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Coordinate viral induction of tumor necrosis factor α and interferon β in human B cells and monocytes

(U937, JY, and Namalwa cells/phorbol ester/lipopolysaccharide/2-aminopurine/cycloheximide)

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ABSTRACT **Human tumor necrosis factor α (TNF-α) gene expression can be induced primarily in cells of the monocyte/macrophage lineage by a variety of inducers, including lipopolysaccharide, phorbol esters such as phorbol 12-myristate 13-acetate, and virus or synthetic double-stranded RNA [poly(I)·poly(C)]. In this paper we show that the TNF-α gene also responds to virus and phorbol 12-myristate 13-acetate in B lymphocytes and that virus is the most potent inducer of TNF-α mRNA in both monocyte and B-cell lines. In addition, we show that viral infection coinduces the expression of TNF-α and interferon β mRNA and that viral induction of both genes is blocked by the kinase inhibitor 2-aminopurine. Inhibition of protein synthesis with cycloheximide had no effect on mRNA expression of the genes in one of three cell lines tested (U937) but blocked the viral induction of both genes in another (Namalwa). Thus, the regulatory factors required for mRNA induction of both genes are present prior to the addition of virus in U937 but not in Namalwa cells. However, in a third cell line (JY), cycloheximide blocked viral induction of the interferon β gene but not the TNF-α gene. Taken together, these observations suggest that viral induction of TNF-α and interferon β gene expression may involve overlapping pathways with both common and distinct regulatory factors.**

Tumor necrosis factor α (TNF-α) is a cytotoxic factor originally identified in the serum of mice primed with tubercle bacilli and then challenged with lipopolysaccharide (LPS) (1). The biological activities of TNF-α are diverse and complex (reviewed in refs. 2–4), and they include the ability to inhibit the replication of both DNA and RNA viruses (5, 6). The antiviral activity of TNF-α can be augmented by class I (α and β) (5) or class II (γ) (6) interferons (IFNs), and the combination of IFNs with TNF-α leads to the induction of a common set of proteins (7). Cooperation between TNF-α and IFN-β in particular has been further demonstrated by showing that antibodies against IFN-β reduce TNF-α-mediated antiviral protection (5) and block the ability of TNF-α to induce an 80,000-Da protein associated with the antiviral state (7). TNF-α can also act with IFN-β to promote the differentiation of a myeloid leukemic cell line (8).

Human TNF-α gene expression is induced by LPS (2, 9, 10), by phorbol esters such as 12-myristate 13-acetate (PMA) (3), and by viruses (6, 11, 12) or synthetic double-stranded RNA molecules such as poly(I)·poly(C) (6). IFN-β gene expression is highly inducible by virus or poly(I)·poly(C), but induction of this gene in human cells by LPS or PMA has not been reported (reviewed in ref. 13). To determine whether viral induction of the human TNF-α and IFN-β genes involves common or distinct regulatory pathways, we examined the viral induction of these two genes in different cell types in the presence or absence of 2-aminopurine (2-AP) or cycloheximide (CHX). 2-AP is a protein kinase inhibitor that specifically blocks the viral induction of IFN-β at the level of transcription (14, 15), whereas CHX, an inhibitor of protein synthesis, does not block the induction of IFN-β in cell types thus far examined (16, 17). We observe both similarities and differences in the effects of these inhibitors on viral induction of TNF-α and IFN-β gene expression in different cell lines. Therefore, viral induction of the TNF-α and IFN-β genes appears to involve overlapping, but distinct, regulatory pathways.

TNF-α is thought to be produced principally by cells of the monocyte/macrophage lineage (2, 3), whereas IFN-β was originally identified as a fibroblast IFN (13). Although both TNF-α and IFN-β respond to viral induction, their coinduction in a single cell type has not been reported. In this study, we show that the TNF-α and IFN-β genes are coinduced by virus in two human B-cell lines, a monocyte-like cell line, and B lymphocytes separated from peripheral blood. These observations extend the tissue-specific expression of TNF-α and IFN-β to include B lymphocytes.

