Involvement of Interferon-System in the Regulation of Cell Growth and Differentiation

Samuel Salzberg  
*Bar Ilan University*

Dalia Hacohen  
*Bar Ilan University*

Sasson David  
*Bar Ilan University*

Sara Dovrat  
*Bar Ilan University*

Sharon Ahwan  
*Bar Ilan University*

See next page for additional authors  
Follow this and additional works at: [https://digitalcommons.usu.edu/microscopy](https://digitalcommons.usu.edu/microscopy)

Part of the Life Sciences Commons

Recommended Citation

Salzberg, Samuel; Hacohen, Dalia; David, Sasson; Dovrat, Sara; Ahwan, Sharon; Gamliel, Haim; and Birnbaum, Miriam (1990) "Involvement of Interferon-System in the Regulation of Cell Growth and Differentiation," *Scanning Microscopy*  : Vol. 4 : No. 2 , Article 27.  
Available at: [https://digitalcommons.usu.edu/microscopy/vol4/iss2/27](https://digitalcommons.usu.edu/microscopy/vol4/iss2/27)
Involvement of Interferon-System in the Regulation of Cell Growth and Differentiation

Authors
Samuel Salzberg, Dalia Hacohen, Sasson David, Sara Dovrat, Sharon Ahwan, Haim Gamliel, and Miriam Birnbaum

This article is available in Scanning Microscopy: https://digitalcommons.usu.edu/microscopy/vol4/iss2/27
In this report we review the current knowledge on the involvement of the interferon (IFN) system in the regulation of cell growth and differentiation. We also summarize our own data which provide evidence for the strong correlation between IFN-mediated growth-arrest of transformed cells and the elevated enzymatic activity of an IFN-induced protein. Similarly, it is demonstrated that elevated levels of IFN-induced proteins accompany the early phases of in vitro cell differentiation. IFN-treatment of NIH/3T3 mouse fibroblasts transformed by Moloney-murine sarcoma virus (MSV) resulted in a significant reduction in the rates of cell growth, protein synthesis and cloning efficiency. In parallel, 2-5A-synthetase activity was induced ten-fold above the background level. Treatment of these cells for 3 days with 450 international units (IU)/ml of IFN followed by its removal, resulted in a gradual increase in all parameters associated with cell growth while the 2-5A-synthetase activity was reduced to its normal level. However, almost no recovery occurred when cells were treated with 1,800 IU/ml. In parallel, 2-5A-synthetase activity remained highly elevated even at 3 days after the removal of IFN. In these cells, the expression of both c-myc and v-mos was reduced rapidly following IFN treatment. Upon removal of IFN after 24 h of treatment, the expression of both genes was resumed but with a different kinetics, suggesting that different mechanisms are responsible for the reduction in gene expression. In rat skeletal muscle cultures which differentiate to form myotubes, the level of both 2-5A-synthetase and protein kinase activities was transiently elevated, reaching a peak at 3 days followed by a decrease to background levels. This peak activity precedes the appearance of the major muscle differentiating proteins.

Key Words: Interferon; Interferon-system; cell growth; differentiation.

*Address for correspondence:
Samuel Salzberg,
Department of Life Sciences,
Bar Ilan University,
Ramat Gan, 52 900 Israel.
Phone No. 972-3-356041

