diated by another factor that reassociates with Fos after guanidine denaturation but is not detectable by [^{35}S]methionine labeling.

The early appearance of Fos after cell-surface stimulation and its nuclear location imply a role in signal transduction systems, perhaps at the level of gene expression. This hypothesis is strengthened by the recent description of transcriptional *trans*-activation in the presence of v-*fos* (*11*) and the evidence presented here for a DNA-binding activity. Although protein binding to DNA cellulose can often result from nonspecific interactions, the release of Fos from isolated nuclei treated with DNase and micrococcal nuclease (Fig. 1B) suggest that this in vitro property reflects a real association. In contrast, recent data obtained from immunofluorescence studies indicate that the protein product of the *myc* oncogene can be released from nuclei by treatment of cells with RNase but not with DNase (*17*).

In studies on c-*fos* induction, we have frequently detected Fos-related antigens with Fos-peptide antibodies (*7, 10, 12, 18*). One of these, the 46K antigen, displays similar biochemical traits to Fos. It is extracted from nuclei by DNase I and micrococcal nuclease but not by RNase A (Fig. 1B), and it binds to DNA cellulose (Fig. 3, A and C). This protein is detected in serum-stimulated fibroblasts, in PC12 cells treated with nerve growth factor and benzodiazepines, and in many other situations in which c-*fos* is induced. The time course of induction of 46K appears to be slightly delayed with respect to Fos (*18*). Thus, Fos may be considered a marker for a family of genes, perhaps functionally and structurally related, that are induced with similar kinetics in response to extracellular stimuli. The ability of these proteins to bind to DNA and the *trans*-activation function associated with Fos suggest that this gene family is analogous to the immediate-early genes of many viruses (*19*). Thus, Fos, and its related antigen, 46K, are candidate regulatory proteins that function in the long-term transcriptional response of cells to growth factors or other external stimuli.

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Tumor Necrosis Factor Reduces c-*myc* Expression and Cooperates with Interferon-γ in HeLa Cells

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The suppression of the c-*myc* nuclear oncogene is associated with growth arrest and may therefore be directly controlled by naturally occurring growth inhibitors. The effect of tumor necrosis factor (TNF) and of interferon-γ (IFN-γ) on c-*myc* expression was investigated in HeLa cells, which respond to these cytokines by a specific arrest in the G_0/G_1 phase of the cell cycle. Northern blot and nuclear transcription analyses indicated that each cytokine reduced within 1 to 3 hours the c-*myc* messenger RNA levels as a result of transcriptional inhibition. Adding the two cytokines together at saturating levels resulted in enhanced inhibition of c-*myc* transcription and of the c-*myc* messenger RNA steady-state levels. While the reduction of c-*myc* messenger RNA by IFN-γ was dependent on new protein synthesis, the inhibitory effect of TNF on c-*myc* messenger RNA was direct and was not abrogated by cycloheximide. The differential effect of the protein synthesis inhibitor and the cooperative inhibitory effects of the two cytokines when added together suggest that IFN-γ and TNF reduce c-*myc* transcription through different molecular mechanisms.

THE ACTIVE EXPRESSION OF C-*myc* IS associated with competence for cell division and is switched off when cells enter the quiescent nonproliferative state during terminal differentiation (*1*). The current concept concerning the activation of c-*myc* in tumorigenesis suggests that it is associated with the loss or disruption of the control elements that enable the normal gene to be switched off when cells stop dividing. Thus, to analyze the molecular basis of abnormal activation of c-*myc* in tumor cells it is important to identify the mechanisms that normally turn off the gene. We recently demonstrated in vitro that an autocrine factor related to interferon-β (IFN-β) that is produced by differentiating hematopoietic cells is part of the mechanism that reduces c-*myc* expression (*2–4*). We based this conclusion on the finding that an antiserum to IFN-β partially abrogates the typical reduction of c-*myc* messenger RNA (mRNA) during differentiation of M1 myeloid cells (*4*). In addition, exposure of certain actively growing hematopoietic cells to purified preparations of exogenous IFN (α and β) selectively reduces the steady-state levels of the c-*myc* mRNA transcripts (*5, 6*).

Together, these data demonstrate that some IFN's may function as natural growth inhibitors that autoregulate c-*myc* expression during terminal differentiation. However, it is clear that the effect of IFN is partial and not sufficient to account for the entire reduction of c-*myc* mRNA during differentiation (*4*). It was therefore interesting to test the possibility that cooperativity between IFN and other potential growth inhibitors might be essential in achieving the complete shutoff of c-*myc*. Two other growth inhibitory substances that have been characterized biochemically and shown to exert antimitogenic functions on certain target cells are transforming growth factor-β (TGF-β) (*7*) and tumor necrosis factor (TNF) (*8, 9*). In this report we describe studies of the possi-

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ble cooperative effects between TNF and IFN in reducing c-*myc* expression.