MATERIALS AND METHODS

Preparation and Analysis of RNA. RNA was prepared as previously described (18) from U937, JY, and Namalwa cells. Uniformly ^{32}P-labeled SP6 RNA probes were prepared as described (18) from SP6 γ actin and SP6 IFN Aha* (16). The TNF-α probe was prepared from a plasmid containing a phage T7 promoter (T7 TNFM Bam). T7 TNFM Bam (Fig. 1*B*) was constructed by first inserting a *Bam*HI linker into PLTNF, a plasmid containing the TNF-α gene (a gift of D. Goeddel, Genentech). A 491-base-pair *Sty* I fragment was then isolated and inserted into the *Eco*RV site in the plasmid Psp 70 (19) (the ends of the *Sty* I fragment were first filled in using the Klenow fragment of DNA polymerase I). Prior to *in vitro* transcription with T7 RNA polymerase, T7 TNFM Bam was linearized with *Xho* I, resulting in a probe that protects a 306-nucleotide fragment. RNA preparations from U937, JY, or Namalwa cells were hybridized simultaneously with the three ^{32}P-labeled probes as described (18). The γ-actin probe was made to have a specific activity that was 5 times less than that of the TNF-α and IFN-β probes. The RNA fragments protected from RNase cleavage were electrophoresed on a 6% denaturing polyacrylamide gel.

Cell Culture and Induction Protocols. U937, Namalwa, and JY cells were grown in RPMI (Hazleton Biologics, Denver, PA) supplemented with 10% fetal calf serum, glutamine, penicillin, streptomycin, and nonessential amino acids, at a density of 0.6–1 × 10^6 cells per ml. Aliquots of 6 × 10^6 cells were left unstimulated or were stimulated by the direct addition of the various inducers as indicated: 5% (vol/vol) Sendai virus (Hazleton Biologics) for 8 hr, PMA (Sigma) at 5 ng/ml for 4 hr, or LPS (*Escherichia coli* 026:B6; Sigma) at 1 μg/ml for 2 hr. CHX (Sigma) at 50 μg/ml and or 10 mM 2-AP (Sigma) were incubated alone or coincubated with the various inducers.

Abbreviations: TNF-α, tumor necrosis factor α; IFN, interferon; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; 2-AP, 2-aminopurine; CHX, cycloheximide.

Isolation of Peripheral B Lymphocytes. Three hundred milliliters of peripheral blood was collected from a healthy donor, and the lymphocyte fraction was separated by Ficoll/Hypaque centrifugation. T cells were depleted with sheep erythrocytes treated with 2-aminoethylisothiouronium bromide. Macrophages were removed by adherence on a plastic tissue culture dish (Falcon) overnight at 37°C. A second stage depletion of residual T cells and macrophages was then performed on the remaining cells by complement lysis employing monoclonal antibodies to T3 (CD3; American Type Culture Collection) and Mo2 (CD14; Coulter). The remaining B cells (1.5×10^7 cells), predicted to be 95–99% pure, were then plated at a density of 0.6×10^6 cells per ml in RPMI (Hazleton Biologics; supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin). Half of the cells were stimulated with Sendai virus as described above, and the other half were left unstimulated. RNA was prepared, and 1.5 μg of RNA was hybridized with the T7 TNFM Bam, SP6 IFN Aha*, and SP6 γ-actin probes and processed as described above except that all three probes were made to be of the same specific activity.

