Posttranscriptional changes in growth factor-inducible gene regulation caused by antiproliferative interferons

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Growth factors stimulate quiescent fibroblasts to progress through G₀/G₁, in part by inducing the expression of genes whose products are necessary or permissive for cell proliferation. Interferons, by contrast, inhibit progress through G₀/G₁ by mechanisms that are poorly understood. We show, in BALB/c murine 3T3 fibroblasts (A31 cells), that α / β -interferon (IFN) had no effect the growth factor-dependent induction of several messenger ribonucleic acids (mRNAs), including those encoding ornithine decarboxylase (*odc*), fibronectin and the *c-fos* and *c-myc* protooncogenes. However, IFN caused an abnormal accumulation of fibronectin and *c-myc* mRNA on polysomes and markedly increased the stability of *c-myc* mRNA. Moreover, despite high, induced levels of mRNA, IFN inhibited the serum-stimulated rise in *odc* enzyme activity and the increased rate of fibronectin protein synthesis. By contrast, IFN had no effect on *c-fos* protein synthesis, nor did it affect the synthesis of most, but not all, proteins detectable by two-dimensional gel electrophoresis. The data suggest IFN inhibits proliferation by suppressing the expression of a subset of growth factor-inducible genes through a selective, posttranscriptional mechanism.

Introduction

The proliferation of differentiated, higher eukaryotic cells is controlled largely by extracellular factors that exert both positive and negative regulation. Polypeptide growth factors are important positive growth regulators. Growth

factors bind to specific cell surface receptors, and ligand binding generates one or more intracellular signals, which in turn induce a number of nuclear and cytoplasmic changes. Growth factors regulate proliferation, principally by stimulating cells to progress through the pre-replicative (G₀ or G₁) phases of the cell cycle. For many cell types, once deoxyribonucleic acid (DNA) replication is initiated, the cell is committed to complete the remainder of the cycle through mitosis (Baserga, 1985).

In vivo, fibroblasts exist primarily in a quiescent growth state (G₀); when stimulated by specific growth factors, they progress through the cell cycle and divide. In culture, fibroblasts enter G₀ when deprived of growth factors or grown to confluence and resume proliferation when given fresh, growth factor-containing medium. Growth factors induce a marked, and often rapid, increase in the messenger ribonucleic acids (mRNAs) for several genes as cells progress from G₀ into S phase. Growth factor-inducible genes include metabolic enzymes (e.g., ornithine decarboxylase), extracellular matrix or cytoskeletal proteins (e.g., fibronectin or actin), and putative growth regulators (e.g., the *c-fos* and *c-myc* protooncogenes) (Baserga, 1985; Denhardt *et al.*, 1986). The translation products of these genes are presumed necessary or permissive for the initiation of DNA synthesis.

In vivo, the effects of growth factors must be tempered to prevent the overgrowth of cells. Several polypeptide growth inhibitors have been identified which, like polypeptide growth factors, bind to cell surface receptors, generating intracellular signals that alter nuclear and cytoplasmic events. Among these are interferons (IFNs), a family of proteins first recognized for their antiviral activity (Lengyel, 1982; Pestka *et al.*, 1987). IFNs modulate many cell functions, particularly within the immune system, and inhibit the proliferation of several cell types, both in vivo and in culture. In quiescent fibroblasts stimulated by growth factors, α - or β -IFN delays the onset of DNA synthesis (Taylor-Papadimitriou, 1985; Lin *et al.*, 1986; Tamm *et al.*, 1987). Thus, IFN dampens and opposes the action of some growth factors. The mechanisms by which

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IFN antagonizes positive growth signals are poorly understood.

IFNs induce a plethora of biochemical and metabolic changes, depending on the type of IFN and the target cell. Two well-characterized effects of IFNs are known to be important for the antiviral activity: 1) IFNs activate a cellular ribonuclease that degrades viral mRNAs and 2) IFNs inactivate the eukaryotic initiation factor 2 (eIF-2), which is needed for viral protein synthesis (Lengyel, 1982; Pestka *et al.*, 1987).

Whether and how the antiviral mechanisms contribute to the antiproliferative effects of IFNs are unclear. For example, IFN reduced the level of *c-myc* mRNA prior to arresting the growth of a continuously proliferating lymphoid tumor cell line (Daudi Burkitt's lymphoma cells). In these cells, α -IFN inhibited *c-myc* transcription (Einat *et al.*, 1985a), but β -IFN acted posttranscriptionally, possibly through ribonuclease activation (Knight *et al.*, 1985). The effects of IFN on the Daudi cells may be unique, however, because in other lymphoid tumor cells, IFNs inhibited proliferation without affecting *c-myc* mRNA (Einat *et al.*, 1985a; Dron *et al.*, 1986). In fibroblasts, IFNs have been reported to slightly increase (Tominaga and Lengyel, 1984) or decrease (Einat *et al.*, 1985b) *c-myc* and *c-fos* mRNA in quiescent cells stimulated by platelet-derived growth factor (PDGF).

