MOLECULAR AND CELLULAR BIOLOGY, Sept. 1983, p. 1656–1664
0270-7306/83/091656-09$02.00/0

Vol. 3, No. 9

Reduced Synthesis of pp60^{src} and Expression of the Transformation-Related Phenotype in Interferon-Treated Rous Sarcoma Virus-Transformed Rat Cells

STANLEY L. LIN,[1] ELLEN A. GARBER,[1] EUGENIA WANG,[1] LAWRENCE A. CALIGUIRI,[1] HUUB SCHELLEKENS,[2] ALLAN R. GOLDBERG,[1] AND IGOR TAMM[1]*

The Rockefeller University, New York, New York 10021,[1] and Primate Center TNO, Rijswijk, The Netherlands[2]

Received 24 March 1983/Accepted 5 July 1983

Treatment of Rous sarcoma virus-transformed rat cells with rat interferon-α (specific activity, 10^6 U/mg of protein) for 24 h caused a 50% reduction in intracellular pp60^{src}-associated protein kinase activity. *Staphylococcus aureus* V8 protease digestion of pp60^{src}, derived from ^{32}P-labeled monolayer cultures incubated with or without interferon, revealed no differences either in the phosphopeptide pattern or in the phosphoserine-phosphotyrosine ratio. However, [^{3}H]leucine pulse-labeling experiments showed that the synthesis of pp60^{src} was reduced by 42 to 48%, relative to the level of bulk protein synthesis, in the interferon-treated cultures. Rat interferon-α also reduced the growth rate of Rous sarcoma virus-transformed rat cells in a dose-dependent manner over a 72-h period. The decrease in growth rate was accompanied by increases in the thickness and number of actin fibers per cell and by a decline in intracellular tyrosine phosphorylation by pp60^{src}. The results suggest that interferon can inhibit the expression of the transformation-related phenotype by selectively reducing the synthesis of the Rous sarcoma virus transforming gene product. However, the interferon effects on the cytoskeletal organization and proliferation of Rous sarcoma virus-transformed cells may be due at least in part to the predominance of interferon-induced phenotypic changes over those caused by pp60^{src}.

The Rous sarcoma virus (RSV) transforming gene product is a 60,000-molecular-weight phosphokinase, denoted pp60^{src} (15), which catalyzes the transfer of the gamma phosphate group from ATP to tyrosine residues (21). The available evidence strongly suggests that pp60^{src} brings about cellular phenotypic changes, collectively denoted as neoplastic transformation, via its tyrosine phosphokinase activity. These changes include dissolution of actin-containing microfilament bundles in RSV-transformed cells (1, 14, 47). Studies with cell lines transformed with temperature-sensitive mutants of RSV have revealed that changes in the distribution and organization of cytoskeletal proteins are among the earliest effects that occur upon downshift to the permissive temperature. Within 15 min after temperature downshift, a seven- to ninefold increase occurs in the percentage of cells accumulating F-actin into newly formed ruffles at the dorsal surface and peripheral edge (7). At 6 to 12 h after a shift to the permissive temperature, cell rounding occurs with concomitant loss of actin-containing microfilaments (7, 48). The rapidity with which these actin-related cytoskeletal alterations occur suggests that pp60^{src} is directly responsible for some of these changes.

Consistent with this is evidence concerning the intracellular distribution and biochemical properties of pp60^{src}, which indicates an association of pp60^{src} with cell membranes. Fractionation of mammalian cells transformed by RSV has revealed that the bulk of pp60^{src}-associated tyrosine phosphokinase activity is associated with the crude membrane fractions, although the principal location of membrane-associated pp60^{src} varies from nuclear membranes, in the case of a subline of RSV-transformed RR1022 rat cells (24, 25), to plasma membranes, in the case of RSV-transformed NRK cells (13). In RSV-transformed NRK cells, immunofluorescence studies have shown pp60^{src} to be localized in adhesion plaques and adhesion junctions (44). The membrane association of pp60^{src} appears to occur via an amino terminal moiety (24, 26), possibly by a mechanism involving the covalent association of lipid with pp60^{src}, as has been found to occur in RSV-transformed chick embryo cells (19, 43). Recovered avian sarcoma viruses, which display decreased membrane as-

sociation of pp60^{src}, also show reduced tumorigenicity in vivo, suggesting that the membrane association of pp60^{src} is crucial to the transforming ability of RSV (23).

