Relative Contribution of Transcription and Translation to the Induction of Tumor Necrosis Factor-α by Lipopolysaccharide*

(Received for publication, May 9, 1997, and in revised form, October 16, 1997)

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The synthesis of tumor necrosis factor-α has been suggested to be regulated at both the transcriptional and translational levels in response to stimulation by bacterial lipopolysaccharide, although the relative contribution of these two mechanisms has not been quantitatively evaluated. Here, using the murine monocytic cell line RAW 264.7 as a model system, we show that steady-state TNF-α mRNA levels increase ~77-fold following treatment with lipopolysaccharide for 2 h and to a maximum of 164-fold after 8 h as measured by an RNase protection assay. The TNF-α gene transcription rate increases ~5-fold following exposure to lipopolysaccharide for 2 h as measured by a nuclear run-on assay. TNF-α mRNA stability did not change in the presence of lipopolysaccharide. A ribosomal sedimentation assay and an RNA transfection assay revealed that the translation rate of endogenous as well as transiently transfected TNF-α mRNAs increases only ~2–3-fold after stimulation with lipopolysaccharide for 2 h. Taken together, these results suggest that the large increase in the level of secreted TNF-α protein in RAW 264.7 cells is due primarily to activation of TNF-α gene transcription.

Tumor necrosis factor-α (TNF-α) is a well characterized cytokine that plays an important role in many inflammatory diseases caused by endotoxins produced by Gram-negative bacteria (1–3). Overproduction of TNF-α contributes significantly to the pathological complications observed in these diseases; therefore, the molecular mechanism of TNF-α induction is of considerable medical interest, and its elucidation will likely contribute to the development of new pharmacological reagents that can prevent TNF-α overproduction. TNF-α is produced mainly in cells of reticuloendothelial origin (e.g. macrophages) and is induced by many external stimuli, the strongest of which is lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria (4, 5). Although TNF-α was first described over 20 years ago, its regulation on the molecular level remains controversial. Depending on the specific study, LPS has been shown to activate TNF-α synthesis primarily through a translational (6–9), or transcriptional (10–14) mechanism. The differences in conclusions reached in those studies may be due to the use of different cell types and experimental conditions that induce TNF-α synthesis by different mechanisms (e.g. mouse versus human macrophages or primary monocytes versus monotypic cell lines). However, it is also possible that the use of different experimental methods to measure transcription and translation accounts for the conflicting conclusions.

LPS-induced changes in the transcription rate of the endogenous TNF-α gene have been measured using nuclear run-on assays and observed to be relatively small (7, 8); however, relatively large changes in the rate, as derived indirectly from measurements of steady-state mRNA levels by Northern blot analysis, have also been observed (8). Translation and transcription changes in the activity of artificial TNF-α promoter-reporter constructs have been measured after either stable (6–9) or transient (10–14) transfection and have likewise yielded conflicting conclusions. On the one hand, using primary mouse macrophages, it has been established that LPS increases the steady-state level of TNF-α mRNA derived from a transiently transfected TNF-α promoter-reporter construct, suggesting a transcriptional activation mechanism. Functional mapping of the TNF-α promoter in this system revealed that LPS induction is dependent on the presence of intact binding sites for the transcription factor NF-κB (10–12). Conversely, using the murine monocytic cell line RAW 264.7, it has been shown that a heterologous stably transfected TNF-α-CAT fusion gene can be strongly induced by LPS at the translational (but not transcriptional) level (7). In this case, LPS has been suggested to function through the Raf/Ras signaling pathway (15) that ultimately targets sequence elements present in the 3′- untranslated region (UTR) of TNF-α mRNA (6, 7).

In this study, we have attempted to measure more precisely the relative contribution of transcription and translation to the induction by LPS of endogenous TNF-α mRNA in RAW 264.7 cells. To determine the relative contributions of transcription and translation quantitatively and to minimize the risk of experimental artifacts, we utilized several functionally distinct transcription and translation assays measuring both endogenous and exogenous TNF-α mRNA levels. We show that it is possible to measure the relative contributions of translation and transcription to the induction not only of transfected TNF-α promoter-reporter constructs, but also of the endogenous TNF-α gene. Our results suggest that in RAW 264.7 cells, the endogenous TNF-α promoter is induced by LPS through a different molecular mechanism as described in studies that used stably transfected TNF-α promoter-CAT constructs (6–9).