Here, we describe how α/β -IFNs affect the expression of several growth-related genes in cultured nontumorigenic murine 3T3 fibroblasts. IFN did not affect the induction of most growth factor-inducible mRNAs, but for selected mRNAs it did alter the polysome distribution and inhibit expression at a posttranscriptional site. Our results suggest that IFNs may exert translational control over selected, growth factor-inducible genes and may thus act to retard the proliferative response to growth factors.

Results

Antiproliferative and antiviral activities of α/β -IFN in murine fibroblasts

We used for our studies A31 cells, a subclone of BALB/c murine 3T3 fibroblasts, because their proliferative behavior, growth factor requirements and pattern of gene expression have been well characterized (Campisi *et al.*, 1984; Ran *et al.*, 1986; Lu *et al.*, 1989). We first determined the antiproliferative and antiviral effects of IFN in these cells.

Subconfluent cells were made quiescent by serum deprivation and stimulated in the absence (control) or presence of 100 U/ml of murine IFN (α/β mixture) with fresh medium con-

taining 10% serum. The onset of DNA synthesis was followed by autoradiography of [³H]thymidine-labeled nuclei. In control cultures, the first cells initiated DNA synthesis 10–12 h after serum stimulation. By contrast, IFN-treated cells initiated DNA synthesis ~16 h after stimulation. Thus, IFN delayed the onset of S phase by 4–6 h (Figure 1A), in agreement with other studies (Taylor-Papadimitriou *et al.*, 1985; Lin *et al.*, 1986; Tamm *et al.*, 1987). The duration of the delay was not affected by higher concentrations of IFN (up to 1000 U/ml) or pretreatment (up to 24 h) (data not shown). The antiviral activity of IFN was assessed by the ability to protect a cell monolayer from lysis by vesicular stomatitis virus. The dose response for inhibition of cellular DNA synthesis and inhibition of viral replication were very similar, showing near maximal effects at 20 U/ml (Figure 1, B and C). IFN was used at 100 U/ml for subsequent experiments.

Effects of IFN on serum-inducible mRNAs

To explain the delay in DNA synthesis, we asked whether IFN delayed or suppressed the induction of growth factor-inducible mRNAs. Quiescent cells were stimulated with serum in the absence or presence of IFN, and the level of nine mRNAs was assessed at various times thereafter (Figure 2). These mRNAs encoded a replication-dependent histone, 3.2; two cytoskeletal proteins, actin and tubulin; the two metabolic enzymes, ornithine decarboxylase (*odc*) (the first and rate-limiting step in polyamine biosynthesis) and major excreted protein (MEP) (a lysosomal protease); the extracellular matrix protein fibronectin; and three protooncogenes, *c-fos*, *c-myc*, and *c-ras-Ha*.

IFN delayed the induction of one of these mRNAs, the one that encodes histone 3.2. It is well established, in these and other cells, that the replication-dependent histones are synthesized only during S phase and that the mRNAs for these proteins are induced nearly coincident with the onset of DNA synthesis (Rickles *et al.*, 1982; DeLisle *et al.*, 1983; Heintz *et al.*, 1983). It was therefore not surprising that IFN delayed the histone 3.2 mRNA induction in parallel with the delayed initiation of DNA replication.

By contrast, IFN neither delayed nor suppressed the serum-dependent induction of the eight other mRNAs that we studied. Actin, tubulin, fibronectin, *odc*, MEP, *c-fos*, *c-myc*, and *c-ras-Ha* mRNAs are all relatively low in quiescent cells, and all are induced by mitogenic growth factors, although to different extents and with different kinetics (Farmer *et al.*, 1983;

Figure 1. Antiproliferative and antiviral activities of murine α/β -IFN in A31 cells. (A) 2×10^4 cells were plated onto 2-cm² wells in 10% serum and, after 24 h, were made quiescent by shifting to 0.4% serum for 48 h. Quiescent cells were stimulated with fresh medium containing 10% serum and [³H]thymidine without (solid line) or with (broken line) 100 U/ml IFN. At the indicated times, radiolabel incorporation was stopped and the cells were processed for autoradiography. (B) Quiescent cells were given serum, [³H]thymidine and the indicated concentrations of IFN. After 16 h, when 45% of the control cells had initiated DNA synthesis, the cells were processed for autoradiography. (C) Cells were grown to confluence on glass coverslips and were mock-infected or infected with VSV in the presence of the indicated concentrations of IFN, fixed and stained to visualize the integrity of the monolayer as described in Methods. In the absence of IFN, VSV caused extensive cell lysis and destruction of the monolayer visible as clear areas on the coverslip.

Campisi *et al.*, 1984; Greenberg and Ziff, 1984; Muller *et al.*, 1984; Frick *et al.*, 1985; Blatti *et al.*, 1988; Lu *et al.*, 1989).

For all eight mRNAs, the initial induction by serum was unaffected by IFN; however, five showed a slight or unusual change in expression. First, actin and tubulin mRNA accumulated to slightly lower levels (about twofold) in the presence of IFN. By contrast, *odc*, *c-myc*, and fibronectin mRNAs accumulated to higher than normal levels in the presence of IFN. These mRNAs normally decline in abundance ~ 8 h after the peak of induction. IFN markedly attenuated the decline. *c-myc* mRNA showed the greatest sensitivity: 16 h after serum stimulation, *c-myc* mRNA was 8- to 10-fold higher in cells that were stimulated in the presence of IFN.

