Cancer Chemother Pharmacol (1992) 30: 12–20

Cancer Chemotherapy and Pharmacology

Posttranscriptional regulation of c-*myc* proto-oncogene expression and growth inhibition by recombinant human interferon-β ser^{17} in a human colon carcinoma cell line*

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Received 26 August 1991/Accepted 13 November 1991

Summary. Recombinant human interferon-β ser^{17} (IFN-β ser^{17}), a cytokine that exhibits both antiviral and antiproliferative activity against a wide variety of cell types, causes a time- and dose-dependent inhibition of monolayer growth and of the expression of the c-*myc* proto-oncogene in DLD-1 Clone A human colon-carcinoma cells. The suppression of c-*myc* expression mediated by IFN-β ser^{17} is due to a posttranscriptional destabilization of c-*myc* mRNA rather than to an inhibition of c-*myc* mRNA transcription. There is evidence suggesting that the selective reduction in the half-life of c-*myc* mRNA in IFN-β ser^{17}-treated cells occurs through an increase in the activity of the 2′,5′-oligoadenylate synthetase/RNase L [2′,5′-oligo (A) synthetase] pathway in DLD-1 Clone A cells. Cotreatment of these cells with IFN-β ser^{17} and the anticancer agent *N*-methylformamide leads to the partial abrogation of 2′,5′-oligo (A) synthetase activity and the stabilization of c-*myc* mRNA. These findings suggest that there is a correlation between the IFN-β ser^{17}-mediated suppression of c-*myc* expression and the induction of 2′,5′-oligo (A) synthetase activity in DLD-1 clone A cells.

Introduction

Our laboratory has been interested in identifying pharmacologic agents that suppress the proto-oncogene c-*myc* in human colon-carcinoma cells. This proto-oncogene, which is the cellular homolog of the transforming gene carried by the avian myelocytomatosis virus (MC29) [22, 49], has been associated with the regulation of proliferation, especially the entry of cells from the G_0 into the G_1 phase of the cell cycle [25], and neoplastic transformation in a number of mammalian cell systems [1]. Several studies have shown that expression of c-*myc* is elevated in up to 70% of human primary colon carcinomas, suggesting that this gene may play some role in the development of this disease [16–18, 53]. It is therefore conceivable that c-*myc*-suppressing agents might exert some selective chemotherapeutic activity against this type of malignancy. We have previously found that high concentrations of *N*-methylformamide (NMF), which induce cytostasis and a more benign phenotype in certain human colon-carcinoma cell lines [11], can decrease the transcription of the c-*myc* gene in these cells [8].

Another class of agents that have been shown to suppress the expression of c-*myc* are the interferons (IFNs), an endogenous group of proteins exhibiting antiproliferative and antiviral activity that fall into three distinct classes: α (leukocyte-derived), β (fibroblast-derived), and γ (lymphocyte-derived) [37]. Several lines of evidence suggest that IFNs may act as negative regulators of c-*myc* expression and cell growth in certain cell systems. IFNs have been shown to cause cytostasis and decrease steady-state levels of c-*myc* mRNA in the Daudi Burkitt's lymphoblastoid cell line, in the mouse embryo AKR-2B line, and in murine sarcoma virus-transformed NIH 3T3 cells [23, 39, 43], among others. Cell lines that are relatively insensitive to the growth-inhibitory actions of the IFNs and sublines that have been selected for resistance to IFNs do not undergo an IFN-mediated loss of c-*myc* expression [15, 31]. Furthermore, it has been demonstrated that platelet-derived growth factor, which causes a relatively rapid induction of c-*myc* in quiescent NIH 3T3 cells, also induces IFN-β expression at a later stage; the elevation of IFN-β coincides with the decline in c-*myc* expression, suggesting that IFN may mediate this decrease as part of a feedback mechanism to control c-*myc* expression and cell growth [57]. This view is strengthened by the finding that IFN-β

* This investigation was supported by PHS grants CA 38630 and CA 20892, by Research Career Development Award CA 01241 awarded by the National Cancer Institute, DHHS, and by grant BC-640 from the American Cancer Society (to T.M.S.). Support was also provided by an Advanced Predoctoral Fellowship Award from the Pharmaceutical Manufacturers Association (to D.C.)

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can act as a negative autocrine factor in some hematopoietic cell lines [40].

In the Daudi lymphoblastoma system, IFN-α and IFN-β have been shown to decrease the levels of c-*myc* transcripts via a posttranscriptional mechanism; i.e., by selectively increasing the rate of degradation of c-*myc* mRNA [13, 27]. Although the mechanism underlying this effect has not been delineated, it is well known that IFNs induce the activity of a double-stranded RNA-dependent 2′,5′-oligoadenylate synthetase [2′,5′-oligo (A) synthetase], which converts adenosine triphosphate (ATP) to the nucleotide polymer 2′,5′-oligo(A); 2′,5′-oligo(A) can in turn activate a latent endoribonuclease (RNase L) [3, 28]. It has been proposed that activation of the 2′,5′-oligo (A)/RNase L pathway might be responsible for the increased rate of degradation of c-*myc* transcripts that occurs in IFN-treated Daudi cells [13, 27]. This apparently is not an obligatory mechanism in all cell types, as elevations of 2′,5′-oligo (A) synthetase can occur in cell lines that do not undergo a suppression of c-*myc* expression on treatment with IFNs [15] or in those whose growth is not inhibited by IFN [54, 56].