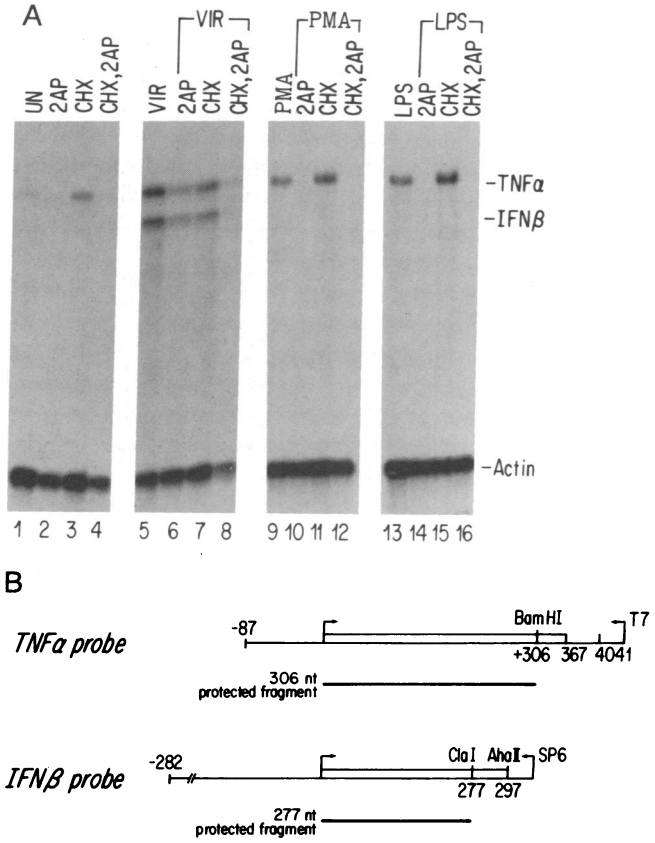

FIG. 1. Induction and inhibition of TNF-α and IFN-β mRNA levels in U937 cells. (*A*) The levels of accurately initiated mRNAs were determined by quantitative RNase protection (18) using the uniformly ^{32}P-labeled RNA probes diagrammed in *B*. An RNA probe complementary to the endogenous γ-actin gene (16) was also used as an internal control for mRNA levels. Assays were carried out at the times of maximal levels of expression observed with each inducer (data not shown). Times postinduction: LPS, 2 hr; Sendai virus, 8 hr; PMA, 4 hr. The level of induction of TNF-α mRNA by virus (lane 5) is higher than the levels achieved after PMA (lane 9) or LPS induction (lane 13). CHX alone induces TNF-α (lane 3) and superinduces the gene in combination with LPS (lane 15). A slight decrease in the level of viral induction of both TNF-α and IFN-β mRNA is observed in the presence of CHX (lane 7). IFN-β is induced by virus (lane 5) but not by CHX, PMA, or LPS (lanes 3, 9, and 13). 2-AP alone had no effect on TNF-α mRNA expression in U937 cells when normalized to actin (lane 2). However, it blocked induction of TNF-α by all of the inducers (lanes 4, 6, 8, 10, 12, 14, and 16) and blocked viral induction of IFN-β (lane 6). un, Uninduced; vir, virally induced. (*B*) Diagram showing the RNA probes used to map accurately initiated TNF-α and IFN-β mRNAs. The rightward arrows indicate the site of initiation of transcription, the open boxes designate transcribed sequences, and the numbers indicate the number of nucleotides (nt) upstream (−) or downstream (+) from the site of transcriptional initiation. The leftward arrows indicate the starting point of transcription of the T7 and SP6 RNA probes. The protected fragment is indicated by the solid line. The SP6 γ-actin probe is described elsewhere (16).

RESULTS

We examined the effects of Sendai virus infection or treatment either with PMA or LPS on the level of TNF-α and IFN-β mRNA expression in Namalwa cells (a Burkitt's lymphoma B-cell line), JY cells (an Epstein–Barr virus-transformed B-cell line), and in the monocyte-like cell line U937. Accurately initiated TNF-α and IFN-β mRNAs were measured by quantitative RNase mapping (18). We found that virus induces TNF-α mRNA in all three cell lines (lane 5 of Figs. 1*A*, 2, and 3), but only the U937 cells express TNF-α mRNA in response to all three inducers (Fig. 1*A*, lanes 5, 9, and 13). LPS did not induce TNF-α mRNA in either of the B-cell lines (data not shown), and PMA only induced the gene in JY cells (Fig. 3, lane 9). Significantly, in U937 cells, the highest level of TNF-α mRNA was observed after viral induction (Fig. 1*A*, lanes 5, 9, and 13). In a parallel study, we found that virus, but not PMA or LPS, induction of U937 cells results in a significant increase in the level of secreted TNF-α protein (data not shown).