Tumor necrosis factor, a 17-kD polypeptide, exerts cytostatic or cytolytic effects on some target cells in culture (*9, 10*). We investigated whether TNF has direct inhibitory effects on c-*myc* expression, determined the level at which the inhibition takes place, and analyzed the effect of cycloheximide on the reduction of c-*myc* expression. Earlier studies showed that, when administered together, TNF and IFN-γ had enhanced antiproliferative effects on certain cell lines (*9*). For our present studies we used HeLa cells, derived from a patient with cervical cancer, and compared the changes in c-*myc* expression after treatment of these cells with each cytokine alone or with the two cytokines in combination.

First we studied the modulation of cell cycle distribution by purified recombinant human TNF (rTNF) and by natural IFN-γ purified to homogeneity. Actively dividing

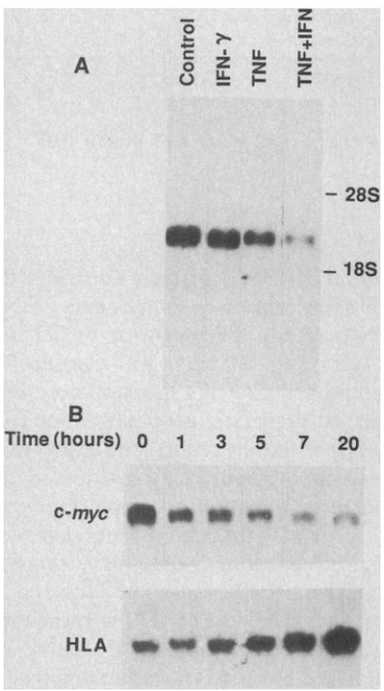

Fig. 1. Reduction of c-*myc* mRNA in HeLa cells by IFN-γ and TNF, alone or in combination. (A) Subconfluent monolayers of HeLa cells were treated for 20 hours with IFN-γ (100 U/ml), TNF (10 ng/ml), or with a combination of the two cytokines in these final concentrations. Total RNA was extracted from the monolayer cells by the urea–lithium chloride procedure and 10-μg samples were analyzed on Northern blots as described (*6*). The probe used was the 1.4-kb Cla I–Eco RI DNA fragment containing the third exon of the human c-*myc* gene (*6*). The autoradiographs were scanned by densitometric tracing. (B) HeLa cells were exposed to the combination of the two cytokines as in (A) and total RNA was extracted at the indicated times (1 to 20 hours). The Northern blots were probed for expression of c-*myc* as in (A) and for class I HLA genes with the HLA-B cDNA clone (*13*).

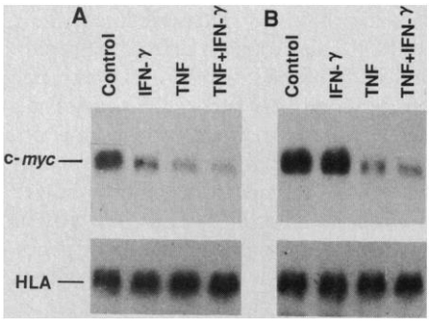

Fig. 2. Effect of cycloheximide on the reduction of c-*myc* by IFN-γ and TNF. Subconfluent monolayers of HeLa cells were incubated for 15 minutes in the presence (B) or absence (A) of CHX (20 μg/ml). Then IFN-γ (100 U/ml) or TNF (10 ng/ml) was added alone or in combination for 3 hours and RNA was extracted and analyzed on Northern blots as in Fig. 1.

subconfluent cultures of HeLa cells were treated for 20 hours with IFN-γ or TNF, or both. The different phases of the cell cycle were then analyzed by flow cytometry. As shown in Table 1, each of the cytokines induced the accumulation of cells in the quiescent G_0/G_1 phase of the cell cycle. The effect of 10 or 100 U of IFN-γ per milliliter (corresponding to 0.25 and 2.5 ng/ml) was more pronounced than that observed after exposing the cells to saturating levels of TNF (100 U/ml, 10 ng/ml). The TNF-mediated change in the proportion of cells in G_0/G_1 was small but reproducible and statistically significant (Table 1). It is interesting that the combination of both cytokines was the most efficient and caused a greater accumulation of cells in the quiescent phase (Table 1). Cell viability remained high in all cultures during the 20-hour treatment period. By trypan blue exclusion test, we found that more than 99% of the monolayer cells were viable. We also measured the cell cycle distribution after incubation of the cytokine-treated cells with a mixture of trypsin and deoxyribonuclease I (DNase I), which hydrolyses DNA in dead cells leaving the viable cells unchanged for DNA analysis (*11*). As shown in Table 1, the relative proportion of cells in the G_0/G_1 phase did not change after treatment with DNase plus trypsin, indicating that the cells that accumulate in the quiescent phase in response to IFN-γ and TNF are viable.