For the endogenous TNF-α message, we demonstrate a 77-fold enhancement of steady-state mRNA levels, an ~5–fold increase in the transcription rate, and only a 2–3-fold increase in the
translation rate following LPS treatment for 2 h. These results suggest that activation of transcription is the predominant mechanism of LPS-mediated TNF-\(\alpha\) induction in RAW 264.7 cells. We therefore suggest that endogenous TNF-\(\alpha\) is induced by LPS in both monocytic cell lines and primary monocytes primarily through a transcriptional mechanism.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—**Murine RAW 264.7 cells were grown as adherent cells in RPMI 1640 medium (Life Technologies, Inc.) with 10% fetal bovine serum (FBS) (Hyclone Laboratories, Logan, UT) and gentamicin (Life Technologies, Inc.) unless indicated otherwise. For RNA isolation, cells were grown in 6-, 10-, or 15-cm tissue culture dishes to 50% confluency and harvested as described below.

**Recombinant DNA and Plasmids—**TNF-CAT hybrid vectors were constructed to permit the in vitro synthesis of capped and polyadenylated TNF-\(\alpha\)-CAT hybrid RNAs that were used in electroporation experiments. A TNF-\(\alpha\)-CAT construct containing the 5′- and 3′-UTRs of TNF-\(\alpha\) was used as template in a polymerase chain reaction with the synthesized primers 5′-CCCCAAATTCGACGAGCTCCCTGACCGA-G3′ and 3′-TTTTTGATCCTTTTTTTTTTTTTTTTTTTT AATTTAAAGCTACCGGCTCC-3′ (Life Technologies, Inc.) to generate a polymerase chain reaction product that represents a full-length cDNA copy of murine TNF-\(\alpha\) mRNA. The TNF-\(\alpha\)-polymerase chain reaction product was linearized with BstXI and BALI, or BstXI and PacI-linearized template plasmid pGEM3Zf(−) (Stratagene, La Jolla, CA). The resulting vector, pBSTNF-CAT, was used for in vitro transcription of TNF-\(\alpha\)-CAT mRNA. The deletion mutations shown in Fig. 5 were derived from this vector by standard techniques (16).

**In Vitro RNA Synthesis—**Typically, 1 \(\mu\)g of pBSTNF-\(\alpha\)-CAT DNA was linearized with BamHI, EcoRI, or PstI and added to an in vitro transcription reaction using T7 bacterial RNA polymerase (Promega, Madison, WI) according to the manufacturer. The CAP structure analogue used, \(\ddot{m}\)GpppG, was obtained from New England Biolabs Inc. (Beverly, MA). After the transcription reaction, RNA synthesis was verified using an RNase-free nondenaturing agarose gel. One \(\mu\)g of transcribed RNA, which was stored in 70% ethanol at −70 °C, TNF-\(\alpha\) and GAPDH antisense RNAs were generated from the plasmid pGEMZf(−) (Promega, Madison, WI) and the commercially available GAPDH plasmid (Life Technologies, Inc., respectively).

**RNA Isolation and RNase Protection Assay—**Isolation of total cytoplasmic RNA and RNase protection assays were performed according to standard procedures (16). Murine TNF-\(\alpha\) was detected with a 212-nucleotide RNA probe derived from the EcoRI-linearized template plasmid pGEMZf(−) TNF. This probe is complementary to the natural 5′-end of the TNF-\(\alpha\) transcript and yields a protected fragment of 189 nucleotides. Murine GAPDH mRNA was detected using a 135-nucleotide RNA probe. This probe is complementary to an internal coding region of human GAPDH and yields a protected fragment of 95 nucleotides. However, since human and mouse GAPDH DNA sequence are not completely identical, incomplete hybridization leads to the generation of a shorter protected fragment of ∼50 nucleotides following RNase treatment as measured by denaturing gel electrophoresis.

**TNF-\(\alpha\) ELISA—**Human TNF-\(\alpha\) protein levels were determined using a specific sandwich ELISA (R&D Systems, Minneapolis, MN) as described by the supplier.