The IFN-β gene is also inducible by virus in all three cell lines, but it is not induced by LPS or PMA in any of them (Figs. 1*A*, 2, and 3). Although class I IFN genes are inducible by LPS in mouse peritoneal macrophages, the effects of LPS on the human IFN-β gene have not been characterized (13). The kinetics of IFN-β and TNF-α mRNA induction by virus are similar but differ significantly from the kinetics of TNF-α mRNA induction by PMA or LPS (data not shown). These observations suggest that viral induction of IFN-β and TNF-α mRNA involves a common and physiologically sig-

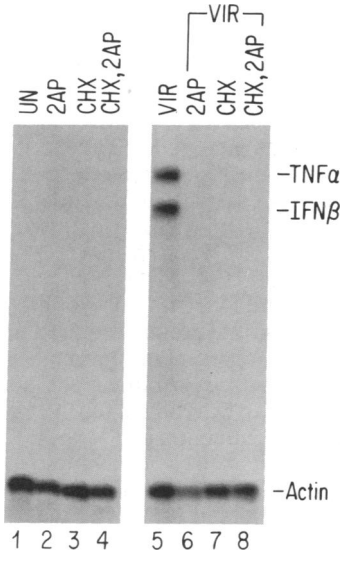

FIG. 2. Induction and inhibition of TNF-α and IFN-β mRNA levels in Namalwa cells: quantitative RNase mapping of TNF-α and IFN-β mRNAs. TNF-α and IFN-β mRNAs were not induced by PMA, LPS (data not shown), or CHX (lane 3) in Namalwa cells. Both genes were induced by virus (lane 5). Viral induction was blocked by 2-AP (lane 6), CHX (lane 7), and CHX plus 2-AP (lane 8). 2-AP alone had no effect on the expression of either gene (lane 2). un, Uninduced; vir, virally induced.

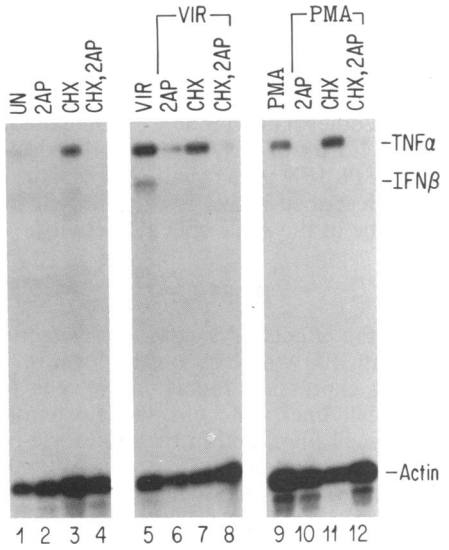

FIG. 3. Induction and inhibition of TNF-α and IFN-β mRNA levels in JY cells: quantitative RNase mapping of TNF-α and IFN-β mRNAs. Both genes were induced by virus (lane 5). IFN-β mRNA was not induced by CHX (lane 3), PMA (lane 9), or LPS (data not shown). TNF-α was not induced by LPS (data not shown) or by CHX when normalized to the level of actin mRNA (lane 3) but was induced by PMA (lane 9). Although CHX blocked viral induction of IFN-β mRNA, it did not block viral induction of TNF-α mRNA (lane 6). In combination with PMA, however, CHX superinduced TNF-α mRNA (lane 11). 2-AP blocked the viral induction of both TNF-α and IFN-β by virus (lane 5). 2-AP also blocked constitutive TNF-α expression (lane 2) and blocked TNF-α induction by PMA (lane 10). un, Uninduced; vir, virally induced.

nificant pathway, which is distinct from the pathways that mediate TNF-α mRNA induction by LPS and PMA.