The addition of interferon (IFN) to cells in culture results both in a reduction in cell proliferation and in profound changes in cell morphology and motile behavior (reviewed in references 35, 46). In contrast to the morphological changes induced by RSV in the neoplastic transformation of cells, IFN-treated diploid human fibroblasts become progressively larger and flatter as proliferation declines (34). Consistent with these morphological alterations, IFN-treated fibroblasts show an increase in the organization of actin-containing microfilaments into bundles (36). Moreover, IFN has been found to demote the expression of the transformed phenotype of tumor cells in culture, as reflected in decreases in saturation density (22), growth in semisolid agar (20), and plasminogen activator secretion (40), and in an increase in microfilament organization (9, 11, 36). IFN could exert its antineoplastic effects by causing a reduction in the level of a transforming gene product or its activity. Alternatively, IFN could suppress the transformed phenotype through causing alterations at a cellular site, such as the plasma membrane (33), that is a target of the transforming gene function.

In the present study, we have examined the effects of rat IFN-α on the expression of the pp60^{src} gene in a clonal line, denoted HB, derived from the RR1022 RSV-transformed rat cell line (29). We report that IFN-α treatment of HB cells results in a selective reduction in the synthesis of pp60^{src}, which correlates with reduced levels of pp60^{src}-associated tyrosine phosphokinase activity in IFN-treated cells. Intracellular tyrosine phosphorylation and cell proliferation declined, and the number of cellular actin fibers increased, during IFN treatment.

MATERIALS AND METHODS

Cells and culture materials. The RR1022 RSV-transformed rat cell line was obtained from the American Type Culture Collection (Rockville, Md.) and was originally derived from an explant of a tumor obtained by intramuscular injection of the Schmidt-Ruppin strain of RSV into an Amsterdam rat (29). The HB clonal line used in these studies was derived from the parental RR1022 line by planting an average of one cell per well into a 96-well cluster dish. Cells from a colony, formed in a well originally containing one cell, were transferred to a 25-cm^2 flask and allowed to grow to confluence. The resultant culture was suspended in semisolid agar, and a resultant colony, denoted HB, was isolated and subcultured. Cells were cultured in 25-cm^2 flasks containing 5 ml of reinforced Eagle minimum essential medium supplemented with 10% calf serum. At confluence, cells were detached from the flask with 0.025% trypsin–0.03% EDTA, diluted 1:40 in culture medium, and planted into new 25-cm^2 flasks. The mean doubling time of HB cells is 14 h. In experiments to determine the synthesis and phosphorylation of pp60^{src}, the intracellular level of phosphotyrosine, and the number of actin fibers per cell (see Tables 2 through 5), cells were planted at a density of 1.3×10^3 cells per cm^2, and all assays were performed 96 h after planting. In the IFN-treated cultures, IFN was added at various times after planting to give the desired duration of exposure. In the control cultures, mock IFN was added 24 h after planting. Cultures were handled in this manner so that at the time of the assay they would all be the same age since their last passage, irrespective of the variable lengths of IFN treatment. This was done to minimize any variation that might arise from culture conditions.

Rat IFN was obtained from a spontaneously transformed rat fibrosarcoma cell line (Ratec) by infection with Newcastle disease virus (39). Precipitation of the serum-free harvest medium with 85% ammonium sulfate or 0.02 M zinc acetate yielded IFN preparations with a specific activity of $\sim 10^6$ U/mg of protein, as measured with vesicular stomatitis virus and expressed in internal laboratory units owing to the absence of an international reference standard (39). Rat IFN was further purified by gel filtration on a Sephadex column to a specific activity of $\sim 10^7$ U/mg of protein. Nucleotide sequences of cloned cDNAs of the IFN mRNAs expressed in the Ratec cells, when compared with other known sequences for IFN-α and IFN-β genes, revealed that the IFN used in the present study belonged to the alpha class (H. Schellekens, unpublished data).

Antiserum directed against rat IFN-α was prepared by first injecting rabbits with IFN-α having a specific activity of 10^6 U/mg of protein and then boosting with IFN-α having a specific activity of 10^7 U/mg of protein. For antibody titrations, dilutions of antiserum were mixed with a constant amount of IFN-α (10 U/ml) before assay for virus inhibition. The neutralization endpoint was defined as the highest dilution at which the serum completely neutralized the antiviral activity of IFN (10 U/ml). The neutralization titer was expressed as the reciprocal of serum dilution at the endpoint times 10. The antiserum used in the present study had a titer of 8,000 U/ml. This antiserum did not neutralize IFN derived from mouse L929 cells, which has been shown to contain a mixture of IFN-α and IFN-β (17).

Determination of cell multiplication. HB cells were planted in 25-cm^2 flasks at a density of 2.5×10^4 cells per flask and allowed to attach for 24 h. The next day, mock IFN or rat IFN-α was added directly to the growth medium, and cell multiplication was quantitated by counting cells on photographic negatives of the same culture areas, which were photographed immediately after IFN addition, and at 24-h intervals thereafter, as previously described (45).