**Nuclear Run-on Analysis—**RAW 264.7 nuclei were isolated and stored frozen as described previously (17). For run-on analysis, nuclei (0.2 ml) were thawed in 120 mM KCl, 1 mM MgCl\(_2\), 2.5 mM dithiothreitol, and 0.1 mM dithiobismuthate (0.2 mCi of \(\alpha\)-\[\text{32P}\]UTP (3000 Ci/mmol; DuPont) in a final volume of 0.4 ml. After 1 h at 30 °C, total RNA was extracted with 3.2 ml of 4 mM guanidinium thiocyanate, 25 mM sodium acetate, pH 5.0, and 10 mM dithiothreitol and separated from proteins and DNA by centrifugation on a 1.3-M cushion of 5.7 mM CsCl and 100 mM EDTA, pH 6.5, at 30,000 rpm in a SW 50.1 rotor for 48 h at 18 °C. The RNA pellet was dissolved in 10 mM Tris-HCl, pH 8.5. RNA was ethanol-precipitated; dissolved in 10 mM EDTA, pH 8.0, adjusted with 0.2 mM NaOH; incubated for 10 min at 0°C, neutralized with HEPEs, and precipitated with ethanol. RNA was then dissolved in water, and an equal amount of labeled RNA was hybridized to slot blots as described previously (18). Hybridization was performed for 4 h in 50% formamide, 750 mM NaCl, 75 mM sodium citrate, 2 mg/ml Ficoll, 2 mg/ml polyvinylpyrrolidone, 2 mg/ml bovine serum albumin (Fraction V; Life Technologies, Inc.), 1 mM EDTA, 10 mM Tris-Cl, pH 7.4, 0.2% SDS, and 0.1 mg/ml salmon sperm DNA at 52 °C with 2 × 10\(^6\) cpm/ml [\(\text{32P}\)]RNA. Blots were washed as described with an RNase step (18) and autoradiographed for 48 h at −70 °C. Signals were quantitated with a Molecular Dynamics Phosphorimager.

**Subsequent Treatment of Ribosomes Bound to Native TNF-\(\alpha\) mRNA—**One 15-cm plate of logarithmically growing RAW 264.7 cells was placed on ice and rinsed with 10 ml of ice-cold 1 × phosphate-buffered saline. The adherent cells were scraped off the plate with a plastic scraper, yielding ∼1–1.5 ml of cell suspension, which was transferred to an RNase-free Eppendorf tube. Cells were pelleted at 3000 rpm for 4 min at 4 °C. The tubes were immediately placed on ice, and the cells were lysed by gentle resuspension in 0.5 ml of ice-cold RNase-free lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 1.5 mM MgCl\(_2\), 10 mM Tris-HCl, pH 8.6, and 20 mM vanadyl-ribonucleoside complexes) followed by a 3–5-min incubation on ice. The lysate was centrifuged at 14,000 rpm in a refrigerated centrifuge at 4 °C for 2 min, and the supernatant was carefully removed and layered directly on top of a prepared and precooled 20–50% sucrose gradient containing 150 mM NaCl, 1.5 mM MgCl\(_2\), 10 mM Tris-HCl, pH 8.6, and 50 \(\mu\)g/ml cycloheximide. The gradient tube was placed into an SW 40 Ti swinging bucket rotor (Beckman Instruments) and centrifuged at 20,000 rpm for 3 h in a Beckman ultracentrifuge. The gradient was subsequently analyzed with a Hoefer gradient analyzer, and gradient fractions (1.5 ml) were immediately frozen. To isolate RNA from the gradient fractions, 20 \(\mu\)g of yeast tRNA and 15 \(\mu\)l of 3 mM sodium acetate were added to the mixture with phenol and chloroform. The RNA was precipitated with ethanol, washed once with 70% ethanol, dried briefly under vacuum, and used in RNase protection assays.

**Electroporation of TNF-\(\alpha\) mRNA into RAW 264.7 Cells—**RAW 264.7 cells (5 × 10\(^5\)) were grown in 20 ml of RPMI 1640 medium supplemented with 10% FBS for 14 h and then scraped off. After washing, the cells were suspended in 20 ml of RPMI 1640 medium from temperature, with or without LPS (1 \(\mu\)g/ml), and divided into two electroporation cuvettes (Bio-Rad). Immediately prior to electroporation, RNA (freshly dissolved in 50 \(\mu\)l of 1 × phosphate-buffered saline) or phosphate-buffered saline alone was added to the cuvettes. Electroporation was performed at 250 V and 960 microfarads. Similar results were obtained when LPS was added after electroporation. The cuvettes were put on ice, and 0.8 ml of RPMI 1640 medium containing 10% FBS was mixed with the cells until the suspension became homogeneous; 0.5-ml aliquots were plated per well in a Primaria six-well plate (Becton Dickinson Labware, Franklin Lakes, NJ), and 3 ml of RPMI 1640 medium containing 10% FBS was added. After 4 h, >70% of the cells readhered to the plate, and the cells were washed once with medium and then harvested for CAT assay.

**CAT Assay—**Cells were scraped off the well; collected in an Eppendorf tube; washed once with 1 × phosphate-buffered saline; and lysed by resuspending in 100 \(\mu\)l of a solution of 0.2 \(\mu\)l Tris-HCl, pH 7.5, followed by one freeze-thaw cycle and a 2-min sonication treatment. Cellular debris was removed by centrifugation, and CAT activity (20 \(\mu\)l of each extract) was measured by diffusion of reaction products into scintillation fluid according to standard procedures (16).