The Effects of 2-AP and CHX on TNF-α and IFN-β Gene Expression. To investigate the relationship between the pathways of viral induction of IFN-β and TNF-α mRNAs, we compared the effects of 2-AP and CHX on viral induction of the two genes. As shown in lane 6 of Figs. 1*A*, 2, and 3, viral induction of both TNF-α and IFN-β mRNA was inhibited by 2-AP in all three cell lines, although there was variation in the extent of inhibition in different cell lines. To rule out the possibility that 2-AP is acting as a general transcriptional inhibitor, we demonstrated that 2-AP does not inhibit heat induction of the endogenous *hsp70* gene in U937 cells and Namalwa cells [data not shown, see also Zinn *et al.* (14)].

The effects of CHX on viral induction of TNF-α and IFN-β mRNA differ between cell types. In contrast to earlier studies in other cell types (16, 17) and in contrast to U937 cells (Fig. 1*A*, lane 7), viral induction of the IFN-β gene is blocked by CHX in Namalwa and JY cells (lane 7 of Figs. 2 and 3). These observations suggest that factors required for viral induction of the IFN-β gene are present in some cells prior to induction but must be synthesized in other cells after induction. A similar conclusion can be reached for the TNF-α gene. CHX does not block viral induction of TNF-α mRNA in U937 and JY cells (lane 7 of Figs. 1*A* and 3) but does block induction in Namalwa cells (Fig. 2, lane 7). Furthermore, the IFN-β and TNF-α genes differ in their sensitivity to CHX in a single cell type, JY cells. In these cells, viral induction of IFN-β but not TNF-α is blocked by CHX (Fig. 3, lane 7). The fact that TNF-α and IFN-β appear to be differentially sensitive to CHX in the same cell line indicates that, although these genes may share a common regulatory pathway, different sets of factors may be required for viral induction in each case.

Previous studies have demonstrated that CHX alone is an inducer of IFN-β mRNA (16, 17). We find that IFN-β mRNA is not inducible by CHX in the cell lines studied here (lane 3 of Figs. 1*A*, 2, and 3). Similarly, the TNF-α gene is not inducible by CHX in Namalwa or JY cells [when the TNF-α band in Fig. 3, lane 3 is normalized to the actin band, the levels of TNF-α mRNA before (lane 2) and after (lane 3) CHX treatment are the same]. However, the TNF-α gene is inducible by CHX alone in U937 cells (Fig. 1*A*, lane 2). CHX induction of gene expression may be a consequence of the loss of a labile repressor that must be constitutively expressed to maintain repression. If this is the case, differences in CHX inducibility of the IFN-β and TNF-α genes in different cell lines may reflect cell line-specific differences in the relative roles of positive and negative regulatory factors. Interestingly, CHX induction of TNF-α mRNA in U937 cells is blocked by 2-AP (Fig. 1*A*, lanes 3 and 4). Similarly, 2-AP blocks the constitutive expression of the TNF-α gene in JY cells (Fig. 3, lane 2). These observations suggest that the activities of factors involved in the constitutive expression of the TNF-α gene are blocked by 2-AP.

In addition to the inhibitory effects of 2-AP on viral induction, we find that this compound also blocks LPS and PMA induction of TNF-α in U937 cells (Fig. 1*A*, lanes 10 and 14) and PMA induction of TNF-α in JY cells (Fig. 3, lane 10). Both PMA and LPS are thought to act through stimulation of protein kinase C (20, 21). Whether 2-AP inhibition of PMA and LPS induction of TNF-α has a direct effect on protein kinase C or on a more distal step in the induction process remains to be determined. In contrast to 2-AP, CHX does not block the induction of TNF-α mRNA by PMA in U937 or JY cells (lane 9 of Figs. 1*A* and 3) and superinduces TNF-α mRNA levels when combined with LPS in U937 cells (Fig. 1*A*, lane 13). In summary, we observe a differential effect of CHX on PMA and LPS induction of TNF-α in U937 cells and, as mentioned above, PMA, but not LPS, induces TNF-α mRNA in JY cells. Therefore, although 2-AP blocks TNF-α induction by both PMA and LPS, these inducers mediate pathways that are at least in part independent.