Immunoprecipitation of radiolabeled pp60^{src} and quantitation of pp60^{src}. Immunoprecipitation of radiolabeled pp60^{src}, protein kinase assay, and partial proteolysis were performed as previously described (18, 25).

Phosphoamino acid analysis of extracted cellular protein. Phosphoamino acid analysis was performed as described by Hunter and Sefton (21) and quantitated as previously described (18). Briefly, subconfluent monolayers of HB cells, grown in 100-mm dishes,

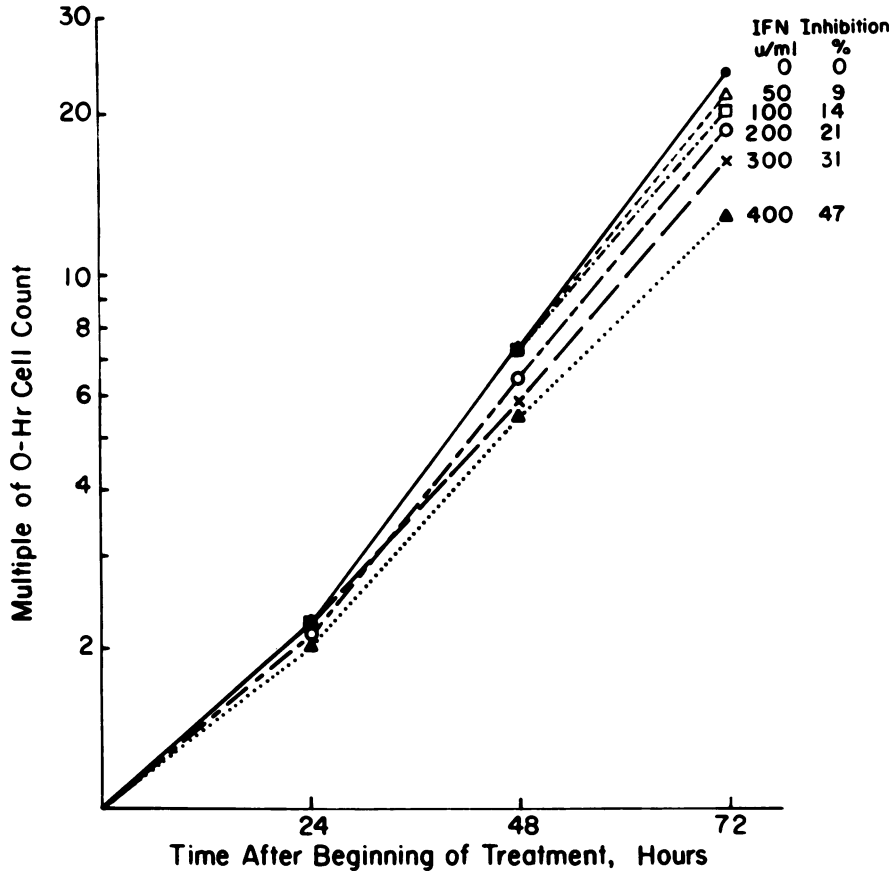

FIG. 1. Inhibition of proliferation of RSV-transformed rat (HB) cells by rat IFN-α. HB cells were planted in 25-cm^2 flasks at ~10^3 cells per cm^2. At 24 h after planting, mock IFN or IFN-α was added directly to the growth medium, and cell counts were made on photographic negatives taken immediately and at 24-h intervals after IFN addition. Curves show results for cells treated with mock IFN (●) and with IFN at 50 (△), 100 (□), 200 (○), 300 (×), and 400 (▲) U/ml.

were incubated for 30 min in 2 ml of phosphate-free Eagle minimal essential medium plus 10% dialyzed calf serum. They were refed with phosphate-free Eagle minimal essential medium plus 10% dialyzed calf serum to which $^{32}P_i$ was added to a final concentration of 1.5 to 2.0 mCi/ml. After incubation for 4 h, monolayers were lysed by the addition of RIPA detergents (25), and the lysates were extracted with phenol, precipitated with ethanol at −20°C, and hydrolyzed with 6 N HCl. The resultant phosphoamino acids were resolved by two-dimensional electrophoresis on thin-layer cellulose plates.