Interferon (IFN) is now well known as a general regulatory molecule which participates in a variety of biological processes within an organism. Increasing evidence suggests that one of the most important functions of IFN regulation concerns the growth and differentiation of different cell systems [Clemens, McNurlan, 1985; Moritz, Kirchner, 1986]. It has been established that the progression of the cell from the stationary phase \( (G_0) \) through the cell cycle is associated with the activation of certain genes [Kelly et al., 1983; Cochran et al., 1984; Greenberg, Ziff, 1984; Bravo et al., 1985]. On the other hand, cell differentiation is accompanied in many cases by the repression of gene expression [Westin et al., 1982; Einat et al., 1985]. The possible involvement of IFN in the regulation of gene expression is not clear, but it has been demonstrated that in some hematopoietic cell systems, IFN is produced in an autocrine manner following induction of differentiation and one of the major IFN-induced proteins - \((2' - 5')\)oligoadenylate synthetase (2-5A-synthetase), is induce during the process [Kimchi, 1981; Friedman-Einat et al., 1982; Yarden et al., 1984; Resnitzky et al., 1986]. The induction is abolished when the cells are incubated with antibodies directed against beta-IFN [Kimchi, 1981; Friedman-Einat et al., 1982]. Moreover, 2-5A-synthetase gene expression is induced in quiescent cells stimulated to grow with a variety of growth factors [Lin et al., 1983; Wells, Mallucci, 1985; Zulla et al., 1985; Garcia-Bianco et al., 1989]. In addition, IFN has been shown to inhibit the expression of certain oncogenes, c-myc in particular, in some treated cells [Jonak, Knight, 1984; Einat et al., 1985; Emanoli-Ravel et al., 1985]. Thus, it is tempting to postulate that at least some of the regulatory functions attributed to IFN is mediated through IFN-induced enzymes, generally referred to as the IFN-system, such as 2-5A-synthetase, 2-5A activated RNase [Jacobson et al., 1983; Krause et al., 1985], and the double-stranded activated protein kinase [Galaibr, Hovanessian, 1985]. The first catalyzes the synthesis of oligomers of adenylic acid in the 2'-5' phosphodiester bond [Kerr, Brown, 1978] which activates a latent endonuclease responsible for the degradation of messenger and ribosomal RNA molecules [Williams et al., 1978; Hovanessian et al., 1979; Wreschner et al., 1981]. Activation of the 2-5A system and protein kinase may, therefore, directly cause inhibition of protein synthesis [Kerr, Brown, 1978; Williams et al., 1978] as manifested by antiproliferative effects.
Indeed, IFN has been shown to inhibit the growth of murine sarcoma virus (MSV)-transformed cells [Bakhanashvili et al., 1983]. In addition, it causes the reversal of the transformed phenotype of cells transformed by retroviruses [Hicks et al., 1981] or by an activated oncogene [Samid et al., 1984]. It has also been demonstrated that 2-5A-synthetase activity is induced to a much greater level in the transformed cells than in their normal counterpart [Bakhanashvili et al., 1983; David et al., 1986]. Furthermore, the activity of both enzymes appears to correlate in certain cases with both the antiviral and the antiproliferative effects of IFN [Bakhanashvili et al., 1983; Samuel et al., 1984; Rice et al., 1985; Reznitzky et al., 1986; Chebath et al., 1987]. It is, thus, obvious that in order to elucidate the role of IFN in the regulation of growth and differentiation, the involvement of the IFN system in these processes should be demonstrated and studied.

Skeletal muscle cultures provide a unique system for the study of morphological and biochemical differentiation. Primary myoblasts can be prepared from the embryonic tissues of a variety of species. With time in culture, the myoblasts fuse to form multinucleated myotubes which later contract spontaneously, a characteristic of differentiated muscle fibers. This event is accompanied by the synthesis of muscle-specific proteins, increase in acetylcholinesterase activity, the appearance of acetylcholine receptor molecules [Fambrough, Rash, 1971; Shainberg et al., 1984; Buckingham, 1977; Salzberg, Brik, 1978], and a decrease in DNA polymerase activity and in DNA synthesis.

The demonstration that the IFN system is activated during the process of muscle differentiation as well as during the arrest of tumor cell growth is essential for understanding the role of the system in the regulation of basic physiological processes within the organism. Our data provide evidence for these phenomena and is presented as part of an overview of the involvement of the IFN system in cell growth and differentiation as discussed at the Scanning Microscopy meetings during May 1989 at Salt Lake City, Utah.

Materials and Methods

Cell cultures

NIH/3T3 mouse fibroblasts transformed by and producing the Moloney strains of MSV and murine leukemia virus (strain 124) [NIH/3T3(MSV) cells] were used throughout this study. Occasionally, we also used the TB cell line (clone 124) producing the same viral complex [Ball et al., 1973] with identical results. The cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% newborn calf serum (Biological Industries, Beit Haemek, Israel). The cultures were subdivided every 3-4 days into 9 cm tissue culture dishes (Nunc, Roskilde, Denmark) and kept at 37°C in a humidified incubator with 5% CO2. Rat skeletal muscle cultures were prepared essentially as previously described [Shainberg et al., 1984]. In all experiments with IFN, the serum concentration was reduced to 2.5%.

Interferon

Mouse beta interferon was used in this study. It was supplied by Lee Biomedical (San Diego, CA) with a specific activity of 5.6x10^7 international units (IU) per mg protein (cat. No. 20171).

Determination of growth rate

About 5x10^4 cells were seeded in 3.5 cm tissue culture dishes (Nunc, Roskilde, Denmark). A few hours after seeding, when the cells were attached to the dish, the medium was replaced with fresh medium containing various concentrations of IFN. At the indicated times after treatment, the cells were removed by trypsin, stained with 0.5% trypan blue (in PBS) and the number of viable cells was determined.