**RESULTS**

**The Transcription Rate of the Endogenous TNF-\(\alpha\) Gene Increases after LPS Addition—**For our studies, we chose the murine macrophage cell line RAW 264.7, which secretes large amounts of TNF-\(\alpha\) following stimulation with LPS and has been extensively characterized (6, 7). Using the RNase protection assay, TNF-\(\alpha\) mRNA could be detected in unstimulated RAW 264.7 cells and was rapidly and strongly induced following addition of LPS (Fig. 1, A and B). The antisense probe used to detect TNF hybridizes only with the first 169 nucleotides of the TNF-\(\alpha\) mRNA molecule that are located at the initiation site upstream of the first splice site, thereby avoiding interference from RNA splicing, and therefore is a more direct measure of TNF-\(\alpha\) promoter strength at early activation time points. This sensitive RNase protection assay coupled with densitometric analysis of the signals representing the protected TNF-\(\alpha\) fragment allowed us to more precisely measure the increase in endogenous TNF mRNA levels. To normalize the measured TNF signal to the relative amount of cytoplasmic RNA loaded in each lane, we used GAPDH mRNA as an inter
nal standard since steady-state GAPDH levels are not affected by LPS addition (Fig. 1A). Quantitation of the TNF-α and GAPDH signal ratios showed that TNF-α mRNA levels increased 77-fold following treatment with LPS for 2 h and 164-fold after 8 h (Fig. 1B).

To determine whether the increase in TNF mRNA levels reflects directly the transcriptional activity of the TNF-α gene, the stability of constitutively expressed and LPS-induced TNF-α mRNA was measured. Actinomycin D, a potent inhibitor of RNA polymerase II-dependent transcription, was added to unstimulated or LPS-stimulated cells, and the steady-state level of TNF-α mRNA was measured at 30-min intervals using an RNase protection assay. Our results (Fig. 1, D–F) indicate that the TNF-α mRNA signal decreases ~2–3-fold after 30 min and 10-fold after 60 min either in the absence or presence of LPS. These results suggest that the LPS-induced change in the steady-state TNF-α mRNA levels reflects primarily a change in the transcription rate of the TNF-α gene, and not a change in RNA stability. If regulation at this level is occurring, then inhibitors of transcription such as actinomycin D (19) or di-chloro-β-D-ribofuranosylbenzimidazole (DRB) (20) should suppress completely the LPS-induced increase in the steady-state TNF-α mRNA levels if added before addition of LPS. As shown in Fig. 2, LPS induction of the TNF-α mRNA was completely blocked by 0.3 μg/ml actinomycin D or 20 μg/ml DRB when added 1 h before LPS addition. GAPDH mRNA levels were not affected by these reagents.

Next, we determined the amount of endogenous TNF-α protein secreted before and after addition of LPS. Although TNF-α can be proteolytically degraded, the half-life of the intact TNF-α monomer in RAW 264.7 cell culture is ~1 day in RPMI 1640 medium/FBS (Ref. 20 and data not shown). In the absence of LPS, the secreted level of TNF-α was 200–300 pg/ml (Fig. 1C). This level was also observed when the cells were cultured in the absence of any additives, such as antibiotics, suggesting that LPS contamination can be excluded from mediating this TNF-α production. This value is highly reproducible and well over the detection limit of the ELISA used (20 pg/ml). These results suggest that a low level of secreted TNF-α protein is constantly being produced in uninduced RAW 264.7 cell cultures. Treatment with LPS increased the steady-state level of