All three classes of IFNs display antiproliferative activity against selected human colon-carcinoma cell lines when they are used either as single agents or in combination with double-stranded RNA [e.g., poly(I):poly(C)] or tumor necrosis factor [4–7, 19, 38, 47, 48]. The ability of these cytokines to decrease c-*myc* mRNA levels has been examined in only a few of these cell lines: human IFN-γ inhibited growth but failed to decrease c-*myc* in the KM12C line [34]; similarly, human IFN-α_2 decreased the growth of the Colo 205 cell line without decreasing c-*myc* expression [21]. However, the present report demonstrates that human recombinant IFN-β ser^{17}, a stable form of IFN-β, suppresses the expression of c-*myc* in the DLD-1 Clone A human colon-carcinoma cell line, and does so by increasing the rate of c-*myc* mRNA degradation. Serendipitously, we also found that the polar solvent NMF partially abrogates both the IFN-β ser^{17}-induced increase in 2′,5′-oligo (A) synthetase activity and the increased rate of c-*myc* transcript turnover in these cells. This finding is consistent with the hypothesis that a 2′,5′-oligo (A) synthetase-sensitive RNase may be mediating this effect.

Materials and methods

Materials. Human recombinant IFN-β ser^{17} was purchased from Triton Biosciences Inc. (Alameda, Calif.).

Cell culture. DLD-1 Clone A human colon-carcinoma cells were cultured in RPMI 1640 media supplemented with 10% fetal calf serum, antibiotics, and buffers in a humidified incubator containing 95% air and 5% CO_2 at 37° C as previously described [11]. In all experiments, DLD-1 Clone A cells (5×10^5) were initially plated in 100-mm tissue-culture dishes (Falcon plastics) in the above-mentioned media. After 24 h incubation, the medium was removed, the cells were washed with physiological saline, and drug-free or drug-containing medium was added. After the appropriate incubation periods, cells were harvested with trypsin-ethylenediaminetetraacetic acid (EDTA), washed with Dulbecco's phosphate-buffered saline, and stored at –80° C for isolation of total cellular RNA.

RNA isolation and Northern blot analysis. Total cellular RNA was isolated using the guanidinium thiocyanate method with cesium chloride modification [8, 9]. RNA was quantitated by absorbance at 260 nm and by comparison with known concentrations of yeast RNA standards that were electrophoresed through an agarose gel and stained with ethidium bromide. Total cellular RNA was electrophoresed through a 1% agarose: 6% formaldehyde gel at 70 V for 8 h. RNA was transferred onto Gene Screen filters (New England Nuclear, Boston, Mass.) for 20 h. The filters were air-dried and baked in vacuo for 4 h at 80° C. Prehybridization was done at 42° C for 16 h in a solution containing 50% deionized formamide, 0.2% polyvinyl-pyrrolidone, 1.0 M NaCl, 0.1% sodium pyrophosphate, 0.2% bovine serum albumin, 0.2% ficoll, 0.05 M TRIS-HCl (pH 7.5), 1.0% sodium dodecyl sulfate (SDS), 10% dextran sulfate, and denatured salmon-sperm DNA (100 μg/ml). Hybridization was performed at 42° C in the same solution containing 10^6 cpm/ml of DNA probes labeled by nick translation [41] with [α-^{32}P]-dATP. Filters were washed twice for 5 min at room temperature with 2 × SSC (1 × SSC = 0.15 M NaCl, 0.0125 M sodium citrate; pH 7.0). This was followed by two 30-min washes in 2 × SSC, 1.0% SDS at 57° C and two 30-min washes in 0.1 × SSC at room temperature. Filters were exposed to preflashed Kodak XAR film with intensifying screens at –80° C. In cases in which a filter was hybridized with more than one probe, the initial hybridized probe was removed from the filters by incubation at 65° C in 0.005 M TRIS-HCl (pH 8.0), 0.0002 M Na_2-EDTA, 0.05% sodium pyrophosphate, 0.002% polyvinylpyrrolidone, 0.002% bovine serum albumin, and 0.002% ficoll for 2 h as recommended by the filter manufacturer.

DNA probes. The c-*myc* probes were prepared from the *ClaI-EcoRI* fragment from plasmid pHSR (purchased from the American Type Tissue Culture Collection, Rockville, Md.). The DNA probe used to measure the expression of GAPDH was isolated from the *PstI-RsaI* fragment of HcGAP3, a partial cDNA clone representing 0.7 kb of the human glyceraldehyde 3-phosphate dehydrogenase gene (GAPDH; kindly provided by Dr. R. Wu, Cornell University).

Assay of transcription rates. The measurement of transcription rates in DLD-1 Clone A cells was modified from various published reports [20, 30, 51] and has been described elsewhere [8]. Harvested cells were washed with phosphate-buffered saline and pelleted at 500 *g*. The cell pellet was resuspended in buffer containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM TRIS-HCl (pH 7.6), 14 mM KCl, 14 mM β-mercaptoethanol, 20% glycerol, and 0.3% (v/v) Nonidet-40 (NP-40), homogenized, and then centrifuged at 1,000 *g* for 5 min at 4° C. The pellet was washed and the nuclei were resuspended in a buffer containing 25% glycerol, 36 mM 4-(2-hydroxylethyl)-1-piperazineethanesulfonic acid (pH 7.6), 3 mM $MgCl_2$, 7 mM dithiothreitol, and 170 mM KCl at a density of 10^7 nuclei/ml. Nuclei (5×10^6) obtained from each experimental group were used for transcription assays. The resuspended nuclei were combined with 1 mM each of ATP, guanosine triphosphate, and cytosine triphosphate; 25% glycerol; 25 mM 4-(2-hydroxylethyl)-1-piperazinethanesulfonic acid (pH 7.6); 2 mM $MgCl_2$; 2 mM $MnCl_2$; 5.5 mM dithiothreitol; and 134 mM KCl. The reaction mixture was incubated for 25 min at 26° C with 100 μCi [α-^{32}P]-uridine triphosphate (UTP)/ml, and the reaction was terminated by the addition of RNase-free DNase (100 units/ml). As an additional control, the inhibitor of RNA polymerase II, α-amanitin, was added (2 μg/ml) to the reaction mixtures.