Determination of the rate of protein synthesis

Cells were seeded and treated with IFN as described above. At the indicated times after treatment, the medium was removed and 1 ml/dish of fresh medium (containing no serum) supplemented with 0.25 micro-Ci of [3H]-leucine (130 Ci/mmole; Amersham International, Amersham, UK) was added. After incubation for 3 h at 37°C, the medium was removed, cultures washed 3 times with cold PBS, and the cells were lysed with 1 ml of 0.5% sodium dodecyl sulfate. Acid-insoluble material was precipitated with 10% cold trichloroacetic acid and the precipitate was collected on glass-fiber filters. The filters were dried, immersed in a toluene-based scintillation liquid and counted.

Determination of cloning efficiency

The cultures were treated with IFN as described above. At the indicated times after treatment, the cells were removed, counted and reseeded in 5 cm dishes at 100 cells per dish in IFN-free medium. After 10-14 days, the cultures were fixed with methanol, stained with Giemsa stain and the number of clones was determined.

Recovery from the IFN effect

Cultures were treated with the indicated IFN concentration, as described above, for 72 h. The cells were then removed with trypsin, washed 4 times with PBS, reseeded at 5x10^4 cells per 3.5 cm dish or at 5x10^5 cells per 10 cm dish (for the determination of 2-5A-synthetase activity), and subdivided into two groups. One group received IFN-free medium, while the other received medium containing identical concentrations of IFN used during the first 72 h. All parameters were then determined as described above.

Preparation of cell extracts (S10)

Cell extracts were prepared after the appropriate treatment by removing the cultured cells with a rubber policeman in PBS. The cells were then centrifuged at 800x g for 5 min, washed twice with cold PBS and resuspended in an ice-cold lysis buffer (buffer A) containing 20 mM Hepes pH 7.5, 5 mM Mg-Acetate, 0.5% NP-40, 1 mM dithiothreitol, 10% glycerol and 1 mM EDTA. The extracts were centrifuged at 10,000x g for 10 min and the soluble fractions (S10) were stored at -70°C until use.

Determination of 2-5A synthetase activity

The activity of 2-5A synthetase in cell extracts (S10) was performed as previously described [Chebath et al., 1987a] with 20 microgram protein in the presence of 50 microgram/ml of poly(I),poly(C) (Pharmacia, Uppsala, Sweden). In some cases, the extracts were first incubated with poly(I),poly(C) agarose beads (Pharmacia), washed and then assayed for 2-5A synthetase activity. The same results were obtained. Hence, the "in solution" method was routinely used. The 32P-labeled 2-5A oligomers were analyzed by high voltage electrophoresis (Chebath et al., 1987a). The radioactive spots were detected by autoradiography and the amount of radioactivity in each spot was determined by immersing the dried
**Interferon-System in Cell Growth and Differentiation**

spots in toluene-based scintillation fluid. The results are presented as pmoles of ATP polymerized based on the radioactivity contained within a 2 μL sample. A volume of electrophoresis sample buffer containing 6% acrylamide (Pharmacia) was added at room temperature for the extraction of 2-5A oligomers formed in the enzymatic assay. First, a mixture of 0.05 units of authentic 5'-oligoadenylyltransferase (Schleicher and Schuell) was incubated at 4°C for 10 min. An equal volume of poly (I), poly (C)-Sepharose beads (Pharmacia) was added to the mixture and incubated at room temperature for 30 min with occasional gentle mixing. The beads were washed several times with buffer B containing 10 mM Hepes pH 7.6, 50 mM KCl, 2 mM Mg-acetate, 7 mM 2-B-mercaptoethanol, 20% glycerol, and then one in buffer C (buffer B supplemented with 5 mM MnCl₂). The final pellet was resuspended in buffer C supplemented with 1 mg-collagenase Y-32p-ATP (50-100 Ci/mmol; New England Nuclear, Boston, MA) and incubated for 90 min at 30°C. After centrifugation, the pellet was washed 3 times with buffer C and resuspended in 2/3 volume of buffer C, and 1/3 volume of electrophoresis sample buffer containing 6% SDS (w/v), 30% glycerol (v/v), 0.02% bromophenol blue (w/v), 200 mM Tris-HCl, pH 6.8 and 250 mM 2-B-mercaptoethanol. The samples were then heated at 90°C for 5 min and centrifuged. The supernatants were collected and analyzed on 10% polyacrylamide slab gels containing SDS. The phosphorylated proteins were detected by autoradiography on Kodak XAR-5 film.