IFN alone, when added to quiescent cells for 6 or 16 h, had little or no effect on the mRNAs

under study, although in some experiments, IFN induced a small (twofold) increase in the level of *c-myc* mRNA (see Figure 3A). In addition, all of the above results were essentially the same whether or not quiescent cells were pretreated with IFN for up to 16 h prior to serum stimulation (Figures 2 and 3 and data not shown). Finally, the effects of either α - or β -IFN alone (100 U/ml each) were similar to the effects of the α/β mixture: the serum-dependent induction of *c-myc* mRNA was unaltered, and each IFN alone caused *c-myc* mRNA to accumulate to higher than control levels 16 h after stimulation (data not shown).

We conclude that, with the exception of histone 3.2 mRNA, IFN neither delays nor appreciably suppresses the induction of several serum-inducible mRNAs, including the mRNAs for three protooncogenes. Moreover, IFN caused some of these mRNAs, particularly

Figure 2. IFN does not inhibit the serum-dependent induction of eight growth factor-inducible mRNAs. 5×10^5 cells were plated onto 150-mm culture dishes in 10% serum and, after 3 d, were made quiescent by shifting to 0.4% serum for 48 h. Quiescent cells were pretreated with IFN for 16 h (Q,I) or not (Q) and then given fresh medium containing 10% serum without (S) or with (S,I) IFN. At the indicated times, RNA was isolated and analyzed for the abundance of *c-fos*, ornithine decarboxylase (ODC) *c-myc*, fibronectin (FBN), *c-ras-Ha*, major excreted protein (MEP), β -tubulin (TUB), β -actin (ACT) and histone 3.2 (HIS) mRNA as described in Methods. In these autoradiograms, laser densitometry showed that 16 h after stimulation *c-myc* mRNA was 9.7-fold higher in IFN-treated cells, whereas actin mRNA was 1.9-fold lower in IFN-treated cells.

c-myc mRNA, to accumulate to substantially higher than normal levels.

Effect of IFN on *c-myc* mRNA induction by purified growth factors

Serum contains many factors, and *c-fos*, *c-myc*, and certain other mRNAs can be induced by at least two, independent growth factor signalling pathways in 3T3 cells (Ran *et al.*, 1986; McCaffrey *et al.*, 1987; Nepveu *et al.*, 1987). We therefore asked whether IFN affected *c-myc* expression when the mRNA was induced by a single pathway.

Quiescent cells were stimulated with purified growth factors in the absence or presence of IFN for 2 or 16 h and the abundance of *c-myc* mRNA was measured. The growth factors that we used were 1) PDGF and the phorbol ester tumor promoter tetradecanoylphorbol acetate (TPA), which utilize a signalling mechanism that

is dependent on protein kinase C (Nishizuka, 1984), and 2) epidermal growth factor (EGF), which, in 3T3 cells, utilizes a signalling mechanism that is independent of protein kinase C and dependent on cAMP (Ran *et al.*, 1986; McCaffrey *et al.*, 1987).

PDGF, TPA, EGF, and serum each induced *c-myc* mRNA equally well in the absence or presence of IFN (Figure 3A). In addition, IFN caused overexpression of *c-myc* mRNA within 16 h after stimulation, whether the cells were stimulated by serum, EGF (Figure 3B), or TPA (Figure 3C). Identical results were obtained whether or not the cells were pretreated with IFN for 16 h.

We conclude that IFN does not impair the ability of either growth factor signalling pathway to induce *c-myc* mRNA and that *c-myc* mRNA accumulates to abnormally high levels in the presence of IFN, regardless of the pathway responsible for its induction.

Figure 3. IFN does not inhibit *c-myc* mRNA induction by growth factors. (A) Cells were made quiescent as described in the legend to Figure 2 (serum-deprived) or by maintenance in 10% serum, feeding every 2–3 d, until they reached confluence. Quiescent cells were untreated (Q) or given IFN (Q,I) for 16 h. Serum-deprived cells, which are responsive to serum or EGF but not to TPA (Ran *et al.*, 1986), were given fresh medium containing 10% serum or 10 ng/ml EGF without (S or E) or with (S,I or E,I) IFN. Confluent cells, which are responsive to serum, PDGF or TPA, but not to EGF (Ran *et al.*, 1986), were given fresh medium containing 40 U/ml PDGF or 10^{-7} M TPA in the absence (P or T) or presence (P,I or T,I) of IFN. After 2 h, RNA was isolated and analyzed for the abundance of *c-myc* mRNA. (B and C) Cells were made quiescent and stimulated as described above. RNA was isolated after 16 h and analyzed for the abundance of *c-myc* mRNA.

Stabilization and accumulation of fibronectin and *c-myc* mRNA on polysomes

IFN inhibits the initiation of translation in virus-infected cells (see Lengyel, 1982; Pestka *et al.*, 1987). We therefore asked whether the elevated levels of mRNA in IFN-treated cells was due to an accumulation in translational initiation complexes or light polyribosomes.

Quiescent cells were stimulated with serum in the absence (control) or presence of IFN for

14 h. A cytoplasmic extract containing polyribosomes was then prepared and fractionated on sucrose gradients. RNA was purified from the gradient fractions and analyzed for the abundance of *c-myc*, fibronectin, and actin mRNA.