After the transcription reaction, nuclei were incubated at 37° C for 1 h in a buffer containing 1% SDS, 5 mM EDTA, 10 mM TRIS-HCl (pH 7.4), and 200 μg proteinase K. The reaction mixture was extracted twice with an equal volume of phenol: chloroform and precipitated by the addition of yeast transfer RNA (10 μg/ml), 200 mM NaCl, and 2.5 vol. ethanol. The labeled pellet was resuspended in a hybridization solution consisting of 10 mM TES (pH 7.4), 0.5 mg yeast transfer RNA/ml, 0.2% ficoll, 0.2% polyvinyl-pyrrolidone, 1% sodium pyrophosphate, and 0.4% SDS. Nitrocellulose filters were prepared by immobilizing 10 μg DNA from the *XhoI-EcoRI* fragment representing exons 2 and 3 of the c-*myc* gene [55], GAPDH, and plasmid PBR 322 using a Bio-Rad dot-blot manifold as suggested by the manufacturer and were then prehybridized for 4 h at 65° C in the hybridization solution described above. Labeled transcription products were added (5×10^6 cpm/ml) and the mixture was hy-

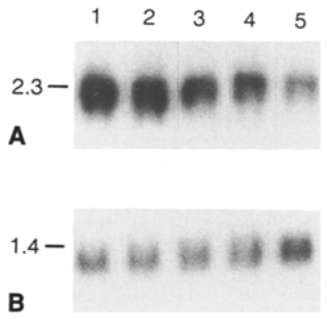

Fig. 1 A, B. Dose-dependent decrease in the expression of c-*myc* in IFN-β ser^{17}-treated DLD-1 Clone A cells. **A** DLD-1 Clone A cells (5×10^5/100-mm tissue-culture dish) were plated as described in Materials and methods. After 24 h, the medium was removed, the cells were washed with physiological saline, and drug-free or drug-containing medium was added. Cells were harvested after 7 days, total cellular RNA was isolated, and Northern blot analysis was performed using 20 μg RNA. *Lane 1*, Untreated DLD-1 Clone A cells; *lane 2*, cells treated with 50 units IFN-β ser^{17}/ml; *lane 3*; cells treated with 75 units IFN-β ser^{17}/ml; *lane 4*, cells treated with 100 units IFN-β ser^{17}/ml; *lane 5*, cells treated with 200 units IFN-β ser^{17}/ml. The filter was hybridized with the *ClaI-EcoRI* fragment representing exon 3 of the c-*myc* gene. The hybridization conditions are described in Materials and methods. Filters were exposed to Kodak XAR film for 48 h at –80° C. **B** The same filter was stripped of the labelled c-*myc* probe as described in Materials and methods and then hybridized with the partial human sequence of GAPDH. Hybridization and wash conditions were the same as those described in **A**. The filter was exposed to Kodak XAR film for 72 h at –80° C. The data were quantitated by excising the appropriate radiolabeled bands from Gene Screen filters, placing them in a vial containing water, and counting the radioactivity in a liquid scintillation counter.

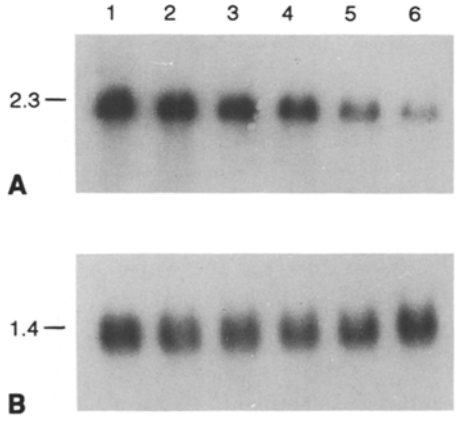

Fig. 2 A, B. Time course of the IFN-β ser^{17}-mediated decrease in c-*myc* in DLD-1 Clone A cells. **A** DLD-1 Clone A cells were plated as described in Materials and methods. After 24 h, the medium was removed, the cells were washed with physiological saline, and fresh medium containing 200 units IFN-β ser^{17}/ml was added. Cells were harvested on days 1 (*lane 2*), 2 (*lane 3*), 4 (*lane 4*), 7 (*lane 5*), and 14 (*lane 6*) after the addition of IFN-β ser^{17}. *Lane 1*, Untreated, exponentially growing DLD-1 Clone A cells. Total cellular RNA was extracted from each group and 20 μg was used for Northern blot analysis. The Gene Screen filter was hybridized with the *ClaI-EcoRI* fragment, which represents the third exon of c-*myc*. The filter was exposed to Kodak XAR film for 48 h at –80° C. **B** The filter used for **A** was stripped of the *myc* as described and then hybridized with the partial clone of human GAPDH. This filter was exposed to Kodak XAR film for 72 h at –80° C. Autoradiograms were quantitated as described in Fig. 1

bridized at 65° C. The filters were washed with several changes of 2 × SSC at 65° C and were then air-dried and exposed to Kodak XAR film with intensifying screens at –80° C. The results were quantitated by excising the spots from the filters and counting them by liquid scintillation spectrometry.

Measurement of 2′,5′-oligoadenylate synthetase activity. The measurement of 2′,5′-oligoadenylate synthetase [2′,5′-oligo (A) synthetase] activity in DLD-1 Clone A cells was adapted from previous published reports [10, 14, 33]. DLD-1 Clone A cells were treated for 96 h with 200 units IFN-β ser^{17}/ml, 170 mM NMF, 200 units IFN-β ser^{17}/ml + 170 mM NMF, or no addition. Cells were harvested as previously described, lysed in a buffer containing 5 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM TRIS-HCl (pH 7.6), 14 mM KCl, 14 mM β-mercaptoethanol, 20% glycerol, and 0.3% (v/v) Nonidet 40 (NP-40, buffer A) and were centrifuged for 5 min at 12,000 *g*. In all, 100 μl cytoplasmic extract corresponding to 150 μg protein was incubated with 30 μl poly(I):poly(C) agarose beads (3.7 mg/ml agarose; P. L. Biochemicals, Milwaukee, Wis.) for 30 min at 37° C. The matrix was washed twice with 5 mM magnesium acetate, 1 mM dithiothreitol, 25 mM KCl, 10% glycerol, and 20 mM TRIS-HCl (pH 8.0), then incubated with 60 μl buffer B (buffer A plus 1 mM dithiothreitol) to which had been added 4.8 mM ATP and 48 μCi 2-[^{3}H]-ATP/ml (Amersham) for 24 h at 37° C. The supernatants were digested with bacterial alkaline phosphatase for 2 h at 37° C. Aliquots of the extracts were spotted onto diethylaminoethyl (DEAE)-cellulose, air-dried, washed with several changes of distilled water, eluted with 0.3 M KCl, and counted in Aquasol scintillation fluid. The protein concentration of the extracts was determined according to Bradford [2]. One unit of enzyme activity was defined as the incorporation of one picomole of ATP per hour at 37° C.