**DNA probes**

The probe for c-myc was a 2.9 kbp BamHI fragment that contained the gag and myc specific sequence of the MC29 viral genome subcloned in pBR322 [Reddy et al., 1983]. The insert DNA was cleaved by BamHI under conditions recommended by the manufacturer (New England Biolabs, Beverly, MA) and purified by agarose gel electrophoresis, electrophoration, and further purification on Elutip-D columns (Schleicher and Schull, Dassel, W. Germany). The probe for detecting v-mos transcripts was a 2.5 kb EcoRI fragment of human DNA which shares a 0.65 kbp region of continuous homology with v-mos. This fragment was also cloned in pBR322 [Prakash et al., 1982]. The insert was cleaved by EcoRI and purified as mentioned above. All the probes were nick-translated as previously described [Rigby et al., 1977] to a specific activity of 1x10⁸ cpm/μg using 32p-dCTP (specific activity – 800 Ci/mm; New England Nuclear, MA) and purified with a labeled precursor. RNA preparations and hybridization.

Total RNA from confluent cultures, either untreated or treated with IFN as indicated for each experiment, was purified by LiCl-urea precipitation and phenol-chloroform extraction as previously described [Ausubel, et al., 1989]. Poly (A)⁺-containing molecules were isolated by passing the RNA preparation through oligo(dT)-cellulose columns [Maniatis et al., 1982]. For slot-blot analysis, samples containing the appropriate amounts of poly (A)⁺ RNA were diluted in 15xSSC (1xSSC = 0.1M NaCl; 0.01M Na citrate), heated at 65°C for 15 min and applied to nitrocellulose membrane filters (Schleicher and Schuell) presoaked in 1xSSC. The filters were washed with 2xSSC, dried and baked at 80°C for 2 h in a vacuum experiment and the spots identified by illumination with a UVSL-25 lamp. In addition, we always used as a positive control cell extracts prepared from IFN-treated NIH/3T3 (MSV) cells. Such extracts contain a high level of active 2-5A synthetase which yields whole series of oligomers.

**Determination of protein kinase activity**

Cell extracts (S10) were prepared as described above. Heparin (50-100 units/ml) was added to cell extract samples each containing 500 microgram of protein. The mixtures were incubated at 4°C for 10 min. An equal volume of poly (I), poly (C)-Sepharose beads (Pharmacia) was added at room temperature for 30 min with occasional gentle mixing. The beads were washed several times with buffer B containing 10 mM Hepes pH 7.6, 50 mM KCl, 2 mM Mg-acetate, 7 mM 2-B-mercaptoethanol, 20% glycerol, and then one in buffer C (buffer B supplemented with 5 mM MnCl₂). The final pellet was resuspended in buffer C supplemented with 1 mg-collagenase Y-32p-ATP (50-100 Ci/mmol; New England Nuclear, Boston, MA) and incubated for 90 min at 30°C. After centrifugation, the pellet was washed 3 times with buffer C and resuspended in 2/3 volume of buffer C, and 1/3 volume of electrophoresis sample buffer containing 6% SDS (w/v), 30% glycerol (v/v), 0.02% bromophenol blue (w/v), 200 mM Tris-HCl, pH 6.8 and 250 mM 2-B-mercaptoethanol. The samples were then heated at 90°C for 5 min and centrifuged. The supernatants were collected and analyzed on 10% polyacrylamide slab gels containing SDS. The phosphorylated proteins were detected by autoradiography on Kodak XAR-5 film.

**Results**

**Susceptibility of NIH/3T3(MSV) cells to growth inhibition by IFN**

NIH/3T3(MSV) cells are highly susceptible to growth inhibition by IFN [Bakhanashvili et al., 1983]. Furthermore, in these cells, both the expression and activity of 2-5A-synthetase are induced to a significant level following IFN-treatment [Bakhanashvili et al., 1983; David et al., 1989]. We therefore reasoned that these cells may serve as a useful model for studies on the involvement of the IFN system in the regulation of cell growth. A typical result of the effect of mouse beta-IFN on the growth characteristics of NIH/3T3(MSV) cells is shown in Fig. 1. In agreement with our earlier findings [Bakhanashvili et al., 1983], it is clearly demonstrated that a significant inhibition of growth in a dose dependent manner is observed in our experimental system. These cells were thus used for further analysis.

**IFN-induced antiproliferative and 2-5A-synthetase activities**

Since we are attempting to establish the involvement of the IFN system in the manifestation of the antiproliferative property of IFN, it was of interest to follow the kinetics of both the growth and the 2-5A-synthetase activity after removal of IFN from the culture medium of NIH/3T3(MSV) cells. It is argued that as long as the enzymatic activity remains elevated, the cells will continue to exhibit growth arrest.

