly with time, as expected, in the case of interferon-treated cells the titer remained about the same as it was right after virus adsorption (1.6×10^5 PFU/ml in this experiment). This indicates that the bulk of the virus titer obtained from interferon-treated cells was apparently due to the parental inoculum and that little, if any, viral progeny was produced.

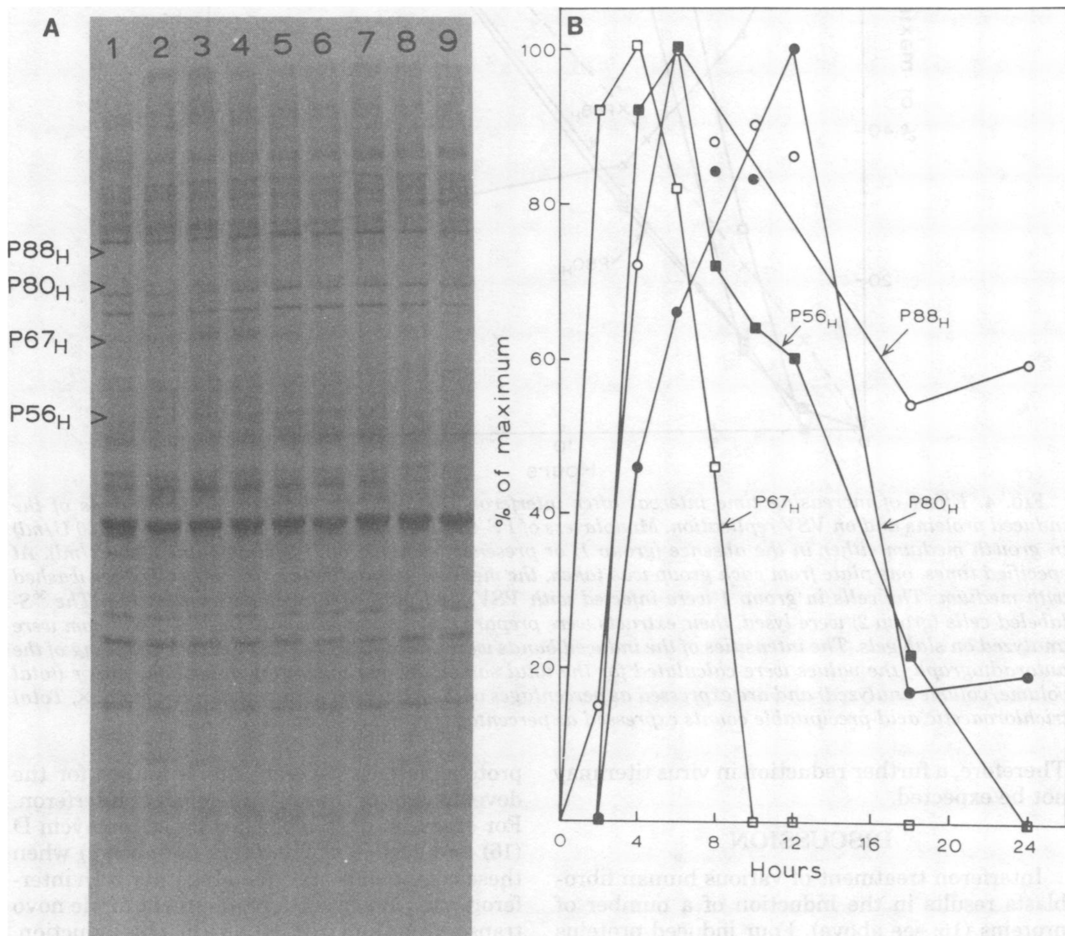

FIG. 3. *Kinetics of accumulation of various induced proteins after interferon treatment. Monolayers of FS-4 cells were incubated in growth medium in the absence or presence of interferon (400 U/ml). At varying times, [^{35}S]methionine (final concentration, 10 μCi/ml) was added, and incorporation continued for 2 h. The medium was then aspirated, and the cells were washed and stored at −80°C. The cell extracts were prepared, and samples containing 1.2×10^4 cpm of each extract were analyzed on slab gels. (A) Fluorograph of the dried gel. Lane 1, Extract of untreated cells labeled for 2 h; lanes 2 through 9, extracts of interferon-treated cells labeled for 2 h at varying times (1, 3, 5, 7, 9, 11, 17, and 23 h, respectively) after the addition of interferon. (B) Relative intensities of the induced bands at different times as determined by densitometric scanning of the fluorograph and expressed as percentages of the maximum obtained for each band. The time points shown represent the medians of the labeling periods (for example, 4 h represents labeling between 3 and 5 h after the addition of interferon).*

