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Molecular and Functional Characterization of Turkey Interferon

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The turkey interferon (TkIFN) gene encodes a signal peptide and a mature protein of 30 and 162 amino acids, respectively. TkIFN mRNA expression was induced by reoviral double-stranded RNA in fibroblasts. The recombinant TkIFN protein possessed species-specific antiviral activity and in synergy with lipopolysaccharide (LPS) induced bone marrow macrophages to produce nitric oxide (NO). LPS or TkIFN alone did not induce bone marrow macrophages to produce significant amounts of NO, which showed that TkIFN provided one of the two signals necessary to induce NO production in turkey macrophages. Unlike the anti-inflammatory nature of mammalian alpha/beta IFNs, TkIFN augmented the LPS-induced expression of interleukin-8, a proinflammatory cytokine. This finding suggests a role for TkIFN in inflammatory conditions.

The interferons (IFNs) represent a family of cytokines that share antiviral, as well as immunomodulatory, and antiproliferative effects on cell functions (4, 31). On the basis of several criteria, the IFNs in mammals have been divided into five distinct classes termed IFN-α, -β, -ω, and -τ (type I IFNs; and hereafter referred to as IFN-α/β) and IFN-γ (type II IFN) (5, 28). IFN was first described in embryonated hens' eggs (12); only recently was the chicken IFN (ChIFN) gene cloned (25). The degree of conservation of the IFN gene among avian species is not known, nor has the cross-reactivity of IFNs been carefully examined. Furthermore, the existence of a division of the avian IFN system into two types is presently not well defined (7). Because of the growing economic importance of the turkey, it has become essential to better understand the innate and the antigen-specific immune system of this animal. Since the biological roles of IFNs are usually species specific, it became necessary to clone and characterize the turkey IFN (TkIFN) gene. In this study, our objective was to clone and express the TkIFN gene and then examine the recombinant TkIFN (r-TkIFN) for species specificity and for antiviral and immunomodulatory activities.

The TkIFN gene was amplified from genomic DNA by PCR (25 pmol of each primer, 200 μmol of each deoxynucleoside triphosphate, 2.5 U of *Pfu* polymerase, 10 μl of 10× *Pfu* polymerase buffer, and 100 ng of template DNA), using primers based on the nucleotide sequence of the ChIFN gene (25). The sequences of the sense and the antisense primers were 5′-ATG GCT GTG CCT GCA AGC CCA-3′ and 5′-AGT GCG CGT GTT GCC TGT GA-3′, respectively. The amplified DNA fragment was cloned into the eukaryotic expression vector pcDNA3 (Invitrogen, San Diego, Calif.). The size of the DNA fragment resulting from the PCR amplification of the turkey genomic DNA was comparable to the size of the coding region for the ChIFN cDNA clone (25). This finding suggested that like IFN-α/β genes of mammalian species (9), the avian IFN genes may lack introns. The nucleotide sequence of the double-stranded DNA template was elucidated by using the Taq DyeDeoxy Terminator Cycle sequencing kit (Applied Biosystems, Foster City, Calif.). The nucleotide sequence and the predicted amino acid sequence of the PCR-amplified product are shown in Fig. 1. Sequence analysis indicated the following: (i) the cloned fragment contained a single open reading frame which had 91 and 82% identity with the ChIFN gene at the nucleotide and amino acid sequence levels, respectively; (ii) the 30-amino-acid hydrophobic signal sequence is present at the amino terminus; (iii) mature TkIFN begins at amino acid residue 31 (cysteine) of the deduced primary translation; and (iv) the mature TkIFN appears to consist of 162 amino acids, including six cysteines at amino acid residues 1, 30, 37, 98, 124, and 137. Four of these six cysteines (at positions 1, 30, 98, and 137) are conserved among most of the mammalian IFN-α proteins (8). With a deduced molecular mass of 18.85 kDa, the mature TkIFN has two potential glycosylation sites at amino acid residues 40 to 42 and 47 to 49. These data strongly suggested that the cloned fragment was the turkey homolog of the ChIFN gene.