The effects of IFN-γ and TNF on c-*myc* expression were then analyzed by measuring both the steady-state levels of c-*myc* mRNA and the c-*myc* transcription rate in isolated nuclei. Total RNA was extracted from HeLa cells after 20 hours of exposure to the cytokines and analyzed for c-*myc* expression by Northern blot transfer and hybridization to the human c-*myc* genomic probe. As shown in Fig. 1A, TNF by itself reduced c-*myc* mRNA levels in HeLa cells by 50%. The maximum inhibitory effect was achieved with 10 ng/ml (100 U/ml) of this purified preparation. The extent of inhibition by IFN-γ (100 U/ml) was much smaller (about 20%), whereas the combination of the two cytokines was the most effective and reduced c-*myc* mRNA by 80% (Fig. 1A). The reduced amount of c-*myc* mRNA could be detected as early as 1 hour after the beginning of the treatment with the combination of the two cytokines and was maximal by 7 hours (Fig. 1B). Each cytokine added alone also had a rapid inhibitory effect on c-*myc* expression (Fig. 2) (*12*). To compare the changes in c-*myc* mRNA with that of other mRNA's, we rehybridized the blot shown in Fig. 1B with the HLA-B cDNA probe (*13*). In contrast to the reduction in c-*myc* mRNA, the levels of the 1.7-kb class I HLA mRNA were not changed up to 3 hours after treatment with TNF and IFN-γ but then gradually increased (Fig. 1B). In other experiments we found that the increase in HLA mRNA was specifically induced by the IFN-γ present in the mixture, whereas exposure to TNF for 3 to 20 hours resulted in no significant stimulatory effect on HLA expression [see also (*14*)].

We then performed nuclear analyses to determine the level at which the down regulatory effects of each cytokine on c-*myc* expression takes place. The bulk rate of

Table 1. The effect of TNF and IFN-γ on cell cycle distribution of HeLa cells. HeLa cells (H229) growing in RPMI 1640 (Gibco) containing 10% heat-inactivated fetal calf serum were cultured in six-well dishes (Costar) at an initial concentration of 2×10^5 cells per well. The specific activity of rTNF was 10^7 U/mg. The natural IFN-γ (specific activity 4×10^7 U/mg) was prepared and purified according to Novick *et al.* (*17*). Specific activities of each cytokine were determined as described (*10, 17*). Cells were incubated for 20 hours, the monolayers were then trypsinized and flow cytometric analysis was performed on the washed cells after staining them with propidium iodide (*6*). A sample of the trypsinized cells was stained with trypan blue to determine percent viability. Results are the average of quadruplicate determinations ± standard error of the mean.

Treatment	Percent G_0/G_1
1. None	52.5 ± 1.2
2. IFN-γ (10 U/ml)	65.5 ± 1.3
3. IFN-γ (100 U/ml)	65.0 ± 1.3
4. TNF*	58.0 ± 1.2
5. IFN-γ (10 U/ml) + TNF*	75.0 ± 1.7
6. IFN-γ (100 U/ml) + TNF*	76.0 ± 1.8
7. IFN-γ (100 U/ml) + TNF*	74.0 ± 1.7†

*The TNF concentration was 10 ng/ml. †Cells from cultures treated with TNF plus IFN-γ were incubated prior to the propidium iodide staining with DNase I (100 μg/ml, Worthington) and trypsin (2.5 mg/ml, Bio-Lab) in phosphate-buffered saline at 37°C for 30 minutes, and then washed in medium supplemented with 10% fetal calf serum.