Immunofluorescence microscopy and measurement of actin fibers. Growth medium from cultures of HB cells grown on no. 1 cover slips was removed by aspiration, and a 3.7% solution of formaldehyde, diluted in calcium-magnesium-free phosphate-buffered saline (PBS-DEF), was added. After fixation for 20 min, cells on cover slips were given three 5-min rinses in PBS-DEF followed by two rapid rinses in distilled water and extracted for 2 min at −20°C in acetone. After drying, the cells were stained for 20 min with 7-nitrobenz-2-oxa-1,3-diazolephallacidin (NBD-phallacidin) (132 ng/ml in PBS-DEF) in a humidified air chamber. The cover-slip cultures were then rinsed once in PBS-DEF and mounted in a 2× PBS-DEF–glycerol (1:1) solution. Actin fibers were observed by immunofluorescence microscopy at an excitation wavelength of 460 to 470 nm. IFN-induced alterations in the actin cytoskeleton were quantitated in a blind examination with coded cultures. Cells were evaluated for the abundance of actin fibers by an observer who otherwise was not a participant in the present study. The observer was instructed to enumerate in each cover slip cells containing less than 6, 6 to 10, and more than 10 fibers per cell. Actin fibers at all planes of the cytoplasm were measured by varying the level of focus. Approximately 1,000 cells were scored in each treatment category.

RESULTS

Dose-response curves for IFN-mediated inhibition of cell multiplication. Figure 1 shows that the extent of growth inhibition was directly related

to the concentration of rat IFN-α in the culture medium, with 9% growth inhibition detectable at an IFN concentration of 50 U/ml and maximal growth inhibition (47%) observed at 400 U/ml. The percentage of inhibition refers to the inhibition of the increase in cell number between 0 and 72 h. Thus, at 400 U/ml, IFN treatment prolonged the doubling time by ~36%, as determined between 24 and 72 h from the beginning of treatment. Similar results were obtained in six experiments in which the IFN preparations used had a specific activity of either 10^6 or 10^7 U/mg. Increasing the IFN concentration to 1,600 U/ml did not result in additional growth inhibition beyond that obtained at 400 U/ml, and no differences in growth rate between untreated and mock-treated cultures were observed. The growth-inhibitory effects could be prevented by antibodies directed against rat IFN-α. Therefore, it appears that the growth inhibition observed was due to IFN itself and not to a contaminant in the preparation.

Effects of IFN on intracellular pp60^{src}-associated phosphokinase activity. Experiments were performed to determine whether there was a correlation between the observed reduction in cell multiplication and the intracellular levels of pp60^{src}-associated phosphokinase activity. pp60^{src} was immunoprecipitated with tumor-bearing rabbit serum from cell extracts made from subconfluent monolayers of HB cells that had been exposed to mock IFN or 400 U of rat IFN-α per ml for 24, 48, or 72 h, and the pp60^{src}-associated phosphorylation of the heavy chain of immunoglobulin G was measured as previously described (25). The results were expressed as counts per minute per milligram of cell extract protein from which the pp60^{src} was derived. A 49 to 51% reduction in intracellular pp60^{src} tyrosine phosphokinase activity, relative to total cellular protein, was oberved in the IFN-treated cultures (Table 1). The reduction in phosphokinase activity appeared to be maximal within 24 h after IFN addition and remained unchanged over the ensuing 48 h, despite the continued presence of IFN and increased inhibition of cell proliferation.

Structural analysis of pp60^{src} by using mutants temperature sensitive for transformation has shown that reduced phosphorylation at the major pp60^{src} tyrosine site is correlated with reduced pp60^{src} kinase activity at the nonpermissive temperature (15). Therefore, the possibilities emerge that IFN treatment could reduce the intracellular level of pp60^{src}-associated kinase activity either by causing alterations in the phosphorylated state of pp60^{src} or by causing a selective reduction in the level of pp60^{src} synthesis. To examine these possibilities, a 4-h pulse of $^{32}P_i$ was administered to subconfluent monolayers of HB cells that had previously been exposed to mock IFN or IFN-α for 24, 48, or 72 h. pp60^{src} was specifically precipitated from cell extracts with tumor-bearing rabbit serum and was isolated as a single labeled band after subjecting the immune complexes to SDS-polyacrylamide gel electrophoresis. Differences in phosphorylation pattern were compared by limited proteolysis of the purified pp60^{src} with *Staphylococcus aureus* V8 protease. The procedure yielded four major phosphopeptides: peptides of 34, 21, and 18 kilodaltons derived from the amino-terminal portion of pp60^{src} and containing phosphoserine and a 26-kilodalton peptide from the carboxy-terminal portion of pp60^{src} and containing phosphotyrosine. Identical phosphopeptide gel patterns were observed in the mock-treated and IFN-treated cultures. The ratio of the sum of counts in the 34-, 21-, and 18-kilodalton bands to the counts in the 26-kilodalton band, i.e., the phosphoserine-phosphotyrosine ratio, showed no significant differences between the control cultures and the cultures treated with IFN for up to 72 h (Table 2). The identical phosphopeptide patterns and similar phosphoserine-phosphotyrosine ratios in control and IFN-treated cultures indicate that reduced levels of pp60^{src}-associated phosphokinase activity in IFN-treated cells are not the result of alterations in the phosphorylation pattern of pp60^{src}. However, the total ^{32}P radioactivity incorporated into pp60^{src} was reduced by approximately 40% in the IFN-treated cultures, suggesting that the synthesis of pp60^{src} was reduced.