Results

IFN-β ser^{17}-mediated suppression of growth and c-myc expression

Initially we examined the effect of a range of recombinant human IFN-β ser^{17} concentrations (100–500 units/ml) on the rate of monolayer growth and cell viability in the DLD-1 Clone A human colon-carcinoma cell line. The results shown in Table 1 indicate that the inhibition of monolayer growth by IFN-β ser^{17} in DLD-1 Clone A cells was dose-dependent. However, there was no greater suppression of growth after 7 days vs 4 days of exposure; because these cultures were refed with fresh IFN every 48 h, this lack of additional response may have been due to IFN-receptor desensitization. These studies also indicate that the viability of DLD-1 Clone A cells did not diminish over the concentrations and time periods examined (Table 1), suggesting that the effect of IFN-β ser^{17} on these cells is cytostatic rather than cytotoxic. These findings are similar to the results described by Chapekar and Glazer [4], who reported that exposure of HT-29 human colon-carcinoma cells to 100 units IFN-β/ml also induced cytostasis after 3 days of treatment.

Since IFN-β has been shown to mediate a repression of c-*myc* expression in Daudi lymphoblastoid cells [23], we investigated whether the same effect was occurring in DLD-1 Clone A cells. DLD-1 Clone A cells were plated and continuously treated for 7 days with 50, 100, 150, and 200 units IFN-β ser^{17}/ml. Total cellular RNA was isolated from each sample after the 7-day treatment period and then analyzed for c-*myc* expression by RNA hybridization. Figure 1 A shows a dose-dependent decrease in the expression

Table 1. Effect of IFN-β ser^{17} on the monolayer growth of DLD-1 Clone A cells

Treatment	Growth inhibition on day 4 (%)	Viability (%)	Growth inhibition on day 7 (%)	Viability (%)
No addition	0	92	0	85
100 units IFN-β ser^{17}/ml	25± 6.2	90	38± 2.7	86
200 units IFN-β ser^{17}/ml	46± 3.9	91	57± 5.5	89
300 units IFN-β ser^{17}/ml	55± 8.8	83	61± 7.4	85
400 units IFN-β ser^{17}/ml	63±11.1	84	67± 5.9	81
500 units IFN-β ser^{17}/ml	71± 6.5	88	78±10.0	85

DLD-1 Clone A cells (1.5 × 10^5/60-mm dish) were plated as described in Materials and methods. After 24 h, the medium was removed, the cells were washed with saline, and drug-containing or drug-free medium was added. Monolayer cultures were refed with drug-free or drug-containing medium every 48 h. Cells were harvested on days 4 and 7 after the addition of drug-containing medium and were tested for monolayer growth using a hemacytometer and for viability by trypan blue exclusion. Data represent mean values ± SD for 3 independent experiments done in triplicate and are expressed as the percentage of inhibition relative to untreated control values

Table 2. Measurement of 2′,5′-oligo (A) synthetase activity in DLD-1 Clone A cells treated with IFN-β ser^{17} in the absence or presence of NMF

Treatment	2′,5′-oligo (A) synthetase activity (units/mg protein)
No addition	7.5±3.6
200 units IFN-β ser^{17}/ml	39.4±5.9*
170 mM NMF	11.5±4.4
200 units IFN-β ser^{17}/ml + 170 mM NMF	21.3±7.7*, **

The measurement of 2′,5′-oligo (A) synthetase activity is detailed in Materials and methods. 2′,5′-Oligo (A) synthetase activity was measured in all treatment groups from DLD-1 Clone A cells that had been treated for 96 h with 200 units IFN-β ser^{17}/ml, 170 mM NMF, or 200 units IFN-β ser^{17}/ml and 170 mM NMF. Data represent mean values ± SD for 3 independent experiments performed in triplicate and are expressed as units per milligram of protein in the cytoplasmic extract
* P <0.001 vs no addition according to Student's t-test; ** P <0.001 vs 200 units IFN-β ser^{17}/ml according to Student's t-test

of the c-*myc* proto-oncogene; i.e., the expression of c-*myc* remained relatively unaffected when cells were treated with 50 units IFN-β ser^{17}/ml but was reduced by approximately 32% at 100 units/ml, by 38% at 150 units/ml, and by 80% at 200 units/ml. Therefore, IFN-β ser^{17} can cause a suppression of c-*myc* expression at concentrations that produce growth inhibition. The filter used in the study illustrated in Fig. 1 A was stripped of the c-*myc* probe and hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Previous studies have indicated that GAPDH is expressed constitutively in several human tissues [12] and therefore serves as a control for amounts of RNA and for general changes in gene expression induced by IFN-β ser^{17}. As indicated in Fig. 1B, the level of GAPDH mRNA in IFN-β ser^{17}-treated cells remained relatively constant.