without LPS; fourth through tenth lanes, cultures with LPS. B, relative levels of TNF-α mRNA. The values shown were obtained from densitometric scanning of the protected bands in A and were normalized to the internal GAPDH control as described under “Experimental Procedures.” Data from one representative experiment out of five are shown. C, induction of TNF-α protein in LPS-stimulated RAW 264.7 cells. Cells were treated with 1 μg/ml LPS. At the indicated time points, the TNF-α protein concentration in cell culture supernatants was determined by ELISA. The results are expressed as the means ± S.E. of four measurements from two independent experiments. The relative stability of TNF-α mRNA in RAW 264.7 cells before and after treatment with 1 μg/ml LPS is shown in D–F. D, actinomycin D added to unstimulated cells (0 h LPS) or to cells stimulated for 2 h with LPS (2 h LPS), and RNase protection assays performed with RNA isolated at 0, 30, 60, and 90 min following actinomycin D treatment. E, overexposure of the autoradiographs in D to visualize TNF-α mRNA levels in the absence of LPS. F, semilogarithmic plot of the data values obtained from densitometric analysis of the autoradiographs in D and E. The half-life times before LPS treatment and after 16 min LPS treatment were obtained by reading the times corresponding to the 50% TNF-α mRNA level (ln[50%] = 3.91 for both slopes). The 90-min points were omitted since they lie outside the linear range. G, nuclear run-on analysis of TNF-α gene activity. Total [32P]RNA isolated from unstimulated or LPS-stimulated RAW 264.7 cells was hybridized to slot blots containing TNF-α DNA, GAPDH DNA, or vector DNA as described under “Experimental Procedures.” Bars represent the average of two independent experiments and were obtained by phosphoimaging of slot blots; they represent the relative transcription rates before (gray bars) or 2 h following (black bars) addition of LPS (1 μg/ml).
TNF-α protein 21-fold after 2 h and 184-fold after 8 h of stimulation. To determine the transcription rate, nuclear run-on assays were also performed. A modest (5-fold) increase in the TNF-α rate was measured in isolated RAW 264.7 nuclei following LPS induction for 2 h (Fig. 1G), and these results are consistent with previous measurements using this assay (17).

Taken together, these results suggest that even in the absence of LPS stimulation, RAW 264.7 cells produce small quantifiable amounts of TNF-α mRNA and protein and that the increase in steady-state TNF-α mRNA and protein levels following LPS addition results primarily from transcriptional activation of the TNF-α gene.

The Average Number of Ribosomes Bound to an Endogenous TNF-α mRNA Molecule Increases 2-3 Fold after LPS Addition—To obtain an estimate of the translation rate of endogenous TNF-α mRNA (i.e. the amount of TNF-α protein produced per RNA molecule/time) before and after LPS addition, we determined the average number of ribosomes bound per endogenous TNF-α mRNA molecule (21). Total cytoplasmic RNA isolated from uninduced or induced RAW 264.7 cells was fractionated on a sucrose gradient (Fig. 3). Under the conditions used for RNA preparation (i.e. in the presence of the translational inhibitor cycloheximide), mRNAs already bound to ribosomes in the intact cells remained complexed with ribosomes, and mRNAs that were not bound to ribosomes remained free of ribosomes throughout the fractionation procedure. Conditions used to fractionate sucrose gradients allowed the resolution of mRNA-ribosome complexes differing by only one bound ribosome. After fractionation, mRNA was extracted, and the amount of TNF-α mRNA present in each fraction was measured directly by the RNase protection assay (Fig. 3) coupled with densitometry (Table I). The densitometric analysis showed that in the absence of LPS, 69% of the total amount of TNF-α mRNA was free of ribosomes, 12% contained one to three ribosomes, and 19% was bound to four to six ribosomes. After induction with LPS, 38% of the TNF-α mRNA was free of ribosomes, 20% contained one to three ribosomes, and 42% was bound to four to six ribosomes. Therefore, this analysis suggested that the average number of ribosomes/TNF-α mRNA molecule increased 2-3 fold following addition of LPS.

The Translation Rate of Transiently Transfected TNF-α mRNA Increases 2 Fold after LPS Treatment—Since the experiments described above did not measure the translation rate of TNF-α mRNA directly, we developed an assay that allows direct measurement of TNF-α mRNA translation in vitro. For this purpose, a TNF-α-CAT construct was engineered that allowed in vitro synthesis of a capped and polyadenylated mRNA molecule that contained the complete 5′- and 3′-UTRs of TNF-α mRNA, but had the TNF-α coding region exchanged for the coding region of CAT (Fig. 4). This mRNA contained most of the regions that are known to play a role in the regulation of TNF-α mRNA translation, namely the ribosome-binding region in the 5′-UTR, the 3′-UTR, and the poly(A) tail. This hybrid mRNA and its derivatives (see below) were synthesized in vitro and transfected transiently into RAW 264.7 cells using electroporation in the presence or absence of LPS. Subsequently, CAT assays were performed to measure directly the amount of translated protein (Fig. 4). Several controls were performed to confirm that CAT levels in this assay measure translation of transfected RNA: (i) omission of the CAP structure in the 5′-UTR, the 3′-UTR, and the poly(A) tail. This hybrid mRNA and its derivatives (see below) were synthesized in vitro and transfected transiently into RAW 264.7 cells using electroporation in the presence or absence of LPS. Subsequently, CAT assays were performed to measure directly the amount of translated protein (Fig. 4). Several controls were performed to confirm that CAT levels in this assay measure translation of transfected RNA: (i) omission of the CAP structure in the 5′-UTR, the 3′-UTR, and the poly(A) tail. This hybrid mRNA and its derivatives (see below) were synthesized in vitro and transfected transiently into RAW 264.7 cells using electroporation in the presence or absence of LPS.
results are from densitometric scanning of the gels shown in Fig 3B.