This possibility was confirmed by isolating pp60^{src} from mock-treated and IFN-treated cultures which had been given a 1- or 4-h pulse of [^{3}H]leucine. In these experiments, radioactivity

TABLE 1. Intracellular pp60^{src}-associated tyrosine phosphokinase activity in control and IFN-treated HB cells[a]

Length of treatment (h)	Phosphokinase activity (^{32}P cpm)		% Reduction
	Control	IFN treatment	
24	65,950	32,290	51
48	62,100	31,630	49
72	65,900	32,950	50

[a] Monolayers of HB cells, mock treated or treated with IFN-α for 24, 48, or 72 h, were lysed in nonionic detergent, and pp60^{src} was specifically precipitated with tumor-bearing rabbit serum. Phosphorylation at a tyrosine residue of the immunoglobulin G heavy chain was quantitated and expressed as total counts per minute per milligram of extracted protein. Standard errors of the percentage reduction values in pp60^{src} kinase activity obtained in these experiments were 4 to 8% of the mean values.

TABLE 2. Intracellular phosphorylation of pp60^{src} in control and IFN-treated cultures[a]

Length of IFN treatment (h)	Amt of phosphoamino acid (cpm/200 μg of total cell extract)		Phosphoserine:phosphotyrosine ratio
	Phosphoserine	Phosphotyrosine	
Control	1,115	385	2.90
24	668	232	2.88
48	650	220	2.95
72	742	261	2.84

[a] Isolated pp60^{src} derived from extracts of control or IFN-treated cells was cleaved with *S. aureus* V8 protease as previously described by Garber et al. (18). The resultant four phosphopeptides, containing either the amino-terminal phosphoserine or the carboxy-terminal phosphotyrosine, were isolated and counted, and the ratio of phosphoserine to phosphotyrosine was calculated.

associated with isolated pp60^{src} and with total trichloroacetic acid-precipitable protein was determined to provide a measure of the synthesis of pp60^{src} relative to the synthesis of total cellular protein. The ratio of counts in pp60^{src} to the counts in total trichloroacetic acid-precipitable protein was reduced by 42 to 48% in the IFN-treated cultures (Table 3). In contrast, bulk protein synthesis in the IFN-treated cultures was reduced by only 20% (Table 3). Like the reduction in kinase activity, the reduction in synthesis of pp60^{src} appeared to be maximal within 24 h after IFN addition. Thus, it appears that the reduction in pp60^{src}-associated kinase activity described above can be accounted for by the selective reduction in the synthesis of pp60^{src}. Furthermore, it should be noted that the synthesis of pp60^{src} was maximally reduced by 24 h from the beginning of IFN treatment, i.e., at a time when growth inhibition was not yet apparent.

Effects of IFN on intracellular tyrosine phosphorylation. Tyrosine phosphorylation in RSV-transformed cells is a highly specific indicator of pp60^{src} activity (21) and is the probable mechanism by which pp60^{src} exerts its transforming effects (41). Therefore, it was of interest to determine the overall level of cellular phosphorylation of amino acids, particularly tyrosine, in proteins of control and IFN-treated cultures. Hydrolysates of total cellular proteins were prepared from extracts of mock-treated or IFN-treated HB cells, and the resultant phosphoamino acids were resolved by two-dimensional electrophoresis on thin-layer cellulose plates by the method of Hunter and Sefton (21). Areas corresponding to phosphotyrosine, phosphothreonine, and phosphoserine were scraped and counted, and the results were expressed for each amino acid as the percentage of total recovered counts. Treatment of subconfluent monolayers of cells with IFN at 400 U/ml caused a significant decline in the level of intracellular tyrosine phosphorylation within 24 h after IFN addition (Table 4). However, the decrease at 24 h was not maximal; instead, the extent of intracellular tyrosine phosphorylation continued to decline with time of IFN treatment and fell to a level 50% of the control value by 72 h after IFN addition.