In extracts from control cells, all three mRNAs were most abundant in the heavy fractions of the gradient (Figure 4). This is consistent with the expectation that these mRNAs are trans-

Figure 4. IFN alters the polysome distribution of *c-myc* and fibronectin mRNA. Cells were made quiescent as described in the legend to Figure 2, given no additions or IFN for 6 h and then stimulated with 10% serum for 14 h in the absence (SER) or presence (SER, IFN) of IFN. Cell lysates were prepared and fractionated on sucrose gradients as described in Methods. Light and heavy fractions, collected from the top and bottom of the gradient, respectively, are indicated. RNA was purified from each fraction and equal volumes from each fraction were analyzed for the abundance of actin (ACT), *c-myc* and fibronectin (FBN) mRNA.

Figure 5. IFN stabilizes *c-myc* mRNA. Serum-deprived cells (Q), prepared as described in the legend to Figure 2, were stimulated with fresh medium containing 10% serum without (S) or with IFN (S,I). After 2, 8, or 16 h, 5 μ g/ml actinomycin D (AMD) was added directly to the culture medium for 30 or 60 min; cytoplasmic RNA was isolated and analyzed for *c-myc* or actin mRNA.

lated in control cells and therefore are associated with many ribosomes; hence, they are found in the heavy polysome fractions of the gradient.

In extracts from IFN-treated cells, actin mRNA was generally lower across the gradient, relative to control extracts. This is consistent with the 2-fold decline in actin mRNA shown in Figure 2, although densitometry of the autoradiogram showed that there was an even greater decline in the actin mRNA associated with polysomes in IFN-treated cells (the range was 4- to 6-fold, except for the heaviest fraction which was >10-fold). However, the polysome distribution of actin mRNA was not altered by IFN and, thus, the actin mRNA on polysomes in IFN-treated cells remained associated predominantly with the heavy polysome fractions (Figure 4). By contrast, IFN caused a marked shift in the polysome distribution of fibronectin and *c-myc* mRNA.

Fibronectin mRNA was most prevalent in the light polysome fractions in extracts prepared from IFN-treated cells, whereas it was most prevalent in the heavy polysome fractions in extracts from control cells (Figure 4). This shift in distribution toward light polysomes is consistent with an IFN-induced accumulation of fibronectin mRNA in initiation complexes; we did not, however, attempt to further characterize the light gradient fractions containing fibronectin mRNA.

Surprisingly, IFN caused an accumulation of *c-myc* mRNA in all gradient fractions (Figure 4). This distribution is similar to the effects of cycloheximide, which blocks translational elongation (Vasquez, 1979). We have shown in A31 cells that cycloheximide increases cytoplasmic *c-myc* mRNA, not by increasing transcription, but by

stabilizing the otherwise labile *c-myc* mRNA; we speculated that accumulation of *c-myc* mRNA on polysomes might render it resistant to ribonuclease attack (Dean *et al.*, 1986).

Indeed, we found a marked stabilization of *c-myc* mRNA in IFN-treated cells (Figure 5). Quiescent cells were stimulated with serum for 2, 8, or 16 h in the absence (control) or presence of IFN. After stimulation, the cells were given actinomycin D, an inhibitor of transcription, for 30 or 60 min. Cytoplasmic RNA was then isolated and analyzed. In control cells, *c-myc* mRNA decayed with a half-life of ~20 min at all times after serum stimulation. In IFN-treated cells, the half-life of *c-myc* mRNA was near normal 2 h after serum stimulation, but had lengthened considerably (>1 h) by 8 and 16 h after serum stimulation.

Taken together, the data suggest that IFN might affect the translation of growth factor-inducible genes.

Effects of IFN on protein synthesis

To directly test the idea that IFN inhibits the translation of cellular mRNAs, we measured the effect of IFN on the rates of accumulation or synthesis of several cellular proteins.

First, the rate of overall protein synthesis was estimated from the incorporation of [3 H]leucine into acid-insoluble material. Four hours after quiescent cells were stimulated with serum, the rate of overall protein synthesis rose, increasing about threefold over the ensuing 12 h. IFN did not affect the basal rate of protein synthesis in quiescent cells. However, IFN partially inhibited, by 60–70%, the serum-dependent rise in protein synthesis, in agreement with the findings of others (Taylor-Papadimitriou, 1985; Lin *et al.*,

1986; Tamm *et al.*, 1987). This inhibition was prevented by antibodies to α - and β -IFN (data not shown).

We next determined the extent to which IFN inhibited the synthesis of specific proteins, particularly those whose corresponding mRNA we had shown was unaffected or elevated by IFN. We focused on three genes whose proteins could be readily assayed by immunoprecipitation or enzymatic activity: *c-fos*, *odc*, and fibronectin. The rates of *c-fos* and fibronectin protein synthesis were determined by pulse-labeling with [35 S]methionine, immunoprecipitating cell lysates with specific antibodies and analyzing immune complexes by denaturing polyacrylamide gel electrophoresis. *Odc* synthesis was inferred from an assay of enzyme activity in cell lysates.