After the nucleotide sequence of the TkIFN gene was confirmed, we tested the ability of the cloned fragment of DNA to express a biologically active protein with antiviral activity. The r-TkIFN was expressed in vitro by transfecting COS cells (generously provided by L. Schiff, University of Minnesota) with the recombinant plasmid pcDNA3 carrying the TkIFN gene downstream of the cytomegalovirus promoter (21). The supernatant from COS cells transfected with the control nonrecombinant plasmid had no IFN activity, whereas supernatants from cells transfected with the IFN plasmid showed high levels of antiviral activity. Three different transfections of COS cells with the TkIFN plasmid produced supernatants with antiviral activities of 12,800, 25,600, and 51,200 U/ml (1 U of IFN is defined as the amount of antiviral activity present in the highest dilution of the COS cell supernatant that conferred complete protection against the cytopathic effect of vesicular stomatitis virus in turkey embryo fibroblasts [TEFs]) when used to neutralize the cytopathic effect of vesicular stomatitis virus on TEFs made from 14-day-old embryos (26). The antiviral activities of the r-TkIFN and IFN from the supernatants of TEFs stimulated with avian reovirus were not affected by overnight exposure to pH 2 (data not shown), suggesting that TkIFN was acid stable. These data indicated that the protein from the in vitro expression of the cloned TkIFN gene had acid-stable antiviral activity similar in nature to that of mammalian IFN-α/β.

Because the biological properties of the r-TkIFN was similar to those of mammalian IFN-α/β, we were interested in TkIFN

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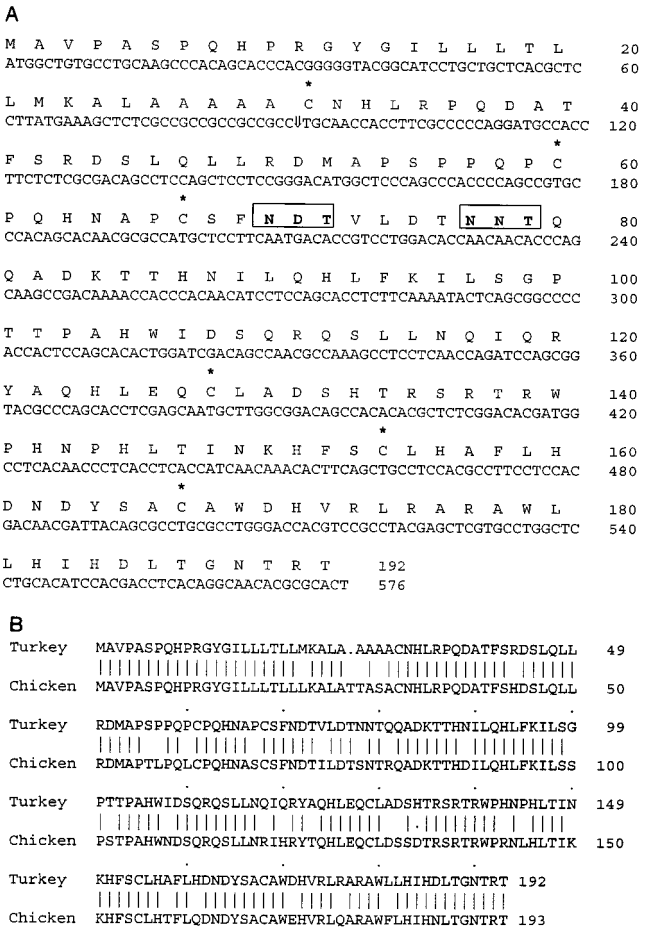

FIG. 1. (A) TkIFN gene nucleotide and predicted amino acid sequences. The nucleotide sequence was determined from two clones derived from two different PCRs. The predicted site for signal peptide cleavage is shown by the arrow. Cysteine residues are indicated by asterisks. The boxes represent potential glycosylation sites. (B) Alignment of chicken and turkey IFN amino acid sequences. Identical amino acid residues are indicated by vertical lines.