Transformed cultures were, therefore, treated with IFN at various concentrations for 72 h. The cells were then removed, replated and divided into two groups. One group received IFN at the same concentrations while the other was refed with IFN-free medium. The rates of cell growth and protein synthesis were then determined. As illustrated in Fig. 2, treatment with IFN at low concentrations (90-450 IU/ml) for 72 h resulted in almost full recovery of the treated cells following removal of IFN. However, as the IFN concentration increased, the recovery was less evident and at 1200-1800 IU/ml, there was almost no recovery, with the kinetics of both cell growth and protein synthesis identical in continuously IFN-treated cultures and in cultures treated with IFN for 72 h only.

Similar conclusions can be drawn from the results on the cloning efficiency of treated cultures summarized in Fig. 3. The cloning efficiency of cells
Figure 1: Phase contrast micrographs of effect of IFN on NIH/3T3(MSV) cultures. (A) Control untreated cultures. Cultures were treated for 3 days with IFN at the following concentrations: (B) 400IU/ml; (C) 800IU/ml; and (D) 1,200IU/ml.

It should be emphasized that in several experiments cultures were treated continuously with IFN for 96 h without being subdivided. Here again, maximum activity was seen at 72 h after treatment, and the same level of inhibition was observed at 96 h (unpublished data). However, the cells tended to detach from the plate’s surface as incubation proceeded. Therefore, the subdivision procedure described above was used in most experiments.

Next, the kinetics of 2-5A-synthetase activity was determined under similar experimental conditions. Cells were treated with either 450 or 1800 IU/ml of IFN for 72 h, then subdivided into two sets. One set received IFN at the same concentration, and the other was fed with IFN-free medium. The activity of 2-5A-synthetase was determined at daily intervals thereafter. The results shown in Fig. 4 clearly demonstrate that the enzymatic activity in continuously treated cultures remained high throughout the entire incubation period in both IFN concentrations used. However, in cells treated with IFN for only 3 days, the level of 2-5A activity was reduced to its normal level following treatment with 450 IU/ml, where the level of the enzymatic activity remains high, although IFN is no longer present in the culture medium. These results correlate well with the data on the recovery from the IFN-induced antiproliferative effect described above (see Fig. 2).

The IFN effect and oncogene expression

The protooncogene c-myc is a cell cycle associated gene shown to be expressed during stimulated cell growth [Kelly et al., 1983] and, when constitutively activated, to block cell differentiation [Coppola, Cole, 1986; Dmitrovsky et al., 1986; Freytag, 1988]. The viral oncogene v-mos is the transforming gene of M-MSV [Blair et al., 1984]. Since in a preliminary experiment we observed that IFN affects the cell cycle distribution of NIH/3T3 (MSV) cells (data not shown) and because these cells...
Figure 2 (at left): Reversibility of the IFN effect on cell growth. Cells treated for 72 h with the indicated IFN concentrations (IU/ml) were subdivided. One set was further treated with the same IFN concentrations (circles), while the other received IFN-free medium (triangles). Cell number (solid lines) and protein synthesis (broken lines) were determined at various times after IFN was removed.

Figure 3 (at right): Reversibility of the IFN-effect on cloning efficiency. Cultures were treated as described in Fig. 2. Cells were cloned in IFN-free medium at various times after removal of IFN and the number of clones was scored 10-14 days later. Squares - set with no IFN treatment after first 72 h; circles - set treated continuously with IFN.

Figure 4. Reversibility of the IFN-effect on 2-5A-synthetase activity. Cultures were treated as described in Fig. 2, at two IFN-concentrations, as indicated in each column. Activity of 2-5A-synthetase was determined at various times after IFN removal and is presented as the ratio between the enzymatic level detected in IFN-treated cells and that detected in untreated cultures.
**Figure 5.** Effect of IFN on the expression of c-myc and v-mos. Cultures were treated with 450 IU/ml of IFN for 24 h. Samples containing 1.5 microgram of poly (A)+ RNA were then analyzed for the presence of either c-myc or v-mos sequences (+IFN). The IFN-containing medium for parallel cultures was removed and replaced with fresh IFN-free medium. At the indicated times thereafter (-IFN), poly (A)+ RNA was isolated and 8 microgram samples were analyzed for c-myc or v-mos sequences.

**Figure 6.** Kinetics of 2-5A-synthetase activity during myogenesis in rat. Rat-skeletal muscle cultures were prepared, and at daily intervals thereafter, 2-5A-synthetase activity was determined in cell extracts. Inset: Appearance of the enzymatic products isolated by high voltage electrophoresis. As a marker (right column), we used a cell extract prepared from NIH/3T3(MSV) cells incubated with 300 IU of mouse beta-IFN for 24 h. This extract was analyzed in parallel with those of muscle origin [Birnbaum et al, 1990].

are transformed by M-MSV, it was important to study the effect of IFN on the expression of these genes in our experimental system. We were particularly interested in following the expression after the removal of IFN from the culture medium because this could provide some clues as to whether IFN acts on the expression of these two genes by similar mechanism.