Of the three genes that we studied, only *c-fos* translation showed nearly identical levels after serum stimulation in the absence or presence of IFN (Figure 6A). The synthesis of the *c-jun* protooncogene protein (p39), which co-immunoprecipitates with *c-fos* (Rauscher *et al.*, 1988), was also largely unaffected by IFN. In addition, the synthesis of neither protein was affected if the cells were pretreated with IFN.

By contrast, the translation products of *odc* and fibronectin showed a reduced rate of synthesis or accumulation in IFN-treated cells (Figure 6, B and C), despite levels of mRNA that were equal or greater than the mRNA level in control cells (Figure 2).

In the case of *odc*, IFN-treated cells accumulated enzyme activity at only 20–30% of the rate of accumulation in control cells, beginning ~4 h after serum stimulation (Figure 6B). The serum-dependent rise in enzyme activity was completely blocked by cycloheximide (Figure 6B), suggesting that the rise in enzyme activity requires de novo protein synthesis. The results support the idea that IFN inhibits the translation of *odc* mRNA, while having no effect on the induction of *odc* mRNA (Figure 2).

IFN also inhibited the serum-stimulated, but not the basal, rate of fibronectin protein synthesis (Figure 6C). Although fibronectin mRNA and protein synthesis rose four- to fivefold in control cells stimulated by serum, only the mRNA increased in cells that were stimulated in the presence of IFN (compare Figures 2 and 6C). In IFN-treated cells, the rate of fibronectin protein synthesis remained at the low but detectable level characteristic of quiescent cells at all times after stimulation.

To obtain a broader view of the specificity and extent to which IFN suppressed cellular protein synthesis, cells were stimulated with serum for

Figure 6. IFN does not affect *c-fos* protein synthesis, but suppresses serum-dependent rise in *odc* activity and fibronectin synthesis. (A) Serum-deprived, quiescent cells were untreated (Q) or given IFN (Q,I) for 16 h and then stimulated with 10% serum in the absence (S) or presence of IFN (S,I) for 30 min. The cells were then pulsed with [35 S]methionine, lysed, and processed for immunoprecipitation of *c-fos* protein as described in Methods. Equal amounts of radioactivity were immunoprecipitated with preimmune (C) and immune serum. (B) Serum-deprived cells were prepared as described in the legend to Figure 2. The quiescent cells were stimulated with 10% serum in the absence (SER) or presence of IFN (SER, IFN) or 2 μ g/ml cycloheximide (SER, CHM). After the indicated intervals, cell lysates were prepared and *odc* enzyme activity was determined as described in Methods. (C) Quiescent cells (Q) were given IFN (Q,I) for 16 h or serum in the absence (S) or presence of IFN (S,I) for the indicated intervals. The cells were then pulsed with [35 S]methionine, lysed, and equal amounts of radioactivity were processed for immunoprecipitation of fibronectin, as described in Methods.

8 h in the absence or presence of IFN and pulse labeled with [³⁵S]methionine for the last 4 h of stimulation. Labeled proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis (Figure 7). Most of the 200–300 labeled proteins resolved by these gels were unaffected by IFN. In fact, we could detect only one protein whose synthesis was consistently reduced in IFN-treated cells. By contrast, a number of new proteins were synthesized in IFN-treated cells, as expected from the studies of other investigators (reviewed in Lengyel, 1982; Pestka *et al.*, 1987; Tamm *et al.*, 1987). Similar results were obtained when the cells were pulse labeled for the last 30 min of stimulation (data not shown).

We conclude that IFN alters the regulation of a subset of growth factor-inducible genes among which are *c-myc*, *odc*, and fibronectin, but not *c-fos*, and suggest that IFN inhibits the expression of selected cellular genes by suppressing translation.

Discussion

It is well established that IFNs can be potent inhibitors of cell proliferation for a number of cell types in vivo and in culture. In quiescent fibroblasts in culture, IFNs antagonize the positive, stimulatory signals provided by growth factors. Thus, IFNs delay or suppress the initiation of DNA synthesis by growth factor-stimulated fibroblasts (Taylor-Papadimitriou *et al.*, 1985; Tamm *et al.*, 1987).

α/β -IFN delayed the onset of DNA synthesis in quiescent A31 cells stimulated by serum by a fixed interval (4–6 h) that did not change with higher IFN concentrations or with pretreatment. However, there was no delay in the induction of eight growth factor-inducible mRNAs. These results argue that IFN does not interfere with intracellular growth factor signals nor with nuclear events that occur subsequent to signal transduction. In addition, our results suggest that ribonuclease activation may not have a major role in the antiproliferative activity. Although a number of studies have shown that IFN can inhibit viral proliferation by degrading viral mRNAs, our results show that IFN does not prevent the accumulation of several growth-related cellular mRNAs.