IFN-induced alterations in the actin cytoskeletons of HB cells. HB cells displayed a flattened, spread form in the IFN-treated cultures, which contrast with the phenotypic features characteristic of transformation by RSV. This raised the possibility that microfilament organization may be altered after IFN treatment of HB cells, as has previously been observed in other cell systems (9, 11, 36, 49). We have investigated the kinetics of induction and extent of alterations in the actin cytoskeleton after IFN treatment. The actin fibers were visualized by NBD-phallacidin staining of fixed cells (2) at various times after IFN addition. For the purpose of quantitating IFN-induced changes, three categories of cells were distinguished: (i) cells containing fewer than six actin fibers spanning the length of the cell (Fig. 2A); (ii) cells containing 6 to 10 fibers per cell (Fig. 2B); and (iii) cells with more than

TABLE 3. Synthesis of pp60^{src} in mock-treated and IFN-treated HB cultures[a]

Length of IFN treatment (h)	Protein synthesis (cpm)		pp60^{src}: cell extract ratio ($\times 10^{-4}$)	% Reduction in pp60^{src} synthesis
	pp60^{src}	Total protein[b]		
Control	259.4	364,200	7.12	0
24	120.8	290,300	4.16	42
48	115.2	298.900	3.85	48
72	122.4	295,200	4.14	42

[a] pp60^{src} was specifically immunoprecipitated from extracts of monolayers given a 4-h pulse of [^{3}H]leucine. Immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis, and pp60^{src} was isolated as a single band before counting.

[b] Total cell proteins were precipitated with ice-cold 10% trichloroacetic acid. The extracts were the same as those from which the pp60^{src} was derived.

TABLE 4. Phosphoamino acid analysis of extracts of control and IFN-treated cells[a]

Length of IFN treatment (h)	Phosphoamino acid	Amt (cpm)	% of total recovered counts
Control	Phosphotyrosine	4,196	2.59
	Phosphothreonine	8,304	5.04
	Phosphoserine	151,983	92.37
24	Phosphotyrosine	2,658	1.97
	Phosphothreonine	7,798	5.69
	Phosphoserine	126,822	92.44
48	Phosphotyrosine	3,520	1.47
	Phosphothreonine	15,009	6.17
	Phosphoserine	208,536	92.36
72	Phosphotyrosine	6,020	1.29
	Phosphothreonine	36,702	7.07
	Phosphoserine	395,384	91.64

[a] Phenol extraction was performed on clarified extracts of control and IFN-treated cells. The extracted proteins were hydrolyzed in 6 N HCl, and phosphoamino acids were separated by two-dimensional chromatography on cellulose plates.

10 fibers (Fig. 2C). Table 5 shows that mock-treated controls contained cells in all three categories, but a large majority (85.6%) of the cells contained only five fibers or fewer (category 1). By 12 h after IFN addition, the fraction of cells with 0 to 5 fibers had decreased to 63%, and there were concomitant substantial increases in cells with 6 to 10 or >10 fibers. With continued IFN exposure, there occurred a gradual, progressive shift of the cell population until by 72 h nearly 75% of the cells contained more than five actin fibers.

IFN treatment also altered the appearance of adhesion plaques, the focal points of cell-substratum contact. As visualized by NBD-phallacidin staining, the adhesion plaques in untreated stationary HB cells were prominent and predominantly located beneath the nucleus (Fig. 2A), whereas in treated cells the plaques were less prominent and were not concentrated in the nuclear region (Fig. 2B and C). In cells with many long, thick actin fibers, the adhesion plaques might be obscured in part by the fibers (Fig. 2C). However, it is likely that in such cells, the individual plaques are probably smaller than those in cells lacking fibers or possessing very few fibers (Fig. 2A).

In HB cells (J. G. Krueger, S. L. Lin, A. R. Goldberg, and I. Tamm, unpublished data) as well as in RSV-transformed NRK cells (44), some of the pp60^{src} is localized in adhesion plaques, which also contain α-actinin, vinculin, and gelsolin in addition to actin (44; E. Wang et al., unpublished data). It has been suggested that the association of pp60^{src} with adhesion plaques plays a role in neoplastic transformation (38). In HB cells engaged in active locomotion, the predominant location of the prominent adhesion plaques changes from the central region of the cell to the leading edge, which indicates the dynamic state of adhesion plaques in cells. Our findings indicate that, upon IFN treatment, a reorganization of adhesion plaques occurs concomitantly with the formation of actin fibers in RSV-transformed rat cells.