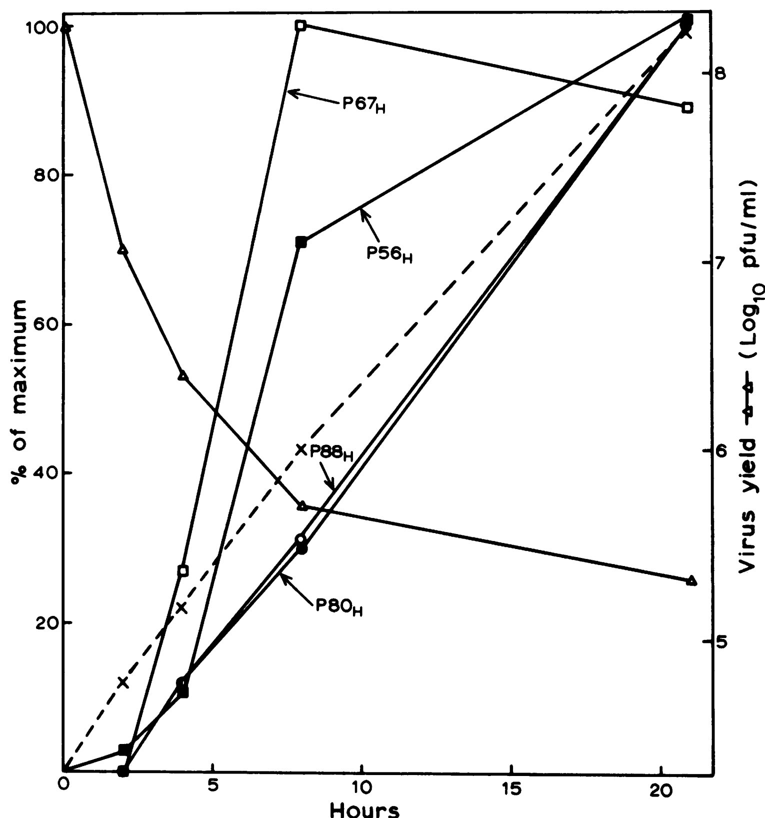

FIG. 4. *Effect of increasing time interval after interferon treatment on the total accumulations of the induced proteins and on VSV replication. Monolayers of FS-4 cells were incubated with interferon (400 U/ml) in growth medium either in the absence (group 1) or presence (group 2) of [^{35}S]methionine (10 μCi/ml). At specified times, one plate from each group was taken, the medium was aspirated, and the cells were washed with medium. The cells in group 1 were infected with VSV, and virus yield was determined (Δ). The ^{35}S-labeled cells (group 2) were lysed, their extracts were prepared, and samples containing 7.4 × 10^4 cpm were analyzed on slab gels. The intensities of the induced bands were quantitated by densitometric scanning of the autoradiograph; the values were calculated for the total sample by multiplying by the volume factor (total volume/volume analyzed) and are expressed as percentages of the maximum obtained for each band.* ×, *Total trichloroacetic acid-precipitable counts expressed as percentages of the value at 21 h.*

Therefore, a further reduction in virus titer may not be expected.

DISCUSSION

Interferon treatment of various human fibroblasts results in the induction of a number of proteins (16; see above). Four induced proteins are detected by direct gel electrophoresis of the S-200 extracts of interferon-treated cells labeled with [^{35}S]methionine, whereas still another protein is detected after fractionation on polyriboinosinic·polyribocytidylic acid-agarose columns followed by gel electrophoresis. Since the four former proteins can be easily quantitated, we analyzed their induction to determine whether this induction correlates with interferon action. The results indicate that the induction of these proteins follows several criteria known for the development of the antiviral effect of interferon. For example, (i) it is blocked by actinomycin D (16) and cycloheximide (data not shown) when these compounds are added together with interferon, thus indicating a requirement for de novo transcription and translation. (ii) This induction, as well as the establishment of the antiviral state, becomes resistant to inhibition by actinomycin D within 2 h after the addition of interferon, thus indicating that under the conditions employed, the transcription event induced by interferon is essentially completed within 2 h. (iii) In agreement with the known species specificity, these proteins are induced by human leukocyte and human fibroblast interferons but not by mouse interferon (16). And (iv) the in-

duction of these proteins and the establishment of the antiviral state show a similar dependence on the concentration of interferon (Fig. 2). These results indicate a correlation between the induction of these proteins and the development of the antiviral state.

Although we routinely used partially purified interferon for these studies, we determined that a highly purified preparation of human leukocyte interferon (specific activity, $\sim 10^8$ reference units per mg of protein; kindly provided by Leo S. Lin and W. E. Stewart II) gave a similar induction of these four protein bands. Furthermore, we observed that similar proteins are induced in several lines of human fibroblasts (16) and osteogenic sarcoma cells (Rubin and Gupta, unpublished data). Moreover, we found an osteogenic sarcoma cell line which did not develop an antiviral state upon treatment with human interferon and also did not show any of the induced bands upon interferon treatment (Rubin and Gupta, unpublished data). This provides support to the conclusion that the induction of these proteins correlates with the development of the antiviral effect of interferon, suggesting that all or some of these proteins may be involved in the antiviral mechanism.

The synthesis of these proteins is induced within a few hours after the addition of interferon (Fig. 3). Figure 1B suggests that their induction is not a sequential process and appears to be coordinated. It is of interest that the rate of accumulation of these proteins reaches a peak which is soon followed by a marked decline, suggesting that the mRNA's for these induced proteins have short half-lives. This may account for the fact that the antiviral state also tends to level off and does not continue to build up. This decline occurred despite the fact that the cells were continuously exposed to interferon, and there was no measurable loss of interferon activity in the medium. This suggests that the interferon-induced synthesis of these proteins is transient and is apparently subject to further control. In this respect, this induction appears to be similar to the induction of interferon itself, which is followed by a shutoff and refractoriness to reinduction (31). The induction of a 56,000-molecular-weight protein observed in chick cells after interferon treatment was also reported to be transient (3).

Interferon treatment has been shown to result in greatly enhanced levels of at least two enzymes, a protein kinase(s) and a 2′,5′-oligoadenylate synthetase. The manner in which the interferon-induced proteins that we have identified may be related to these induced enzyme activities is presently under investigation. Whatever the relationship with these enzymes, the evidence suggests that the appearances of these induced proteins can be used as markers to study the events involved in the induction and regulation of cellular genes by interferon in relation to the development of its biological effects.

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