Thus, cultures were treated with IFN for 24 h, the medium was then replaced with IFN-free medium and the kinetics of both c-myc and v-mos expression was determined by slot-blot analysis of poly (A)+-containing RNA at different times thereafter. The results, summarized in Fig. 5, indicate that IFN treatment caused complete disappearance of both c-myc and v-mos transcripts at 24 h after the treatment was initiated. However, following removal of IFN, the level of c-myc RNA transcripts increased gradually as a function of time. On the other hand, the kinetics of v-mos expression under the same conditions was different. Although by 24 h after the addition of IFN similar results to those observed with c-myc were evident, by 6 h after IFN-removal, an increase in the amount of transcripts was clearly visible, which was followed by a gradual decrease toward the background levels observed in untreated cultures (not shown). Under similar conditions no alteration in beta-actin gene expression was observed before or after IFN removal (Ahwan et al., submitted for publication).
Activation of the IFN system during myogenesis

Terminal differentiation is associated with growth arrest of the differentiated cells. We showed that the level of 2-5A-synthetase activity correlates well with the antiproliferative activity of IFN. Therefore, it was now important to clarify whether the IFN system is also activated during the differentiation process. Myoblasts obtained from rat, mouse, or chick embryonic muscle provide a unique model for the study of differentiation. Within a week after seeding, these cells fuse to form myotubes, which contract spontaneously while synthesizing muscle specific proteins. In order to study the involvement of the IFN system in myogenesis, rat embryonic muscle cultures were prepared and, at daily intervals after seeding, 2-5A-synthetase activity was determined in cell extracts. The enzymatic products included the various 2-5A oligomers formed in the assay. These were separated by high-voltage electrophoresis, and the radioactive spots were identified by autoradiography. The results are demonstrated in the Inset to Fig. 6. The scanning analysis of the radioactive spots in illustrated in the major section of Fig. 6 which shows that 2-5A-synthetase activity was transiently elevated during myogenesis. After a low basal level on the first day in culture there was a gradual increase to a peak activity on the third day and a decline on the following days. The major enzymatic product detected in muscle cell extracts was the trimer form of 2-5A, although traces of the dimer form could also be observed. The same result was obtained with muscle cell extracts from the other animal species used in this study. In contrast, a series of oligomers was observed with cell extracts prepared from IFN-treated NIH/3T3(MSV) cells which served as a marker (Fig. 6 Inset, right column).

Next, we decided to determine the kinetics of the activity of double-stranded activated protein kinase, a well known and characterized IFN-induced protein [Galabru et al., 1984; Galabru, Hovanessian, 1985]. Similar kinetics to that observed with 2-5A-synthetase will support the assumption that several proteins known to be induced by IFN are elevated in a characteristic manner during the differentiation of skeletal muscle cultures. Cell extracts were prepared at daily intervals after seeding and the protein kinase activity was assayed in each extract. In Fig. 7, the appearance of the major phosphorylated protein, P68, most likely the enzyme molecule itself [Galabru, Hovanessian, 1985], is shown in the inset and its tracing registered by a scanning densitometer is demonstrated in the main part of the figure. Similar to the pattern observed with 2-5A-synthetase, the activity of protein kinase was transiently elevated in rat skeletal muscle cultures, reaching a peak activity on the third day in culture.

Discussion

The mechanism involved in the antiproliferative effect of IFN is still not completely understood. However, since the antiviral property of IFN appears to be mediated through proteins induced in IFN-treated cells [Baglioni, 1979], it is tempting to postulate that most biological effects of IFN are also mediated through similar sets of proteins. Indeed, published data demonstrate a correlation between the sensitivity of cell growth to IFN and the inducibility of 2-5A-synthetase by IFN in these cells [Czarniecki et al., 1981; Kimchi, 1981], although some reports do not always support this conclusion [Vandenbussche et al., 1981].