RNA production by isolated nuclei unchanged at 3 hours after exposure of HeLa cells to either IFN-γ or TNF or both factors together. However, measurements of the relative rate of c-*myc* mRNA transcription at 3 hours revealed a marked reduction by TNF (10 ng/ml) or by IFN-γ (100 U/ml) (Fig. 3). The TNF-mediated transcriptional inhibition exceeded slightly the reduction by IFN-γ (70% and 55% inhibition, respectively), and the combined effects of the two cytokines together were greater (80% inhibition) than the effect of each agent alone (Fig. 3). As intrinsic control, the same mRNA's that were transcribed in vitro were hybridized to the HLA-B cDNA probe. The HLA mRNA transcription was almost unchanged by TNF and was slightly increased by IFN-γ and by both factors together (1.2-, 1.7-, and 2.5-fold increases, respectively). These results illustrate the selectivity of the inhibitory effects on c-*myc*. The increase in HLA transcription at 3 hours after exposure to IFN-γ was relatively small, consistent with the delayed kinetics of the IFN-γ mediated increase in HLA mRNA steady-state level detected on Northern blots (Figs. 1B and 2) and with previous reports showing that HLA induction by type II IFN is much slower than by type I IFN (*15*). The data in Figs. 1 and 3 show that TNF by itself reduces c-*myc* mRNA levels in HeLa cells as a result of selective transcriptional inhibition. The IFN-γ in this cell system exerts a similar effect, although at a lower efficiency, and together the two cytokines show enhanced inhibitory effects on c-*myc* expression.

The effect of cycloheximide (CHX) on the TNF- and IFN-γ–mediated c-*myc* inhibition was studied to determine whether synthesis of new proteins is required for the reduction in c-*myc* expression. The CHX (20 μg/ml; Sigma) was added to the cells 15 minutes before the addition of the two factors, total RNA was extracted at 3 hours, and equal amounts of RNA were analyzed on Northern blots. As shown in Fig. 2, CHX had no effect on the TNF-mediated reduction of c-*myc* mRNA; the reduction by TNF was 50 and 85% in the absence and presence of CHX, respectively. In contrast, the inhibition exerted by IFN-γ was completely abrogated in the absence of new protein synthesis, and even the threefold potentiation in c-*myc* mRNA caused by the CHX by itself was not prevented by IFN (Fig. 2). Hybridization with the HLA-B cDNA probe used as an intrinsic control illustrates again that the down modulation as well as the CHX-mediated potentiation of expression in control cells are all specific for the c-*myc* gene system, whereas the expression of the class I HLA genes remains unchanged after the different treatments (Fig. 2). We therefore conclude that while continuous protein synthesis is required for IFN-γ–mediated c-*myc* inhibition, the inhibitory effect of TNF is direct and does not depend on protein synthesis.

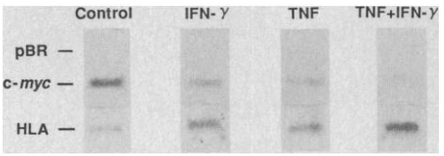

Fig. 3. Analysis of gene transcription after treatment of HeLa cells with IFN-γ or TNF. HeLa cells were treated with each cytokine alone or in combination for 3 hours as described in Fig. 1. Nuclear runoff analysis was performed as detailed elsewhere (*6*). In general, nuclei were isolated from 5×10^7 cells as before (*6*) except that 0.5% Nonidet P-40 (NP-40) was added to the hypotonic buffer. Nuclei (2×10^7) were incubated for 20 minutes at 25°C as described previously (*6*). Recombinant plasmids containing the c-*myc* third exon or the HLA-B cDNA were linearized with Eco RI, and heat denatured. Samples of 10 μg were spotted on nitrocellulose filters and then treated with 0.5*M* NaOH. Hybridization with the in vitro synthesized ^{32}P-labeled RNA (10^7 cpm/ml) and washing of the filter were performed as described previously (*6*). The radioactivity was visualized by autoradiography.

The TNF and IFN-γ molecules interact with different cell surface receptors and exert a different spectrum of biological activities on cells. Here we demonstrate that in HeLa cells each of these cytokines selectively reduces c-*myc* levels as a result of transcriptional inhibition. The differential effect of CHX on the IFN-γ– and the TNF–mediated c-*myc* inhibition suggests that each of the cytokines operates on c-*myc* expression through different molecular mechanisms. Also, the enhanced effects of the two cytokines when administered together support the possibility of different intracellular mediators.

By analyzing cell variants resistant to the suppressive effect of IFN we concluded previously that c-*myc* inhibition is linked to the subsequent IFN mediated G_0/G_1 arrest of cells (*6*). The potentiation of c-*myc* inhibition by the two cytokines added together could be the cause of the enhanced increase in the percentage of cells that accumulate in the G_0/G_1 resting phase of the cell cycle (Table 1). TNF exerts different effects on the cell cycle depending on the type of target cells. In L cells a specific arrest in the G_2 phase has been detected (*11*). In normal fibroblasts, TNF functions as a growth stimulatory rather than inhibitory factor (*9, 16*). It will be of interest to study the effects of TNF on c-*myc* expression in the different cell systems and to analyze in this way the molecular basis of the bifunctional properties of this polypeptide with respect to cellular growth regulation.

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