Cell Type Specificity of TNF-α and IFN-β Gene Expression. According to current dogma, TNF-α is produced primarily in cells of the monocyte/macrophage lineage, whereas the closely linked cytotoxic factor TNF-β (lymphotoxin) is derived from activated T lymphocytes (reviewed in refs. 2 and 3). Although a previous study demonstrated that cells of B-lymphocyte lineage can produce cytotoxic factors in response to PMA, these factors were not identified as TNF-α (22). We have shown that TNF-α mRNA is transcribed in B-lymphocyte cell lines constitutively (Fig. 3, lane 1) as well as in response to virus (lane 5 of Figs. 2 and 3) and PMA (Fig. 3, lane 9). We have also demonstrated that TNF-α protein increases upon viral induction in these B-cell lines (data not shown). To determine whether primary B cells also produce TNF-α mRNA, we separated B cells from peripheral blood and stimulated them with virus. As shown in Fig. 4, the TNF-α gene is highly inducible by virus in peripheral B cells.

The IFN-β gene is thought to be expressed primarily in fibroblasts, although its expression has also been demonstrated in virally induced unseparated peripheral blood leukocytes and Namalwa cells (23, 24) and in murine monocytes/macrophages (13). We have shown that the IFN-β gene is inducible by virus in the human monocyte-like U937 cell line (Fig. 1*A*, lane 5) and in the two human B-cell-derived lines JY and Namalwa (lane 5 of Figs. 2 and 3). As shown in Fig. 4, the IFN-β gene is also highly inducible in peripheral B cells. Viral induction of IFN-β gene expression has also been observed in another independently derived human monocyte line, BT4 cells (A.E.G., B. Walker, R. Schooley, and T.M., unpublished results) and in the T-cell tumor line HPBALL (data not shown).

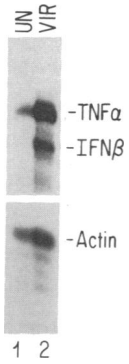

FIG. 4. Viral induction of TNF-α and IFN-β mRNA levels in human B lymphocytes: quantitative RNase mapping of TNF-α and IFN-β mRNAs. RNA prepared from uninduced (lane 1) and virally induced (lane 2) B lymphocytes was analyzed as described in Figs. 1–3. Uninduced peripheral B cells (lane 1) produce a small amount of TNF-α mRNA. Both the TNF-α and IFN-β genes are highly inducible by virus (lane 2). The top portion of the gel is a longer autoradiographic exposure than the bottom portion.

DISCUSSION

We have demonstrated that the human TNF-α and IFN-β genes are coordinately induced by virus in a monocyte-like cell line, two B cell lines, and in circulating peripheral B lymphocytes. The kinetics of viral induction of TNF-α and IFN-β are similar, and induction of both genes is blocked by the kinase inhibitor 2-AP. Viral induction of both genes is blocked by CHX in one cell line (Namalwa) and not blocked in another (U937). Taken together, these observations suggest that both genes may be activated by virus through the same regulatory pathway. However, in one cell line (JY) induced with virus, IFN-β mRNA expression is blocked by CHX, but TNF-α mRNA expression is not. Thus, although the regulatory pathways may overlap, at least one of the regulatory factors required for IFN-β gene expression is not required for the expression of the TNF-α gene. Although the TNF-α gene does contain a repeated hexamer motif sequence in its first intron, which is similar to sequences found in the IFN-β promoter necessary for viral induction (25), these sequences are not required for viral induction of TNF-α (A.E.G. and T.M., unpublished data). We conclude that early steps in the viral induction pathway may be shared by the two genes, but the regulatory elements that mediate viral induction and the factors that bind to these elements are, at least in part, distinct.

The CHX inhibition studies described above suggest that cell lines differ in their ability to constitutively produce regulatory proteins involved in viral induction. In Namalwa cells, for example, the observation that viral induction of TNF-α and IFN-β is blocked by CHX indicates that the regulatory factors required for mRNA expression of both genes are produced only after induction. However, previous studies have shown that viral induction of the IFN-β gene in fibroblast cell lines is not prevented by CHX (15, 19), and we find that CHX does not prevent the viral induction of both genes in U937 cells. Thus, in these fibroblast and U937 cells, the transcription factors essential for mRNA expression are present prior to induction. Cell type differences in the effects of CHX on gene expression are not unprecedented. For example, CHX blocks the activation of a number of IFN-inducible genes in HeLaM cells but has no effect in other cell types (26).