expression in TEFs upon exposure to reoviral double-stranded RNA (dsRNA). To examine the induction of TkIFN mRNA transcription and its relationship to the secretion of TkIFN protein, RNA and conditioned medium from reovirus-stimulated TEFs were collected at various time points from 0 to 24 h poststimulation. Total cellular RNA was extracted from reovirus-induced TEFs by using TRIzol reagent (GIBCO/BRL, Gaithersburg, Md.). Approximately 10 μg of total RNA was fractionated by electrophoresis in a 1% agarose gel containing 2.2 M formaldehyde and transferred to nylon membranes in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (24). Hybridization was performed with ^{32}P-random primer-labeled TkIFN DNA at 2×10^6 cpm/ml overnight. Filters were washed twice with 2× SSC–0.1% sodium dodecyl sulfate (SDS) at 42°C for 30 min and with 0.2× SSC–0.1% SDS at 42°C for 30 min before autoradiographic exposure. After probing of blots for IFN mRNA, the blots were reprobed with chicken β-actin cDNA (15). The autoradiographic signals were quantitated by densitometry (Molecular Dynamics Personal SI; using I. P. Lab gel software version 1.5 h for the Macintosh) and corrected on the basis of β-actin mRNA concentration in the same lane. The results of the Northern (RNA) blot analysis to quantitate mRNA expression at different intervals after reovirus stimulation are depicted in Fig. 2. The data show that the TkIFN gene was expressed in TEFs upon stimulation with UV-inactivated avian reovirus. The steady-state levels of mRNA for TkIFN increased in the reovirus-stimulated TEFs in a time-dependent fashion, being first detectable at 5 h, attaining peak levels at 14 h, and then rapidly declining thereafter. After 24 h of exposure to reovirus, the cellular expression of TkIFN mRNA was barely detectable. No TkIFN mRNA was detected in the mock-stimulated TEFs at any time. We also examined if TkIFN mRNA induction was dependent on protein synthesis by Northern blot analysis of the RNA extracted from reovirus-exposed TEFs (10 and 14 h postexposure) with or without cycloheximide (50 μg/ml) in the medium. Cycloheximide abrogated the induction of TkIFN gene expression (data not shown) by reoviral dsRNA in TEFs, suggesting that protein synthesis may be necessary for the induction of steady-state levels of TkIFN mRNA, which is similar to what has been reported for ChIFN (25).

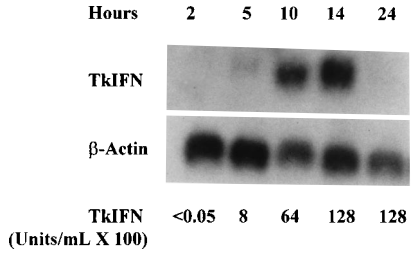

FIG. 2. Kinetics of TkIFN and TkIFN mRNA expression in TEFs exposed to UV-inactivated avian reovirus.

The conditioned medium from reovirus-exposed TEFs was examined for antiviral activity by the IFN assay. The results of the IFN assay shown in Fig. 2 indicate that IFN activity was first detectable in the TEF supernatants at 5 h, with peak IFN levels being attained at 10 to 24 h after reovirus exposure. Taken together, these data indicated that TkIFN mRNA and protein expression attained peak levels concomitantly in TEFs exposed to reovirus. Thus, TkIFN is strikingly similar to IFN-β of mammals, a type I IFN (also known as fibroblast IFN), induced in fibroblasts by viruses, lipopolysaccharide (LPS), and double-stranded RNA (28).

We were also interested in the relationship of TkIFN expression with that of other cytokines induced by reovirus in TEFs. IFN-β has been reported to downregulate interleukin-8 (IL-8) expression in human fibroblasts (20). 9E3/CEF4 is an avian cytokine induced in chicken embryo fibroblasts and chicken monocytes by inflammatory stimuli (3) and LPS, respectively (2). 9E3/CEF4 is suspected to be the avian homolog of the mammalian IL-8 (3, 29). We examined the induction of 9E3/CEF4 gene expression in reovirus-stimulated TEFs. RNA from reovirus-stimulated TEFs was collected at various time points from 0 to 24 h poststimulation and analyzed by Northern hybridization as described above for the induction of TkIFN mRNA by dsRNA. The cDNA for 9E3/CEF4 kindly provided by H. Hanafusa (The Rockefeller University, New York, N.Y.) was used as a probe. As shown in Fig. 3, the steady-state level of 9E3/CEF4 mRNA was not detected at 2 h, first became detectable at 5 h, and attained peak levels at 24 h, the last time point examined. Attainment of peak levels of 9E3/CEF4 mRNA coincided with the occurrence of two other events: (i) IFN mRNA declining to undetectable levels and (ii) the attainment of high levels of IFN activity in the medium. Studies with human fibroblasts showed that exogenous IFN-β inhibited the poly(I-C)-induced accumulation of IL-8 mRNA