We then investigated whether there was a time-dependent decrease in c-*myc* expression in DLD-1 Clone A cells treated with 200 units IFN-β ser^{17}/ml. The expression of c-*myc* was suppressed by 23%, 28%, 44%, 85%, and >90% after 24, 48, and 96 h and 7 and 14 days of treatment, respectively (Fig. 2A, lanes 2–6, respectively). It should be noted that the inhibition of c-*myc* expression mediated by IFN-β ser^{17} parallels the growth inhibition in DLD-1 Clone A cells up to only 96 h of treatment. For example, at 24 h, cell growth was inhibited by 18%, whereas c-*myc* expression was suppressed by 23%; however, on day 7, the expression of c-*myc* was inhibited by 85% (Fig. 2A, lane 5), whereas the monolayer growth of DLD-1 Clone A cells that were also treated with 200 units IFN-β ser^{17}/ml was suppressed by 57% (Table 2). There was no appreciable change in the expression of GAPDH over the time course of IFN-β ser^{17} treatment (Fig. 2B).

Mechanism of the IFN-β ser^{17}-mediated decrease in c-myc expression

There are two general mechanisms by which the expression of c-*myc* can be suppressed in transformed cells: (1) by decreasing the stability of the c-*myc* mRNA or (2) by inhibiting the rate of c-*myc* transcription. In the Daudi lymphoma system, it has been reported that IFN-β decreases the stability of c-*myc* [27], whereas IFN-α also affects the rate of c-*myc* gene transcription in this cell line [15]. Initially we investigated whether IFN-β ser^{17} was affecting the stability of c-*myc* mRNA. DLD-1 Clone A cells were treated for 96 h with 200 units IFN-β ser^{17}/ml, after which the medium was removed and replaced with fresh medium containing 5 μg/ml of the transcriptional inhibitor actinomycin D. Samples were harvested at 10, 20, 40, 80, and 120 min after the addition of actinomycin D, total cellular RNA was isolated, and Northern blot analysis was performed.

The data quantitated from Northern blots (Fig. 3A) demonstrated a reduction in the half-life of the c-*myc* mRNA in IFN-β ser^{17}-treated cells as compared with untreated DLD-1 Clone A cells ($t_{1/2}$ control = 29 ± 5.2 min, n = 3; $t_{1/2}$ IFN-β ser^{17}-treated = 15 ±3.5 min, n = 3; Fig. 3A). The half-life of GAPDH mRNA was not significantly altered during this treatment period (Fig. 3B). These results indicate that IFN-β ser^{17} reduced the levels of c-*myc* via a posttranscriptional mechanism. However, it is possible that IFN could also have affected the rate of c-*myc* transcription. Nuclear runoff assays were performed to

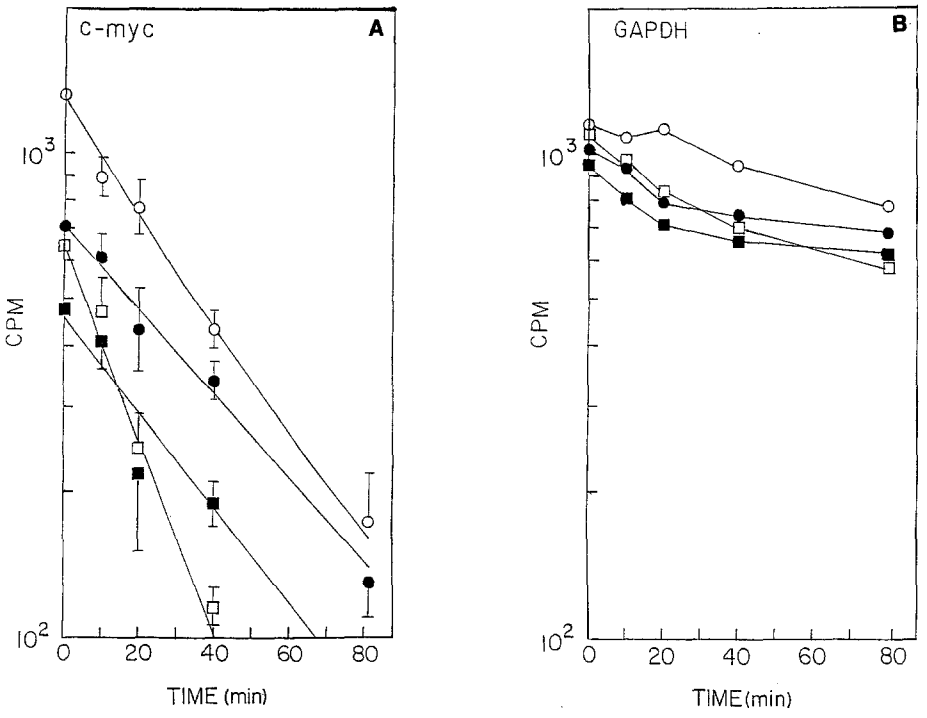

Fig. 3 A, B. Effect of IFN-β ser[17] on the stability of c-*myc* mRNA. DLD-1 Clone A cells were plated as described in Fig. 1. After 24 h, the medium was removed and medium containing 200 units IFN-β ser[17]/ml, 170 mM NMF, or 200 units IFN-β ser[17]/ml and 170 mM NMF was added. These cells were incubated for 96 h, at which time the medium was removed and fresh medium containing 5 μg/ml actinomycin D was added. The cells were incubated for an additional period of up to 120 min in medium containing actinomycin D. Total cellular RNA was isolated at each indicated time point and Northern blot analysis was performed using 20 μg RNA and probed with the *ClaI-EcoRI* fragment representing the third exon of the c-*myc* gene. **A** The half-life for c-*myc* mRNA from control cells (○), IFN-β ser[17]-treated cells (□), NMF-treated cells (■), and DLD-1 Clone A cells treated with IFN-β ser[17] and NMF (●) in Northern blot analysis was quantitated by excising the appropriate radiolabeled bands from the Gene Screen filter membrane, placing them in a vial containing 5 ml water, and counting the radioactivity in a liquid scintillation counter. Data were plotted using the method of least-squares fit. Each point represents the mean value ± SD for 3 independent experiments. **B** Northern blot analysis was performed as described above, and the Gene Screen filters were hybridized to the DNA representing the partial cDNA sequence of human GAPDH to determine the half-life of this constitutive gene. The data were quantitated and plotted as described in **A** and represent the average values for 3 independent experiments. The symbols are defined as shown in **A**

address this question. Nuclei were isolated from DLD-1 Clone A cells that had been treated for 96 h with 200 units IFN-β ser[17]/ml. The rate of c-*myc* transcription from IFN-β ser[17]-treated cells was reduced by only 7% relative to untreated controls (Fig. 4). There was no significant change in the rate of transcription of GAPDH in IFN-β ser[17]-treated cells as compared with untreated control cells (Fig. 4). Therefore, the suppression of c-*myc* expression by IFN in DLD-1 Clone A cells apparently occurs at the level of mRNA stability and not by altering the rate of transcription of the c-*myc* proto-oncogene.