DISCUSSION

Neoplastic transformation of fibroblasts by RSV results from the expression of the viral *src* gene whose product, denoted pp60^{src}, has been shown to be a tyrosine phosphokinase (21). Available evidence suggests that it is the tyrosine phosphokinase activity of pp60^{src} that is responsible for the transforming effect of RSV (41). Although the mechanism by which pp60^{src} exerts its transformation is unclear, studies utilizing mutants of RSV have indicated a correlation between tyrosine phosphorylation of vinculin, loss of actin-containing stress fibers, and the ability to grow in soft agar (38, 42). This suggests that the altered growth characteristics of transformed cells result partly from alterations in the cytoskeleton brought about via a mechanism involving the phosphorylation of vinculin at tyrosine residues. Although effects on the cytoskeleton might not be a direct consequence of pp60^{src} action, the fact remains that the dissolution of actin fibers is a consequence, be it direct or indirect, of *src* gene expression.

We have found that in IFN-treated cells the intracellular level of pp60^{src}-associated phosphokinase activity drops to one-half of the control level within 24 h from the beginning of treatment, which can be largely attributed to a selective decrease in the synthesis of pp60^{src} relative to the synthesis of cellular proteins in bulk. These results are surprising in view of previous studies, which have shown that IFN treatment of murine cells exogenously or endogenously infected by murine leukemia virus does not affect either the transcription of the viral genome or the synthesis of viral proteins, although virus release is inhibited (5, 6, 16, 37). Furthermore, Oxman and colleagues have demonstrated that the pretranslational mechanisms responsible for simian virus 40 T antigen expression are sensitive to IFN-mediated inhibition when the T antigen mRNA is derived from independent simian virus 40 genomes but are resistant to inhibition when the genetic information for simian virus 40 T antigen is incorporated into the host cell genome, as in the case of transformed cells, or into an adenovirus

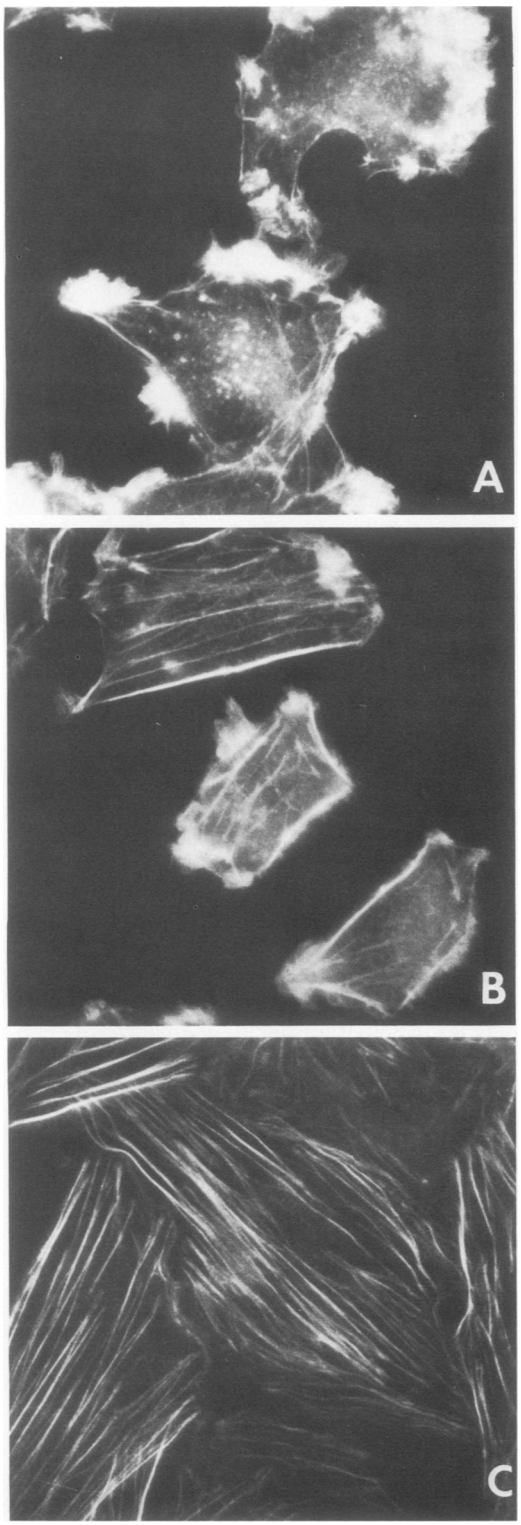

FIG. 2. Actin-containing cytoskeletons in control and IFN-treated HB cells. Photographs of cells representative of the three categories of cytoskeletal organization defined in the text: (A) cells with fewer than 6 fibers per cell, (B) cells with 6 to 10 fibers per cell, and (C) cells with more than 10 fibers per cell. Cells in (A) were not treated with IFN, whereas cells in (B) and (C) had been treated with IFN at 400 U/ml for 3 days. F-actin was stained with NBD-phallacidin as described in the text.