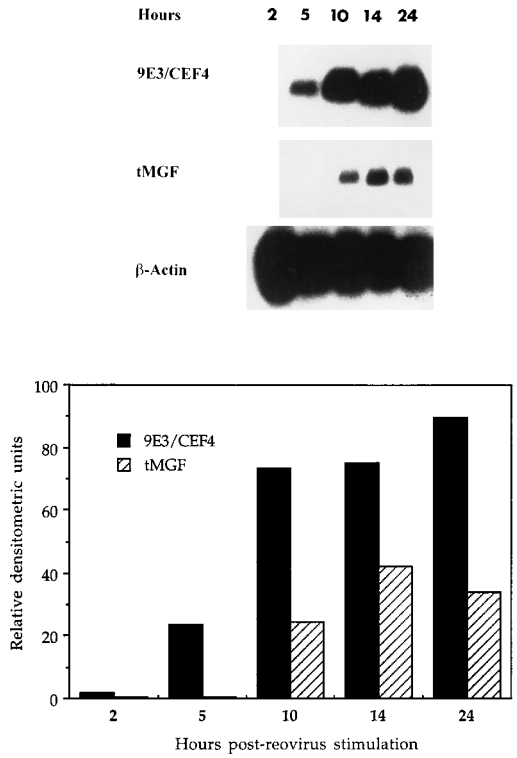

FIG. 3. Kinetics of the induction of 9E3/CEF4 and tMGF mRNAs in TEFs exposed to UV-inactivated avian reovirus.

(20). We theorize that like reovirus, poly(I-C), a potent inducer of IFN-β (20), may stimulate the expression and accumulation of IL-8 mRNA in human fibroblasts, quite possibly in the presence of endogenous IFN-β. However, in the presence of exogenous IFN-β during the initial stimulation with poly(I-C), IL-8 mRNA was not detected (20). More studies are necessary to critically examine the autocrine effects of TkIFN on its own mRNA or other cytokine mRNAs.

IL-6 mRNA levels have been shown to increase in fibroblasts upon exposure to dsRNA (23). We examined in reovirus-stimulated TEFs the expression of the turkey myelomonocytic growth factor (tMGF), a homolog of the cytokine chicken MGF (cMGF). cMGF is an avian cytokine related to IL-6 that can be induced in chicken macrophages by bacterial LPS (16). Experiments in a chicken liver cell assay have shown that recombinant cMGF can act like IL-6 to induce acute-response genes (16). Total cellular RNA was extracted from reovirus-induced TEFs at various intervals as described for 9E3/CEF4 and examined for tMGF mRNA levels by Northern hybridization as described above. The cMGF probe (388 bp) used to detect tMGF was generated by reverse transcription-PCR using RNA isolated from LPS-stimulated HD-11 cells (a retrovirus-transformed chicken macrophage cell line obtained from K. Klasing, University of California, Davis) and primers designed from the published cDNA sequence for the cMGF (16). Our studies revealed that reovirus dsRNA induced the expression of tMGF in TEFs (Fig. 3). tMGF mRNA was expressed at very high levels at 10, 14, and 24 h poststimulation, similar to 9E3/CEF4 mRNA. This observation is in agreement with the report that dsRNA was a potent inducer of IL-6 expression in fibroblasts (23).

IFNs generally have been considered host species specific (13). The species-specific activities of r-TkIFN and r-ChIFN

TABLE 1. Cross-species activity of r-TkIFN and r-ChIFN and the effect of anti-ChIFN antibody on the antiviral effect[a]

Recombinant IFN source	Antibody	IFN titer (U/ml) in embryonic fibroblasts from:	
		Turkey	Chicken
Turkey	NRS	640	40
	Anti-ChIFN	<10	<10
Chicken	NRS	640	1,280
	Anti-ChIFN	<10	10

[a] A 50-μl volume of a serial dilution of either r-ChIFN or r-TkIFN was mixed with an equal volume of a 1:100 dilution of normal rabbit serum (NRS) or rabbit anti-ChIFN antibody and incubated at 37°C for 4 h. At the end of the incubation period, the mixtures were transferred onto secondary TEFs and examined for protection against vesicular stomatitis virus-induced cytopathic effect by the IFN bioassay.

(kindly provided by Syntro Research Laboratories, San Diego, Calif.) were examined by testing their antiviral activities on TEFs and CEFs by the standard vesicular stomatitis virus-based IFN assay (26, 27). As shown in Table 1, TkIFN exhibited species-specific antiviral activity. The titers of TkIFN on CEFs and TEFs were 40 and 640 U/ml, respectively. ChIFN showed a lesser degree of species specificity; the titers of ChIFN on CEFs and TEFs were 1,280 and 640 U/ml, respectively.