One mechanism by which the decrease in c-*myc* stability may occur involves the IFN-β ser[17]-mediated activation of 2′,5′-oligo (A) synthetase. The induction of this enzyme results in the formation of 2′,5′-oligoadenylate, which in turn activates an endoribonuclease (RNase L) [3, 28, 33]. This RNase may play a pivotal role in the cytostatic action and/or the destabilization of c-*myc* mRNA mediated by IFN-β ser[17]. We therefore investigated whether this system was activated in DLD-1 Clone A cells. DLD-1 Clone A cells were treated for 96 h with 200 units IFN-β ser[17]/ml, after which the cells were harvested, cytosolic extracts were prepared, and 2′,5′-oligo (A) synthetase activity was measured. The data shown in Table 2 demonstrate a >5-fold increase in 2′,5′-oligo (A) synthetase activity in DLD-1 Clone A cells treated with IFN-β ser[17] as compared with untreated control cells. In addition, time-course studies (not shown) demonstrated that 2′,5′-oligo (A) synthetase is induced at as early as 6 h after the initiation of treatment, i.e., preceding the observed decrease in c-*myc* expression at 24 h posttreatment. These results suggest that the activation of this enzyme system in IFN-β ser[17]-treated DLD-1 Clone A cells may account in part for the decrease in the stability of c-*myc* mRNA.

Effects of N-methylformamide on the action of IFN-β ser[17]

We have previously shown that high concentrations (170 mM) of the anticancer agent *N*-methylformamide (NMF) inhibit the expression of c-*myc* in DLD-1 Clone A human colon-carcinoma cells by affecting the rate of transcription (a 42% decrease in c-*myc* transcription occurs after a 96-h exposure to NMF) via a mechanism that has not been elucidated [8]. We hypothesized that combining an agent that inhibits the transcription of c-*myc*, i.e., NMF, with an agent that decreases the $t_{1/2}$ value for c-*myc* transcripts, i.e., IFN-β ser[17], may result in a greater than addi-

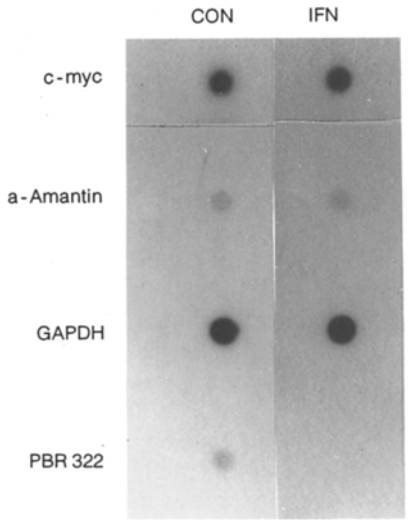

Fig. 4. Measurement of the rate of c-*myc* transcription in DLD-1 Clone A cells. Transcription assays were performed as previously described [8]. In all, 5×10^6 nuclei from cells treated with 200 units IFN-β ser^{17}/ml were used for the transcription reaction. Labeled RNA transcripts were hybridized as previously reported [8] to 10 μg c-*myc*, PBR 322, and GAPDH DNA that had been immobilized onto nitrocellulose filters using a Bio-Rad Dot-Blot manifold. PBR 322 DNA was not included in the IFN-β ser^{17}-treated samples. The c-*myc* DNA represents the *XhoI-EcoRI* fragment of exons 2 and 3 of the *myc* gene. Filters were exposed to Kodak XAR film for 48 h at –80° C. The results were quantitated as described in Fig. 1

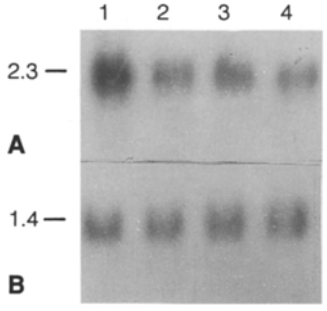

Fig. 5 A, B. Suppression of c-*myc* proto-oncogene expression in DLD-1 Clone A cells treated with IFN-β ser^{17} in combination with NMF. **A** DLD-1 Clone A cells were plated, washed, and treated with 170 mM NMF (*lane 2*), 200 units IFN-β ser^{17}/ml (*lane 3*), or 200 units IFN-β ser^{17}/ml and 170 mM NMF (*lane 4*) for 96 h as previously described. Total cellular RNA was isolated, Northern blot analysis was performed, and the Gene Screen filter was hybridized to the *ClaI-EcoRI* fragment representing the third exon of the c-*myc* gene. *Lane 1*, Untreated, exponentially growing DLD-1 Clone A cells. The filter was exposed to Kodak XAR film for 72 h at –80° C. **B** The Gene Screen filter used for **A** was stripped of the c-*myc* probe and hybridized to the DNA representing the GAPDH gene. This filter was exposed to Kodak XAR film for 96 h at –80° C. The data were quantitated as described in Fig. 1

tive effect on the levels of c-*myc* and the growth of DLD-1 Clone A cells.