Despite normal mRNA induction, IFN markedly altered the polysome distribution of fibronectin and *c-myc* mRNA. The polysome distribution of fibronectin mRNA suggests that eIF-2 inactivation might play a role in the inhibition of fibronectin protein synthesis, whereas the polysome distribution of *c-myc* mRNA suggested that IFN might inhibit the translation of this mRNA at a different, or at least an additional, site, possibly one affecting translation elongation. The stabilization of *c-myc* mRNA on polysomes, which probably accounts for the accumulation of *c-myc* mRNA in IFN-treated cells, was similar to the effect of cycloheximide (Dean *et al.*, 1986), a protein synthesis inhibitor

Figure 7. Effect of IFN on the synthesis of cellular proteins resolvable on 2D gels. Cells were made quiescent as described in the legend to Figure 6, were untreated (control) or given IFN for 14 h. Control cells were stimulated with fresh medium containing 15% serum (SER); IFN-treated cells were given fresh 15% serum and IFN (SER, IFN). After 4 h, the cells were pulse-labeled with [³⁵S]methionine for 4 h and equal amounts of radioactively labeled proteins were analyzed by two-dimensional polyacrylamide gel electrophoresis, as described in Methods. The open-headed arrow in each panel indicates the actin proteins (MW, ~43 kDa; pI, 5.4–5.45) (Garrels and Gibson, 1976), more basic proteins are located to the right of the actins. Proteins induced by IFN are indicated by empty squares in the SER panel and solid arrow heads in the SER, IFN panel. A protein whose synthesis is suppressed by IFN (MW, ~40 kDa; pI, ~5.4) is indicated by a solid arrow head in the SER panel and an empty square in the SER, IFN panel.

that blocks elongation (Vasquez, 1979). We could show directly that IFN inhibited fibronectin protein synthesis, but the low level of *c-myc* protein in A31 cells made it difficult for us to clearly demonstrate an effect of IFN on this protein. However, it is intriguing that transfected 3T3 cells expressing high levels of *c-myc* mRNA have shown to be resistant to growth inhibition by IFN (Einat and Kimchi, 1988).

There were several interesting features of the translational inhibition by IFN. First, it was very selective. *c-fos* translation was virtually unaffected, and, in fact, the synthesis of most of the cellular proteins detectable by conventional two-dimensional gels were unaffected. By contrast, the synthesis of fibronectin, *odc* (inferred from enzyme activity) and at least one other protein of unknown function was sensitive to IFN. Although our list of IFN-sensitive proteins is undoubtedly incomplete, and may well include *c-myc*, it is clear that it is restricted to a small fraction of cellular proteins. The basis for this selectivity is not known. However, transfection of a *c-myc* gene lacking the first exon rendered cells resistant to IFN (Einat and Kimchi, 1988), raising the possibility that structural features at the 5' end of the *c-myc* mRNA, and possibly other mRNAs, may target these messages for translational control by IFN. Alternatively, the high levels of *c-myc* mRNA in the transfected cells could drive the synthesis of sufficient *c-myc* protein to permit proliferation.

Our data also show that IFN inhibited translation only after growth factor stimulation. Thus, fibronectin protein synthesis in quiescent cells was not affected by IFN, even after a 16 h exposure. However, IFN strongly inhibited the rise in fibronectin protein synthesis after the mRNA level had risen in response to serum. Lin *et al.* (1986) have shown that IFN inhibits the stimulated, but not basal, synthesis of two PDGF-inducible proteins. The effect of IFN on the mRNAs for these proteins was not studied, but our results suggest that, like fibronectin and presumably *odc*, IFN may suppress their expression by inhibiting translation. The IFN-sensitive proteins are all growth factor-inducible with somewhat slower kinetics than the induction of *c-fos*. It is possible, therefore, that proteins such as *c-fos* or p39/*c-jun* are insensitive to IFN because the inhibitory translational mechanism requires an hour or so of growth factor stimulation to develop. Alternatively, the *c-fos* and p39 mRNAs may simply be poor targets for translational inhibition by IFN.

Finally, our results indicate that the inhibitory effects of IFN on overall and specific protein synthesis are partial. This finding emphasizes

the importance of an optimal rate of translation for progression through G₁. Pardee and co-workers have proposed that G₁ progression is regulated by the accumulation of a serum-inducible, labile protein of ~68 kDa (p68) (Croy and Pardee, 1983). We did not find a protein of this size whose synthesis was inhibited by IFN on our two-dimensional gels, but the gel system may not have been of sufficiently high resolution to identify p68. p68 is postulated to regulate the induction of S phase-specific genes (Coppock and Pardee, 1987). This is particularly interesting in light of our finding that IFN delays the induction of histone mRNAs. Since the replication-dependent histone genes are expressed only during cellular DNA replication, IFN may delay the expression of histone, and possibly other S phase-associated genes, by retarding the accumulation of a regulatory protein such as p68.

A number of recent studies have emphasized the importance of antiproliferative molecules in balancing the positive actions of growth factors, thus ensuring tissue homeostasis (Keski-Oja and Moses, 1987; Kohase *et al.*, 1986, 1987). Mitogenic growth factors generally activate a large number of growth-related genes and often cause a large rise in the levels of cytoplasmic mRNA. Although less is known about how growth inhibitors suppress proliferation, in several cases it appears that antiproliferative molecules may be more subtle and selective in their effects on gene expression. For example, transforming growth factor- β has been shown to inhibit *c-myc*, but not *c-fos*, mRNA in murine keratinocytes (Coffey *et al.*, 1988), and heparin has been shown to selectively suppress *c-myc* and *c-fos* mRNA by protein kinase C-dependent stimulation but not by stimulation through other growth factor-linked protein kinases (Wright *et al.*, 1989). Some growth factors have been shown to induce the expression of IFNs (Kohase *et al.*, 1987), which then presumably act as negative feedback regulators of the proliferative response. Many growth factors act at the level of transcription to increase the expression of growth-related genes. Our data suggest that the negative feedback control by IFN may act at the level of translation to down-regulate the expression of a subset of these genes.