The neutralizing capacity of rabbit anti-ChIFN antibody (kindly provided by Syntro Research Laboratories) against ChIFN and TkIFN was measured by the constant-antibody-concentration method (14). Data in Table 1 also show that a 1:200 dilution of anti-ChIFN antibody completely abrogated the antiviral activity of TkIFN at all dilutions on both TEFs and CEFs. The same dilution of the anti-ChIFN antibody completely neutralized the activity of ChIFN on TEFs. However, the lowest dilution of ChIFN was not neutralized by anti-ChIFN antibodies on CEFs, which correlates with the observed twofold higher antiviral activity of ChIFN on CEFs compared with TEFs. These data show that the TkIFN and ChIFN are antigenically similar.

Mammalian IFNs have been demonstrated to possess immunomodulatory activities (18). Activated rodent macrophages inhibit microorganism and tumor cell growth through a high output of nitric oxide (NO). The induction of high levels of NO in rodent macrophages has been demonstrated to require stimulation with IFN-γ and LPS, a priming and a triggering stimulus, respectively (17). However, there is no report to our knowledge that turkey macrophages produce NO. We examined the r-TkIFN for its ability to induce NO in turkey bone marrow (BM) mononuclear cells (MNCs). BM MNCs were obtained from the femur bone of turkeys according to published procedures (22), and 10^6 viable MNCs were seeded in triplicate in a 100-μl volume in RPMI 1640 medium (Sigma) supplemented with 5% fetal bovine serum (RPMI-5; Sigma). Serial twofold dilutions of either r-TkIFN or LPS (*Escherichia coli* O111:B4 LPS; Sigma) singly or together in a 100-μl volume in RPMI-5 were added to the cells, which were then incubated for 48 h at 41°C. At the end of incubation period, supernatants were assayed for nitrite levels by using the Greiss reagent as described previously (19, 30). r-TkIFN alone at all concentrations tested failed to induce the production of detectable levels of NO in BM MNCs (Fig. 4A). LPS alone at various concentrations induced the production of very low amounts of NO in BM MNCs (Fig. 4B). However, r-TkIFN and LPS together induced high levels of NO production in the BM MNCs (Fig. 4). The optimum concentrations of IFN and

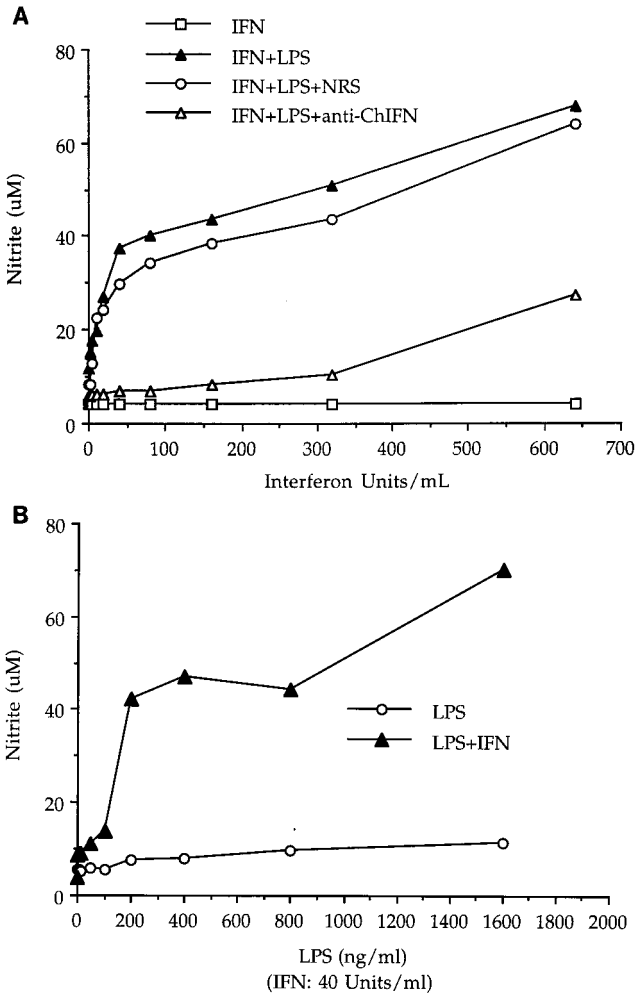

FIG. 4. (A) Synergistic induction of NO in turkey BM MNCs by TkIFN and LPS and its inhibition with anti-ChIFN antibodies. LPS alone at 100 ng/ml resulted in the accumulation of 12.08 μM nitrite in the medium. The concentration of nitrite in the unstimulated culture was 4.02 μM. NRS, normal rabbit serum. (B) Effect of LPS concentration on the synergistic effect of TkIFN on NO induction in BM MNCs.