**Table I**

| Polysomal profile of TNF mRNA before and after induction with LPS |
|---------------------------------------------------------------|
| No. of ribosomes/TNF mRNA molecule | 0 | 1 | 2 | 3 | 4 | 5 | 6 |
| Fraction No. | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 |
| % total TNF mRNA in absence of LPS | 3 | 11 | 31 | 24 | 5 | 5 | 2 | 9 | 8 | 2 |
| Combined % TNF mRNA in absence of LPS | 69 | | | | | | | | |
| % total TNF mRNA in presence of LPS | 2 | 22 | 10 | 4 | 7 | 7 | 6 | 16 | 17 | 9 |
| Combined % TNF mRNA in presence of LPS | 38 | 20 | 42 |

**Fig. 4.** Translation rate of transiently transfected TNF-α mRNA. TNF-CAT mRNA was introduced into RAW 264.7 cells by electroporation as described under "Experimental Procedures." CAT activity was measured 4 h after electroporation following a variety of treatments. -LPS, shown is the CAT activity observed in the absence of LPS; +LPS, shown is the CAT activity observed in the presence of LPS (1 μg/ml); DNA only, the DNA template used for generation of RNA was used instead of RNA; no CAP, the CAP structure analogue was omitted from the in vitro transcription reaction; 5μg RNA, instead of 1 μg, 5 μg of TNF-α-CAT mRNA was electroporated in the presence of LPS. Results of four independent experiments are expressed as the means ± S.E.

**vitro** RNA synthesis reactions completely abolished any CAT activity above background levels, indicating that it is indeed the RNA and not residual amounts of TNF-α DNA that accounted for CAT activity; and (ii) in addition, the DNA template used for in vitro transcription cannot by itself induce any CAT activity because it contains no eukaryotic promoter, but only the bacterial promoter used for in vitro transcription. In this system, the CAT activity depends largely on the amount of TNF-α-CAT mRNA added to the cells; electroporation of 5 μg of TNF-α-CAT mRNA resulted in a ~5-fold increase in CAT activity (Fig. 4).

Interestingly, the CAT message could be easily translated in the absence of any exogenous stimulus. These observations are consistent with our previous finding that the endogenous TNF-α message is complexed with ribosomes even in the absence of LPS (Fig. 3). To exclude the possibility that some step in the electroporation procedure induced CAT expression, we measured the effect of electroporation on endogenous TNF-α levels by ELISA with or without LPS stimulation. These experiments did not reveal any effect of electroporation on TNF-α production (Ref. 9 and data not shown). LPS addition consistently resulted in ~1.8-fold higher CAT levels as compared with unstimulated cells, indicating an ~2-fold higher rate of translation. This result is consistent with the 2–3-fold increase in the number of ribosomes bound to the endogenous TNF-α message observed after LPS addition (Fig. 3).

Translational induction of transiently transfected TNF-α mRNA is thought to be mediated by interaction of 5′- and 3′-UTRs, as studies have suggested that both 5′- and 3′-UTRs are required for translational activation of the TNF-α message (9). To test this hypothesis further, using this electroporation technique, we generated several deletion mutants of the basic TNF-α-CAT construct (Fig. 5). Only the basic construct, which contained both an intact 5′- and 3′-UTR, showed an LPS-induced effect on the level of translation, and all mutations in the 5′- and/or 3′-UTR had reduced or abolished induction levels following LPS treatment (Fig. 5). Taken together, these results suggest that LPS inducibility of TNF-α translation requires intact 5′- and 3′-UTRs.

**DISCUSSION**

In this study, we have attempted to re-examine the mechanism of TNF-α induction by LPS. A series of studies investigating LPS-induced TNF-α synthesis in primary mouse monocytes (10–12) and in the mouse monocytic cell line P388D1 (13) have indicated primarily a transcriptional mechanism, whereas another series of studies in the mouse monocytic cell line RAW 264.7 have provided evidence for a predominantly translational mechanism (6–9). Our results suggest that these differences are likely due to the measurement of endogenous or transiently transfected TNF-α mRNA (10–14), in contrast to measurements made with cells containing stably integrated TNF-α mRNA (6, 7, 9). Our results support a predominantly transcriptional mechanism of LPS-mediated TNF-α production in both primary macrophages and RAW 264.7 cells when endogenous or transiently transfected TNF-α is measured in contrast to stably transfected TNF-α. Our results suggest that the transcription rate of the endogenous TNF-α gene in RAW 264.7 cells, as measured indirectly using RNase protection assays, increases 77-fold 2 h after addition of LPS, whereas the transcription rate increases ~5-fold using the nuclear run-on assay. During the same time period, the transcription rate of endogenous TNF-α mRNA increases ~2–3-fold. We measured the transcription rate additionally through quantitation of steady-state TNF-α mRNA levels during the early activation phase. This approach was possible since the stability of steady-state TNF-α mRNA in LPS-stimulated and unstimulated cells treated with actinomycin D did not change (Fig. 1). These results suggested that the observed rapid increase in steady-state TNF-α levels was due the accumulation of newly synthesized mRNA, and not due to an increase in mRNA stability.
suggesting that induction is due to transcription rather than to translation rates of TNF-α. We observed that the increase in the endogenous TNF-α mRNA, that these nuclear run-on experiments do not accurately measure TNF-α gene activity levels.