Methods

Cell culture

Nontumorigenic BALB/c A31 cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 4 mM glutamine and 10 or 15% calf serum in a 10% CO₂/90% air atmosphere at 37°C, as described (Lu *et al.*, 1989).

Autoradiography of nuclei

Cells were given 1–2 $\mu\text{Ci/ml}$ ^3H -[methyl]-thymidine (60–70 Ci/mmol), incorporation was stopped with 6–8 drops of 1 M ascorbic acid, and the cells were washed with phosphate-buffered saline (PBS) and fixed with methanol. NTB2 emulsion was applied, developed after 2 days with D-19 and fixed with Rapid-Fix. After staining with Giemsa, 200–500 nuclei were counted at 200 $\times$ magnification.

Virus infection

4×10^4 cells were plated in 10% serum on 16-mm coverslips in 2-cm² wells. After 4 d, the monolayers ($\sim 2 \times 10^5$ cells) were washed with PBS, given 0.1 ml 10% serum (mock infection) or vesicular stomatitis virus (VSV, 10^5 pfu) in 10% serum for 1 h at 37°C, washed again and given 0.5 ml 10% serum containing the indicated amounts of IFN. After 72 h, the cells were washed with PBS, fixed with 10% formaldehyde in PBS and stained with 1% crystal violet in 25% ethanol.

RNA isolation and Northern analyses

RNA was isolated by either of two methods, with similar results. Total cellular RNA was isolated by lysing cells in 4 M guanidine isothiocyanate and pelleting the RNA through a CsCl gradient, as described by Chirgwin *et al.* (1979). Alternatively, cytoplasmic RNA was isolated by lysing cells in nonionic detergent and removing nuclei by centrifugation, as described by Favaloro *et al.* (1980). After extraction with phenol and chloroform, the RNA was precipitated with ethanol and salt, dissolved in water and stored frozen. RNA was quantified by absorbance at 260 nm; purity was assessed by absorbance at 280 and 310 nm. Quantitation was by laser densitometry.

The RNA (15 μg) was fractionated by electrophoresis through 1% agarose-6% formaldehyde gels, transferred by blotting onto nitrocellulose or nylon membranes and fixed to the membranes by baking. The membranes were hybridized, washed, and subjected to autoradiography, as previously described (McCaffrey *et al.*, 1987).

Probes

Probes were generated by labeling plasmids or plasmid inserts with [^{32}P]dCTP using random oligonucleotide priming, except for the *c-ras-Ha* probe which was a [^{32}P]UTP-labeled antisense RNA. *v-fos* sequences were from Dr. I. Verma, Salk Institute; a partial murine *odc* cDNA was from Dr. O. Janne, Rockefeller University; a full length murine *c-myc* cDNA was from Dr. K. Marcu, State University of New York, Stony Brook; a partial rat fibronectin cDNA was from Dr. Richard Hynes, Massachusetts Institute of Technology; *v-ras-Ha* sequences were subcloned into the *Sac I-EcoRI* sites of pGEM-I; murine MEP sequences were from Dr. M. Gottesman, National Institutes of Health; partial rat β -actin and β -tubulin cDNAs were from Dr. S. Farmer, Boston University Medical School; a partial murine histone 3.2 cDNA was from Dr. W. Marzluff, University of Florida, Tallahassee.

Polysome isolation and distribution

Cells ($\sim 5 \times 10^7$) were washed with ice cold PBS containing 10 $\mu\text{g/ml}$ cycloheximide and lysed in cold buffer containing 150 mM KCl, 10 mM MgCl_2 , 10 mM Tris HCl (pH 7.6), 0.5% Triton X-100, and 10 $\mu\text{g/ml}$ cycloheximide. The lysate was spun at $12\,000 \times g$ for 10 min and the supernatant was fractionated on sucrose gradients as described by Morgan *et al.* (1971). Briefly, postmitochondrial supernatants were made 1% in Triton X-100, layered onto 15–40% sucrose

gradients in lysis buffer and spun at $26\,000 \times g$ in a SW28 rotor for 3.5 h. Eighteen fractions were collected from each gradient and each fraction was precipitated with ethanol. Precipitates were dissolved in 20 mM Tris HCl, 0.2% sodium dodecyl sulfate (SDS) and extracted with phenol and chloroform. The RNA was precipitated with salt and ethanol, dissolved in water and the equivalent of equal gradient fraction volumes were loaded for Northern analysis.

Protein synthesis measurements

Cells were pulsed with [^3H]leucine (5 $\mu\text{Ci/ml}$; 58 Ci/mmol) for 30 min and quickly washed three times with cold PBS. Cold trichloroacetic acid (TCA, 1 ml) was added for 10 min; a sample of the TCA was removed to determine the acid-soluble radioactivity. The TCA precipitate was washed twice with cold TCA and solubilized in 0.5 ml 0.2 N NaOH, 0.2% SDS. TCA-soluble and TCA-insoluble radioactivity was determined by liquid scintillation counting.