We initially examined the effect of the combination of these agents on monolayer growth after 96 h and on clonogenicity in semisolid medium. The combination yielded equivocal results; 170 mM NMF failed to potentiate the effect of 200 units IFN-β ser^{17}/ml on the growth of DLD-1 Clone A cells grown in monolayers; however, NMF did

Table 3. Effect of IFN-β ser^{17} and NMF on the monolayer growth and clonogenicity of DLD-1 Clone A cells

Treatment	Monolayer growth[a]	Clonogenicity (%)
No addition	0	29 ± 3.1
200 units IFN-β ser^{17}/ml	47 ± 4.8	16 ± 2.7
170 mM NMF	43 ± 6.1	19 ± 1.7
200 units IFN-β ser^{17}/ml + 170 mM NMF	75 ± 9.7	4 ± 1.1

The monolayer growth of DLD-1 Clone A cells treated for 96 h with 200 units IFN-β ser^{17}/ml, 170 mM NMF, or 200 units IFN-β ser^{17}/ml plus 170 mM NMF was measured as described in Table 1. Data are expressed as the percentage of inhibition as compared with untreated control values. The clonogenicity of DLD-1 Clone A cells was measured as previously described [11]. Briefly, 3×10^4 cells/35-mm tissue-culture dish were used for this study. Dishes were scored for colony formation after 14 days of incubation. Colonies containing ≥ 50 cells were used for determinations of cloning efficiency under a light microscope. Data from both studies represent mean values ± SD for 3 independent experiments performed in triplicate

[a] Values are expressed as the percentage of growth inhibition

potentiate the inhibitory effect produced by IFN-β ser^{17} on the clonogenicity of this cell line in soft agar (Table 3). The expression of c-*myc* was determined in these cells after 96 h treatment with 200 units IFN-β ser^{17}/ml, 170 mM NMF, and 200 units IFN-β ser^{17}/ml plus 170 mM NMF. Whereas both IFN-β ser^{17} alone and NMF alone inhibited the expression of c-*myc* by 55% and 47%, respectively, as compared with untreated control cells (Fig. 5 A, lane 1 vs lanes 2 and 3), there was no apparent potentiation of the effect when both agents were used together, i.e., a 70% reduction in the expression of c-*myc* (Fig. 5, lane 4 vs lanes 2 and 3). The level of GAPDH expression remained relatively constant in each of these samples (Fig. 5 B).

Although the basis for the lack of potentiation between IFN-β ser^{17} and NMF is unclear, one possibility could be that NMF may interfere with the IFN-β ser^{17}-mediated destabilization of c-*myc*. The half-life of c-*myc* mRNA in IFN-β ser^{17}-treated (200 units/ml) DLD-1 Clone A cells is 15 min (Fig. 3 A, approximately half that of control cells ($t_{1/2}$ = 29 min; Fig. 3 A). We determined that in the presence of 170 mM NMF, the half-life of c-*myc* in IFN-β ser^{17}-treated cells increases from 15 ± 3.5 min (n = 3) to 25 ± 5.5 min (n = 3; Fig. 3 A). These findings may be explained if NMF was interfering with the IFN-β ser^{17}-mediated induction of the RNase L enzyme pathway that is activated by 2′,5′-oligoadenylate. Indeed, NMF treatment attenuated the IFN-β ser^{17}-mediated induction of 2′,5′-oligo (A) synthetase by 45% in these cells (Table 2). Therefore, the lack of potentiation observed for the combination of IFN-β ser^{17} and NMF might be partially explained by the ability of NMF to interfere with the induction of 2′,5′-oligo (A) synthetase and the degradation of c-*myc* mRNA in IFN-β ser^{17}-treated DLD-1 Clone A cells.

Although these data indicate that NMF and IFN-β ser^{17} do not constitute a rationally based chemotherapeutic drug combination, they do provide some insights into the mechanism of action of IFN-β ser^{17} in these cells. The finding

that NMF partially blocks the IFN-β ser^{17}-activated induction of 2′,5′-oligo (A) synthetase and the IFN-β ser^{17}-mediated decrease in the half-life of c-*myc* mRNA suggests that these two events are causally related. If so, this represents evidence that in DLD-1 Clone A cells, the IFN-β ser^{17}-mediated induction of 2′,5′-oligo (A) synthetase activity could be triggering a latent 2′,5′-oligo (A) synthetase-responsive RNase that is responsible for the decreased stability of c-*myc*.

Discussion

This study demonstrated the following: (1) that IFN-β ser^{17} inhibits the growth of the DLD-1 Clone A human colon-carcinoma cell line in a dose-dependent manner; (2) that at concentrations that produce growth inhibition, there is a suppression of the expression of the c-*myc* proto-oncogene; (3) that the IFN-β ser^{17}-induced suppression of c-*myc* expression occurs via a posttranscriptional mechanism rather than an inhibition of c-*myc* transcription; and (4) that this posttranscriptional mechanism may involve the 2′,5′-oligo (A) synthetase/RNase L cascade.

Is c-*myc* suppression an important mechanism by which IFNs exert their antiproliferative action? With regard to this, most malignant cells fall into two categories. In the first category are cells such as the Daudi lymphoblastoid line in which a profound depression of c-*myc* levels occur at growth-inhibitory concentrations of IFNs [23]. That Daudi cells selected for resistance to IFNs do not undergo decreases in c-*myc* levels suggests but does not prove that a causal relationship exists between these events [15]. Another example, at least in the hands of some investigators, is represented by BALB/c 3T3 cells. A G_0/G_1 block and a suppression of c-*myc* occur in this cell type in response to IFN-α or -β [26]. If the c-*myc* gene is transfected into BALB/c 3T3 cells, the entry of these transfected cells into the S phase is less affected by IFN treatment than is that of untransfected BALB/c 3T3 cells [26]. These data suggest a cause-and-effect relationship between IFN-triggered suppression of c-*myc* expression and growth inhibition in some cell lines.