Our results with RAW 264.7 cells differ in several regards from previous studies with this cell type, which also showed a relatively small (~3–5-fold) increase in the transcription rate of endogenous TNF-α after LPS induction using nuclear run-on assays (7, 9). While in our study we measured transcription of the endogenous TNF-α gene using a TNF-α probe derived near the transcription initiation site in both steady-state mRNA and nuclear run-on assays, other investigators have measured transcription rates in isolated nuclei using TNF-α probes derived farther downstream of the initiation site (7, 9). In addition, as discussed above, most of the measurement of transcription and translation rates by these authors was performed with stably transfected TNF-α-CAT constructs. The random integration of TNF-α-CAT DNA into the genome may in some cases generate artifactual effects; indeed, the half-lives of these hybrid mRNAs are much longer than those of the endogenous TNF message (>3 h versus 19–24 min, respectively), and the steady-state levels of RNA expressed from various TNF-α-CAT constructs in the absence of LPS vary for unknown reasons up to 17-fold, although the promoter is identical in all constructs (9). Despite these differences, a relatively modest increase in TNF-α transcription rate is observed following LPS treatment in RAW 264.7 cells and in primary murine monocytes. In an attempt to account for the large increase in both steady-state TNF-α mRNA and protein levels and this apparent discrepancy with nuclear run-on measurements, we measured steady-state mRNA levels at relatively early time points using an RNase probe derived near the initiation site. Our results suggest that the large increase in steady-state TNF-α mRNA levels is primarily due to increased gene transcription and further suggest that these nuclear run-on experiments do not accurately measure TNF-α gene activity levels.

Our results with RAW 264.7 cells are consistent with recent data indicating that LPS induces simultaneously two signaling pathways in RAW 264.7 cells (22). In that study, induction of the post-transcriptional Raf-1/mitogen-activated protein kinase-dependent pathway by itself was observed to only produce a low level of TNF-α protein induction, whereas additional activation of a putative transcriptional NF-κB-dependent pathway was observed to result in an ~20-fold enhancement (22). The primary role of transcription in LPS-induced TNF-α expression is consistent with the observation that intact NF-κB-binding sites in the TNF-α promoter are necessary for proper induction of TNF mRNA in primary bone marrow macrophages (10–12). In this regard, it is interesting to note that nuclear NF-κB DNA-binding activity is strongly and rapidly induced in RAW 264.7 cells as early as 15 min following LPS addition (23); these kinetics are consistent with the time course we observed for TNF-α mRNA induction. Recently, treatment of primary human monocytes with LPS for 1 h has been shown to increase the rate of TNF-α gene transcription ~4-fold, to increase steady-state TNF-α mRNA levels ~9-fold, to cause an increase in the fraction of TNF-α mRNA associated with large polysomes, and to have no effect on TNF-α mRNA stability (i.e., the measured half-life of TNF-α mRNA is 25 min in the presence or absence of LPS) (24). Together with our results in RAW 264.7 cells showing similar TNF-α mRNA half-lives, these observations suggest that the primary effect of LPS is to rapidly activate TNF-α gene transcription, which results in an increase in steady-state TNF-α mRNA levels, the translation of which

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**Fig. 5.** LPS inducibility of CAT activity depends on the presence of intact TNF-α 5’ and 3’-UTRs. The TNF-α-CAT mutant RNAs with deletions in the 5’- or 3’-UTR shown in A were electroporated into RAW 264.7 cells. CAT activity with and without LPS treatment was measured as shown in B after 4 h. Results of four independent experiments are expressed as the means ± S.E.

following LPS treatment. Furthermore, low concentrations of two different transcriptional inhibitors were shown to completely block the LPS-mediated induction of TNF-α mRNA, suggesting that induction is due to transcription rather than to an increase in TNF-α mRNA half-life (Fig. 2). The relatively modest increase in transcription rate as measured by the nuclear run-on assay is consistent with previous data observed for the LPS-induced TNF-α gene in RAW 264.7 cells (17). More important, we observed that the increase in the endogenous TNF-α protein level as measured by ELISA was only 1.8-fold higher than the increase in the endogenous TNF-α mRNA level, directly suggesting that translation of the endogenous TNF-α message is not induced more than ~2-fold by LPS.