LPS necessary to induce NO production were determined by a checkerboard titration (data not shown). At a constant LPS concentration, r-TkIFN induced NO production in BM MNCs in a dose-dependent manner (Fig. 4A). Similarly, at a constant TkIFN concentration, LPS stimulated macrophages to produce copious amounts of NO in a dose-dependent fashion (Fig. 4B). These data clearly demonstrated that TkIFN was necessary but not sufficient to induce the production of significant amounts of NO by turkey BM MNCs, quite possibly the macrophages. The results also indicated that TkIFN can provide one of the two signals necessary for the activation of turkey macrophages to produce NO in vitro. Our results are consistent with the reports that IFN-β synergistically augmented the production of NO triggered by LPS in a mouse macrophage cell line (10) and enabled LPS-hyporesponsive murine inflammatory macrophages to produce NO (32).

The ability of anti-ChIFN antibodies to neutralize the synergistic effect of r-TkIFN with LPS to induce NO in BM MNCs was studied by the constant-antibody-concentration method (14). Serial twofold dilutions (50 μl) of r-TkIFN in RPMI-5

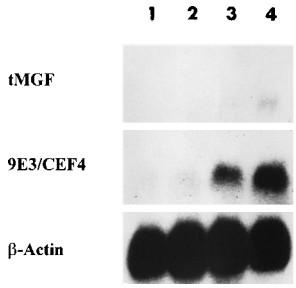

FIG. 5. Augmentation of LPS-induced expression of tMGF and 9E3/CEF4 mRNA by TkIFN in BM MNCs. Lane 1, medium alone; lane 2, TkIFN alone (128 U/ml); lane 3, LPS (1 μg/ml); lane 4, TkIFN (128 U/ml) plus LPS (1 μg/ml).

were incubated with 50 μl of a 1:100 dilution of rabbit anti-ChIFN or normal rabbit serum in RPMI-5 for 4 h at 37°C. At the end of incubation, 50-μl aliquots of the TkIFN-antibody mixtures were mixed with 50 μl of LPS (400 ng/ml) to give a final LPS concentration of 100 ng/ml and then added to 10^6 BM MNCs in 100 μl of RPMI-5. After incubating for another 48 h at 41°C, the supernatants were analyzed for nitrite concentrations as described above. The anti-ChIFN antibody neutralized the NO-inducing ability of TkIFN on BM MNCs except at the lowest dilution of r-TkIFN (Fig. 4A). These data established the specificity of NO induction by TkIFN in conjunction with LPS in turkey BM MNCs.

We studied the expression of 9E3/CEF4 mRNA in BM MNCs stimulated with LPS and TkIFN. To study the induction of cytokine mRNA by IFN and LPS, 2×10^7 BM MNCs in RPMI-5 were incubated with medium, LPS (1 μg/ml), r-TkIFN (128 U/ml), or LPS (1 μg/ml) plus r-TkIFN (128 U/ml). Total cellular RNA was isolated at 4 h poststimulation and analyzed for cytokine mRNA by Northern hybridization and autoradiography as described earlier. As shown in Fig. 5, LPS alone induced the expression of 9E3/CEF4 at 4 h poststimulation. Although TkIFN by itself did not stimulate the expression of detectable amounts of 9E3/CEF4 mRNA, it potentiated the effect of LPS to induce higher levels of 9E3/CEF4 mRNA. This is in striking contrast to the inhibitory effect of IFN-α/β on IL-8 mRNA expression by human peripheral blood MNCs and myelomonocytic cells (1). On the basis of molecular and biological characteristics, we assume that TkIFN is a type I IFN and 9E3/CEF4 is the avian homolog of IL-8. On the basis of these assumptions, our results demonstrated one striking dissimilarity between the mammalian and avian IFNs in the regulation of IL-8. The results as shown in Fig. 5 show that as was found for NO induction, the low level of the LPS-induced expression of tMGF mRNA in BM MNCs was potentiated by TkIFN. These data show that two signals were necessary to induce high levels of NO and the mRNAs for two proinflammatory cytokines, 9E3/CEF4 and tMGF. This is in striking contrast to the ability of mammalian IFN-α/β to inhibit inflammation after local LPS injection (11).

Nucleotide sequence accession number. The GenBank accession number for TkIFN is U28140.

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