Immunoprecipitations

Cells were pulsed with [^{35}S]methionine (100 $\mu\text{Ci/ml}$ for fibronectin; 300 $\mu\text{Ci/ml}$ for *c-fos*) (1200–1400 Ci/mmol) for 20 min in methionine-free DME containing dialyzed serum and/or IFN, as appropriate. Cells were quickly washed three times with cold PBS and lysed by scraping in cold lysis buffer. For *c-fos* analysis, 2×10^6 cells on 60 mm dishes were lysed in 10 mM Tris HCl (pH 7.6), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate (DOC), 0.1% SDS and 1 mM phenylmethylsulfonyl fluoride (PMSF), as described by Curran *et al.* (1984); for fibronectin, 5×10^5 cells on 35 mm dishes were lysed in 20 mM Tris HCl (pH 8.3), 150 mM NaCl, 2 mM ethylenediamine tetraacetic acid (EDTA), 1% Triton X-100, 2 mM PMSF, 2 mM iodoacetamide, 2 mM *N*-ethylmaleimide and 0.2% bovine serum albumin, as described by Bolmer and Wolf (1982). Lysates were clarified by centrifugation and equal amounts of TCA-precipitable radioactivity were incubated with primary antiserum (affinity purified rabbit IgG against *fos* peptides; rabbit polyclonal antiserum against human fibronectin) for 2–4 h at 4°C. Antibody complexes were recovered with formalin-fixed *Staphylococcus aureus* or goat anti-rabbit Immunobeads and washed extensively. Antigens were released with SDS sample buffer and analyzed on 7.5% (*fos*) or 5% (fibronectin) SDS-polyacrylamide gels, essentially as described by Laemmli (1970). Labeled proteins were detected by fluorography onto preflashed XAR-5 film.

Ornithine decarboxylase enzyme assays

Cells were detached from the culture dish with isotonic trypsin (0.05%)-0.2 mM EDTA, quickly diluted into cold DME containing 10% serum and 0.5% soy bean trypsin inhibitor and collected by centrifugation at $500 \times g$ for 5 min. Cell pellets were washed with cold PBS and suspended in cold lysis buffer (50 mM Tris HCl (pH 7.2), 0.1 mM EDTA, 0.05 mM pyridoxal phosphate, 10 mM dithiothreitol) at 1.5×10^6 cells per 0.1 ml. Cells were disrupted by freezing and thawing. Lysates were clarified by centrifugation at $13\,000 \times g$, 4°C, for 10 min and assayed essentially as described by Chen *et al.* (1976). Briefly, duplicate 50 and 100 μl samples of lysates were incubated for 60 min at 37°C with 10 μl [^{14}C]ornithine (0.05 μmol ; 7–8 $\mu\text{Ci}/\mu\text{mol}$) in capped 1.5 ml microtubes containing a penicillin disc impregnated with NCS-toluene tissue solubilizer suspended from the cap by an 18 G needle. The reaction was terminated by injecting 200 μl 10% TCA; incubation was continued for an additional 90 min at 37°C to ensure complete absorption of the liberated $^{14}\text{CO}_2$. Radioactivity absorbed to the discs was mea-

sured by liquid scintillation counting. Little or no radioactivity was released from boiled cell lysates.

Two-dimensional polyacrylamide gel electrophoresis

Cells were labeled with 200 μ Ci/ml [35 S]methionine, as described for immunoprecipitations. After labeling, the cells were lysed and analyzed by two-dimensional polyacrylamide electrophoresis, as described by O'Farrell (1975). The first dimension contained ampholines having pH ranges of 5 to 7 and 3.5 to 10 in a 4:1 ratio. The second dimension was an SDS-8% polyacrylamide gel. Detection was by autoradiography onto preflashed XAR-5 film.

Materials

Serum was from Gibco Laboratories (Grand Island, NY) or Sigma Chemical Co. (St. Louis, MO.) Murine α/β -IFN was from Sigma (4.2×10^5 U/mg protein) or Lee Biomolecular Research Laboratories San Diego, CA (4.7×10^5 U/mg protein or 4×10^6 U/mg protein); murine α - (1×10^6 U/mg protein) or murine β - (1×10^7 U/mg protein) IFN and IFN antibodies were from Lee. [32 P]nucleotides, [35 S]methionine (Trans-label) and Biotyne nylon membranes were from ICN Biomedicals (Costa Mesa, CA); [3 H]thymidine and [14 C]ornithine were from New England Nuclear (Boston, MA); photographic emulsion and film were from Kodak (Rochester, NY). Enzymes for generating probes were from New England BioLabs (Beverly, MA). Nitrocellulose and penicillin discs were from Schleicher and Schuell (Keene, NH). Fibronectin antibody and EGF (receptor grade) were from Collaborative Research (Lexington, MA); Immunobeads were from BioRad (Rockville Center, NY). NCS-toluene tissue solubilizer was from Amersham (Arlington Heights, IL).

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