However, in a number of cell lines, IFNs can exert growth-inhibitory action without producing a concomitant reduction in c-*myc* expression. For example, the growth of human colon-carcinoma cell lines Colo 205 and KM12C is inhibited by human IFN-α_2 and -γ, respectively, without any effect being observed on the expression of this proto-oncogene in these cells [21, 34]; the same is true for NCI-H146 and NCI-H82 human small-cell lung carcinomas that are treated with IFN-β [36]. In human HeLa cells, IFN-α causes a decrease in c-*myc* mRNA levels, whereas IFN-γ increases c-*myc* expression, although the inhibition of cell growth is greater following treatment with IFN-γ [24]. Furthermore, murine IFN-β can block the bombesin-stimulated growth of Swiss 3T3 cells and the platelet-derived growth-factor-triggered growth of quiescent BALB/c 3T3 cells without inhibiting the elevations of c-*myc* that normally accompany treatment with these growth factors [32, 52]. The c-*myc*-independent mechanism(s) by which IFNs exert their growth-inhibitory action are not known. One possibility in some cell systems involves the inhibitory effect produced by IFNs on protein synthesis; this is thought to occur via an activation of a protein kinase that phosphorylates and inactivates the α-subunit of eukaryotic protein synthesis initiation factor eIF-2 [44, 45].

DLD-1 human colon-carcinoma cells appear to follow the Daudi model. There is an inhibition of c-*myc* expression at IFN concentrations known to inhibit the growth of this cell line. In Daudi cells, c-*myc* is regulated by IFN-α and -β via a posttranscriptional mechanism [13, 27]. IFN-α (100 units/ml) decreases the half-life of c-*myc* mRNA from approximately 49 to 20 min; it has been suggested that this occurs through the activation of the 2′,5′-oligoadenylate/RNase L system [13]. It has also been conjectured that c-*myc* mRNA, which exhibits a considerable degree of secondary structure [42], may itself serve as the source of double-stranded RNA that is normally required to activate 2′,5′-oligo (A) synthetase [13]. Similarly, in DLD-1 Clone A cells, IFN-β ser^{17} reduced the half-life of c-*myc* transcripts from 29 to 15 min (Fig. 3 A).

There is evidence that this effect occurs via the 2′,5′-oligoadenylate/RNase L cascade. First, a 5-fold increase in 2′,5′-oligo (A) synthetase activity accompanied the reduction in the half-life of c-*myc* (Table 2; Fig. 3 A). When the IFN-β ser^{17}-mediated induction of this enzyme activity was partially blocked by cotreatment with NMF, a corresponding increase occurred in the half-life of c-*myc* relative to that observed following treatment with IFN alone (Fig. 3 A). This finding is consistent with the hypothesis that the 2′,5′-oligo (A) synthetase-sensitive RNase L is mediating the increased rate of c-*myc* transcript degradation in DLD-1 Clone A cells. However, it should be noted that a direct cause-and-effect relationship between the IFN-β ser^{17}-induced loss of c-*myc* expression and its cytostatic action in the DLD-1 Clone A cell line has not been firmly established.

IFN-induced elevations in 2′,5′-oligo (A) synthetase activity are not necessarily coupled to a decrease in c-*myc* suppression. For example, c-*myc* levels in HL-60 human promyelocytic leukemia and U937 human histiocytic lymphoma cells treated with IFN-α either remain unaltered or are only slightly increased despite an approximately 15-fold induction of 2′,5′-oligo (A) synthetase activity in each case [15]. The same holds true for Daudi cells selected for resistance to IFN-α [15]. In a similar vein, Chapekar and Glazer [5] observed that treatment of the HT-29 human colon-carcinoma cell line with IFN-γ caused both an induction of 2′,5′-oligo (A) synthetase and growth inhibition; it was concluded that these events were unrelated, since no 2′,5′-oligo (A) could be detected and no increase was observed in the rate of ribosomal RNA degradation.

The "missing link" in at least some of these systems may be the availability of double-stranded RNA. Schillbach et al. [50] have described a neuroblastoma cell line whose expression of N-*myc*, a homolog of c-*myc*, is normally not affected by IFN-β despite elevations in 2′,5′-oligo (A) synthetase; however, when these cells are transfected with a plasmid vector expressing antisense N-*myc*, IFN-β produces a marked decrease in N-*myc* expression. It was presumed that in these transfected cells, sense/an-

tisense N-*myc* hybrids served as a source of double-stranded RNA for activation of the 2′,5′-oligo (A) synthetase activity induced by IFN-β, leading to an increase in RNase activity [50]. This may explain in part why the combination of IFNs and double-stranded RNA produces a much greater antiproliferative effect than does either agent alone [4, 6, 7]. Intriguingly, some cell types naturally produce antisense c-*myc* transcripts [35], which raises the possibility that IFNs may stimulate the degradation of c-*myc* mRNA in cell types that exhibit active transcription of antisense c-*myc*; this hypothesis remains to be tested.

The finding that the polar solvent NMF can partially block the action of IFN-β ser^{17} on the rate of c-*myc* degradation was serendipitous. We had predicted that NMF, which inhibits the expression of c-*myc* in DLD-1 Clone A cells at the transcriptional level [8], would potentiate the c-*myc*-suppressing actions of IFN-β ser^{17}, which acts at the posttranscriptional level in these cells. However, the effect of the combination of NMF and IFN-β ser^{17} on c-*myc* levels in DLD-1 Clone A cells was less than additive (Fig. 5); subsequently, we determined that cotreatment with NMF inhibited the IFN-β ser^{17}-mediated induction of 2′,5′-oligo (A) synthetase as well as the decrease in c-*myc* stability. It is apparent that NMF was interfering with some aspect of the IFN-β ser^{17} signaling pathway(s) that trigger the induction of 2′,5′-oligo (A) synthetase. In any case, recombinant IFN-β ser^{17} has been used in phase II clinical trials to treat patients presenting with advanced colorectal carcinoma, but has yielded only a low response rate [29, 46, 49]. Therefore, studies delineating the mechanism by which this IFN exerts its antineoplastic action on colon carcinoma cells might provide insights that lead to a more efficacious use of this agent.

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