TABLE 5. Distribution of cells within various categories defined by the organization of the actin-containing cytoskeleton

Length of IFN treatment (h)	% of cells with no. of actin-containing fibers:		
	0–5	6–10	>10
Control	85.6	10.1	4.3
12	62.6	29.1	8.3
24	59.4	32.3	8.3
48	52.0	27.6	20.4
72	24.5	58.3	17.2

genome, as in adenovirus-simian virus 40 hybrid viruses (30, 31, 32). Thus, at present there is no apparent parallel in other viral systems to our finding of suppression of the synthesis of a transforming gene product by IFN treatment. On the other hand, selective inhibition of specific cellular enzymes has been previously observed in cells after IFN treatment. For example, dexamethasone induction of tyrosine aminotransferase is reduced much more than bulk protein synthesis after IFN treatment of rat hepatoma tissue culture cells (3), and IFN pretreatment of chick retinal tissue prevents the induction of glutamine synthetase by hydrocortisone without affecting the levels of lactate dehydrogenase and acetylcholine esterase (28). Thus, the effect of IFN treatment on the expression of $pp60^{src}$ in RSV-transformed rat cells appears to be analogous to its effects on the expression of certain normal cellular genes.

We have evaluated $pp60^{src}$ function in IFN-treated cells by using two parameters, i.e., by measuring intracellular tyrosine phosphorylation and by enumerating fibers (bundles) of actin-containing microfilaments. In contrast to the rapid and maximal reduction in $pp60^{src}$-associated phosphokinase within 24 h after IFN addition, the decrease in tyrosine phosphorylation in the treated cells appears to be more gradual, as indicated by 24, 43, and 50% reductions in the phosphotyrosine fraction of total phosphoamino acid-associated radioactivity at 24, 48, and 72 h after IFN addition and labeling with ^{32}P for 4 h. It may be noted that the decrease (50%) in tyrosine phosphorylation that occurs upon IFN treatment is much less than the

nearly complete inhibition observed in cells transformed by temperature-sensitive mutants of RSV upon shift to the nonpermissive temperature. Although tyrosine phosphorylation occurs on several proteins in RSV-transformed cells (4, 12, 27), the tyrosine phosphorylation relevant to growth or neoplastic transformation may comprise only a small fraction of the total phosphorylation of tyrosine residues.

IFN treatment also increases the abundance and thickness of actin fibers. We have observed changes as early as 12 h after IFN addition, and we found a progressive increase in the frequency of cells containing more than five fibers each throughout the 72-h period following IFN addition. This progressive change qualitatively parallels the reduction in tyrosine phosphorylation in IFN-treated cells. With the appearance of increased numbers of actin fibers, there is a concomitant decrease in the number of prominent adhesion plaques. Previous studies have shown that IFN treatment of normal untransformed human cells also increases both the number and thickness of actin fibers (36). Therefore, the possibility exists that the increase in actin fibers which we have observed after IFN treatment of RSV-transformed rat cells is not the result of decreased intracellular levels of pp60^{src} phosphokinase activity but rather reflects the ability of IFN to induce the formation of actin fibers by a mechanism that predominates over the expression of phenotypic changes in the cytoskeleton.

As for the mechanism of growth inhibition of RSV-transformed rat cells by rat IFN-α, at least two possibilities need be considered. First, IFN could inhibit cell cycling by selectively inhibiting the expression of certain genes critical for cell proliferation, such as the calmodulin gene (8, 10). Second, proliferation might be inhibited in consequence of the increased organization of actin filaments, which may affect the distribution and function of proliferation-related surface proteins in cells or may impede cycling through physical effects (45, 46). Present data do not enable us to distinguish between these possibilities. It will be of interest to determine the precise mechanism whereby IFN treatment decreases the level of pp60^{src} in cells, to explore the possible role of the cytoskeleton in the regulation of pp60^{src}-catalyzed tyrosine phosphorylation in vivo, and to delineate the role of the cytoskeleton in cell proliferation.

ACKNOWLEDGMENTS

This work was supported by U.S. Public Health Service research grants CA18608, CA13362, and AG03020 and by Biomedical Research Support grant SO7 RR07065, awarded by the National Institutes of Health. Rat IFN production was sponsored by a grant from the Koningin Wilhelmina Fund. L.A.C. was an American Cancer Society Scholar supported by grant SG-114 from the American Cancer Society. E.A.G. was the recipient of postdoctoral fellowship CA06664 awarded by the National Institutes of Health. S.L.L. was a postdoctoral fellow under training grant AI07233 awarded by the National Institutes of Health.

We appreciate the assistance of Lawrence Huang in counting actin fibers and of Amy Berg in preparing photographs.

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