Previous studies have shown that 2-AP blocks viral induction of IFN-β and the serum induction of the c-*fos* and c-*myc* cellular oncogenes (14, 15). In this paper, we show that 2-AP blocks viral induction of TNF-α and IFN-β mRNA. Although the mechanism by which 2-AP blocks viral induction of TNF-α and IFN-β gene expression is not understood, it is possible that it acts by inhibiting a protein kinase. This possibility is based on the following observations: (*i*) 2-AP has been shown to inhibit two kinases *in vitro*, the heme-dependent and the double-stranded RNA-dependent eukaryotic initiation factor 2α kinases (27). (*ii*) Double-stranded RNA is the putative intracellular signal that activates IFN expression upon viral induction (28). (*iii*) In certain cell lines the IFN-β gene is not inducible by virus, but this block to induction can be overcome by priming the cells with IFN-α or -β (16). The double-stranded RNA-dependent eukaryotic initiation factor 2α kinase is inducible by IFN-α or -β and activated by double-stranded RNA (13). Although 2-AP may also inhibit other kinases, it does not appear to be a general kinase inhibitor (C. Baglioni, personal communication). In summary, 2-AP may block viral induction of TNF-α and IFN-β by inhibiting a protein kinase that modifies factors required for the expression of the two genes.

We note that 2-AP also blocks induction of TNF-α mRNA by LPS and PMA. Although both of these inducers activate the protein kinase C pathway (20, 21), we do not know if 2-AP directly blocks protein kinase C activity or a later step in the pathway that may be shared with the viral induction pathway. Although our data do not rule out the possibility that the pathways that mediate LPS and PMA induction of TNF-α mRNA overlap with the viral induction pathway, they do demonstrate that the pathways are distinct. Moreover, the pathways that mediate LPS and PMA induction of TNF-α mRNA are themselves differentiable.

We have shown that TNF-α mRNA is highly inducible by virus in B lymphocytes. We have also shown that IFN-β mRNA is highly inducible by virus in human cell lines of monocyte and B-cell derivation as well as in peripheral B lymphocytes. The expression of these genes in numerous cell types suggests that their biological roles are broader than previously believed. For example, TNF-α is a potent inducer of the B-cell growth factor interleukin 6 (29, 30). Viral induction of TNF-α in B cells could therefore lead to lymphocyte proliferation by means of the production of interleukin 6. TNF-α may in this way enhance the growth of cells in which it is produced and may also play a role in the stimulation and maintenance of elevated levels of circulating lymphocytes associated with viral infection.

The observation that TNF-α and IFN-β share a regulatory pathway in the same cell types is particularly interesting in light of the shared and synergistic activities of their gene products. TNF-α can act with IFN-β to inhibit viral replication (5, 6), to promote the differentiation of a myeloid leukemic cell line (8), and to induce a common set of proteins (7). Virus may therefore provide an important inductive signal for other biological activities of these gene products. Additional functions attributed to TNF-α include inhibition of lipoprotein lipase (2, 31), stimulation of leukocyte adhesion (32) and procoagulant (33, 34) properties of vascular epithelium, induction of hematopoietic growth factors (29, 35–37), and mediation of septic shock (reviewed in refs. 2 and 38). In the cell lines studied here, virus is a more potent inducer of TNF-α mRNA and protein than is LPS. This suggests that TNF-α alone may not be the major protein mediator of septic shock in humans. It is also intriguing to speculate that TNF-α could be involved in the shock, wasting, or inflammatory syndromes that accompany some viral infections. Exploration of the transcriptional regulation of TNF-α and IFN-β should provide new insights into viral pathogenesis as well as shed light on the general mechanisms by which eukaryotic genes are regulated by virus, PMA, and LPS.

Note Added in Proof. After this manuscript was submitted for publication, we became aware of independent studies that demon-

strate the constitutive production and mitogen inducibility of TNF-α mRNA and protein in human B cells (39).

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