There is no doubt that various types of cells differ in their sensitivity to IFN and in the inducibility of 2-5A-synthetase, indicating that IFN acts through various mechanisms [Esteban, Pazv, 1985]. However, in a few cell lines derived from NIH/3T3 mouse fibroblasts, a defect detected in the 2-5A-system was responsible for the resistance of the cells to, at least, the IFN-induced antiviral effect [Epstein et al., 1981; Salzberg et al., 1983]. We have demonstrated that a few MSV-transformed mouse fibroblast clones possess an unusually high basal level of 2-5A-synthetase activity. These cells are relatively resistant to cytolytic viral infection even in the absence of IFN [David et al., 1989]. Hence, it seems important to establish whether it is possible to attribute an essential role to an IFN-induced enzyme in its antiproliferative effect as well. In the present study, a correlation between the level of 2-5A-synthetase activity and the antiproliferative effect manifested by IFN on MSV-transformed NIH/3T3 cells was demonstrated. IFN strongly inhibited the growth of the transformed cells in a dose-dependent manner, while 2-5A-synthetase activity was induced to a significantly high level under the same experimental conditions. Furthermore, we demonstrated for the first time that as long as the activity of this enzyme is not reduced following the removal of IFN from the culture medium (e.g., with 1800 IU/ml), the antiproliferative effect of IFN remains constant. However, when lower IFN concentrations were used (450 IU/ml), recovery was clearly observed, and this was accompanied by a reduction in 2-5A-synthetase activity.

The role of the 2-5A system in the development of the antiproliferative effect is not clear. Certainly, 2-5A-synthetase could be just one of a variety of factors, including IFN-induced protein kinase [Galabru, Hovanessian, 1985], which may function during this process and lead to the regulation of gene expression.

The protooncogene c-myc has been shown to be involved in differentiation and growth arrest in several cell systems [Gonda, Metcalf, 1984; Lachman, Shoulchi, 1984; Dean et al., 1986; Siebenlist et al., 1988]. During these processes, c-myc expression is reduced, apparently by a two-step mechanism leading to an initial post-transcriptional block which is followed by inhibition of c-myc transcription [Siebenlist et al., 1988]. On the other hand, c-myc as well as other nuclear protooncogenes are transiently activated when cells are induced to proliferate [Campisi et al., 1984; Cochran et al., 1984; Greenberg et al., 1985; Kelly et al., 1983]. It is, thus, obvious that the level of expression of c-myc is an indication of the availability of the cell for replication. This fact is supported by our data which demonstrate that, in NIH/3T3(MSV) cells, IFN-treatment results in a rapid reduction in the expression of c-myc. In this respect, these cells behave similarly to those of other IFN-sensitive cell lines, particularly Daudi cells, which have also shown a reduction in c-myc expression following IFN treatment [Einat et al., 1985; Jonak, Knight, 1984; Dani et al., 1985].

V-mos is the transforming oncogene of M-MSV
[Blair et al., 1984], and its expression is driven by the viral promoter present in the viral long terminal repeat. Therefore, this expression may be regulated by a mechanism that is not cellular gene control.

At least two possibilities are immediately feasible in explaining the mechanism involved in the inhibition of c-myc or v-mos expression by IFN in our experimental system. First, a shut-off of the transcription machinery is apparently a target of IFN action, as has been shown by Einat and colleagues [Einat et al., 1985]. However, a post-transcriptional control, which affects the stability of RNA transcripts rather than the transcription rate, must also be considered, especially since such a mechanism of IFN-action has been reported [Jonak, Knight, 1984; Dani et al., 1985]. Finally, a more complex mechanism which combines both possibilities cannot be ruled out. For example, one can argue that IFN first causes pretermination of transcription at the non-coding sequence of the first myc axon [Nepveu, Marcu, 1986], and only then a block in the transcription process itself. However, in our experimental system there was a difference in the kinetics of reappearance of c-myc and of v-mos transcripts upon removal of IFN. In the case of c-myc, a gradual increase in the level of mRNA specific molecules was observed, which, we suggest, is an indication of the reduction of the transcriptional activity that is blocked in the presence of IFN. However, in the case of v-mos, an immediate increase in the level of RNA transcripts was evident, followed by a decrease toward control levels. One possible explanation for this phenomenon is that IFN affects the stability of the transcript rather than the rate of transcription with the binding of DNA polymerase to the gene promoter. When the block is removed, mRNA molecules are immediately available in great abundance, as active transcription never ceases. This is followed by a stabilization step concomitant with the reappearance of a regular cell phenotype.

It has been shown that IFN stimulates various morphologic and biochemical changes in several cell systems. The best examples studied thus far are the hematopoietic cell systems [Rossi et al., 1981; Tomida et al., 1982; Fisher et al., 1983; Hattori et al., 1983; Harris et al., 1985]. Indeed it has been demonstrated that IFN is released in an autocrine fashion in some hematopoietic cells [Gonda, Metcalf, 1984; Harris, Metcalf, 1985]. It has also been shown that the enzymatic activities of both 2-5A-synthetase and double-stranded RNA-activated protein kinase in vivo are reduced to basal levels in fully differentiated myotubes, indicating that these enzymes are no longer needed at this stage of the differentiation process. An identical trimer form of 2-5A with similar kinetics was observed during chondrogenesis in vivo [Nepveu et al., in preparation].

The exact role that the IFN system may fulfill during differentiation remains unclear. However, it is evident that nuclear protooncogenes, particularly c-myc, are essential for cell-growth and are switched off during the differentiation of several cell systems, including hematopoietic cells [Gonda, Metcalf, 1984; Lachman, Shoulchi, 1984]. In some cases, for example in the murine embryonal carcinoma cell line F9 induced to differentiate with retinoic acid and cyclic AMP, the expression of c-myc is regulated post-transcriptionally [Dean et al., 1986]. One interpretation is that the stability of c-myc mRNA transcripts is reduced in this system, which could certainly be the result of nuclease activity induced by 2-5A oligomers, the enzymatic products of 2-5A-synthetase [Baglioni, 1979]. The fact that the enzymatic product of 2-5A-synthetase has been detected in IFN-treated cultures. Although it is certainly possible that this similarity in kinetics is coincidental and that these enzymes have no functional role in myogenesis, it is reasonable to consider that the IFN system may play an important role in the initiation of the differentiation process. This hypothesis is strengthened by the fact that the enzymatic product of 2-5A-synthetase present in myoblasts is the trimer form of 2-5A, which is unique and may reflect an activity of a more specialized enzyme. Indeed, various forms of 2-5A-synthetase have been detected in IFN-treated cells [Chebath et al., 1987a]. Another indication of the importance of the IFN system is the fact that the enzymatic activities of both 2-5A-synthetase and protein kinase are reduced to basal levels in fully differentiated myotubes, indicating that these enzymes are no longer needed at this stage of the differentiation process. An identical trimer form of 2-5A with similar kinetics was observed during chondrogenesis in vivo [Maoor et al., in preparation].

Based on the results of this study, we suggest that the IFN system may be involved in the differentiation process of cells of various lineages and may indeed be crucial for the normal progression of differentiation. An altered expression of the IFN system or a lack of response to its signals may therefore lead to the development of malignancy. However, more direct proof is needed before the role of the IFN system in myogenesis can be established unambiguously.

Acknowledgements

This study was supported by grants from the Israel Cancer Research Fund and the Mitzl Dobrin Cancer Foundation.

References


Interferon-System in Cell Growth and Differentiation


of U937 cells: Comparison with other agents that promote differentiation of human myeloid or monocyte-like cell lines. J. Clin. Invest. 72, 237–244.


Interferon-System in Cell Growth and Differentiation

differentiation of histiocytic lymphoma U937 cells. EMBO J. 3, 969-973.

Discussion with Reviewers

P.M. Grimley: How specifically does your data contribute to a better understanding of the effects of interferon on differentiation? For example, the adipocyte model has already been examined in considerable detail by Grossberg et al. In view of previous data, how can you postulate a significant role of 2-5A-synthetase, since you did not assay for RNAase or ribosomal cleavage activity in your experiments?

Authors: The discussion brings data that support the hypothesis that the activation of the IFN system occurs both in IFN-growth arrested cells and during terminal differentiation. In both cases, cells enter the G0 phase in the cell cycle. It is true that these findings are only correlative and may be fortuitous. Also, we point out that in different differentiating systems like myogenesis or chondrogenesis, the appearance of 2-5A-synthetase or protein kinase activities occur in a specific manner. Certainly, only when specific inhibitors for 2-5A-synthetase or antisense RNA will be available, a more direct approach can be utilized.

B. Silverman: What is the rationale for adding heparin to the protein kinase assays? Hovanessian showed that heparin activates the kinase.

Authors: Heparin inhibits the activity of most protein kinases not related to the IFN-effect.

J. Chebath: Did you determine the levels of c-myc and v-mos expression in cells treated for 3 days by 1500 U/ml of IFN?

Authors: The recovery of c-myc and v-mos expression after IFN-removal was studied on cultures treated for 24 h only with IFN. We are planning to perform a more detailed analysis on the recovery of cells treated for extended periods of time with different concentrations of IFN.

J. Chebath: Did you try to identify further the radioactive oligomers of ATP formed in the muscle cell extracts in the course of differentiation as authentic 2-5A trimer?

Authors: We used two sets of markers for the identification of the 2-5A oligomers, including authentic molecules. We believe that those markers are sufficient to positively identify the enzymatic products, particularly when the experiments were performed at least 6 times with identical results.