We have attempted to estimate changes in relative translation rates of TNF-α mRNA after LPS induction in vivo. For this purpose, we determined the average number of ribosomes bound per endogenous TNF-α mRNA molecule before and after addition of LPS (Fig. 3). Assuming that all TNF-α mRNA-bound ribosomes translate equally well before and after LPS addition, the average number of ribosomes/TNF-α mRNA molecule should be directly proportional to the translation rate. We found that the number of ribosomes bound per TNF-α molecule increased 2–3-fold after addition of LPS; therefore, we suggest that the translation rate also increases 2–3-fold. This view is supported by our observation that the translation rate of transiently transfected TNF-α-CAT mRNA increased ~2-fold after LPS stimulation (Fig. 4). Since the TNF-α transcription rate increased ~77-fold 2 h after LPS addition and continued to increase for another 6 h, we conclude that the synthesis of TNF-α is induced by LPS in RAW 264.7 cells primarily at the transcriptional level.

Our results with RAW 264.7 cells differ in several regards from previous studies with this cell type, which also showed a relatively small (~3–5-fold) increase in the transcription rate of endogenous TNF-α after LPS induction using nuclear run-on assays (7, 9). While in our study we measured transcription of the endogenous TNF-α gene using a TNF-α probe derived near the transcription initiation site in both steady-state mRNA and nuclear run-on assays, other investigators have measured transcription rates in isolated nuclei using TNF-α probes derived farther downstream of the initiation site (7, 9). In addition, as discussed above, most of the measurement of transcription and translation rates by these authors was performed with stably transfected TNF-α-CAT constructs. The random integration of TNF-α-CAT DNA into the genome may in some cases generate artifactual effects; indeed, the half-lives of these hybrid mRNAs are much longer than those of the endogenous TNF message (>3 h versus 19–24 min, respectively), and the steady-state levels of RNA expressed from various TNF-α-CAT constructs in the absence of LPS vary for unknown reasons up to 17-fold, although the promoter is identical in all constructs (9). Despite these differences, a relatively modest increase in TNF-α transcription rate is observed following LPS treatment in RAW 264.7 cells and in primary murine monocytes. In an attempt to account for the large increase in both steady-state TNF-α mRNA and protein levels and this apparent discrepancy with nuclear run-on measurements, we measured steady-state mRNA levels at relatively early time points using an RNase probe derived near the initiation site. Our results suggest that the large increase in steady-state TNF-α mRNA levels is primarily due to increased gene transcription and further suggest that these nuclear run-on experiments do not accurately measure TNF-α gene activity levels.

Our results with RAW 264.7 cells are consistent with recent data indicating that LPS induces simultaneously two signaling pathways in RAW 264.7 cells (22). In that study, induction of the post-transcriptional Raf-1/mitogen-activated protein kinase-dependent pathway by itself was observed to only produce a low level of TNF-α protein induction, whereas additional activation of a putative transcriptional NF-κB-dependent pathway was observed to result in an ~20-fold enhancement (22). The primary role of transcription in LPS-induced TNF-α expression is consistent with the observation that intact NF-κB-binding sites in the TNF-α promoter are necessary for proper induction of TNF mRNA in primary bone marrow macrophages (10–12). In this regard, it is interesting to note that nuclear NF-κB DNA-binding activity is strongly and rapidly induced in RAW 264.7 cells as early as 15 min following LPS addition (23); these kinetics are consistent with the time course we observed for TNF-α mRNA induction. Recently, treatment of primary human monocytes with LPS for 1 h has been shown to increase the rate of TNF-α gene transcription ~4-fold, to increase steady-state TNF-α mRNA levels ~9-fold, to cause an increase in the fraction of TNF-α mRNA associated with large polysomes, and to have no effect on TNF-α mRNA stability (i.e., the measured half-life of TNF-α mRNA is 25 min in the presence or absence of LPS) (24). Together with our results in RAW 264.7 cells showing similar TNF-α mRNA half-lives, these observations suggest that the primary effect of LPS is to rapidly activate TNF-α gene transcription, which results in an increase in steady-state TNF-α mRNA levels, the translation of which
accounts for the observed dramatic increase in secreted TNF-α protein.

**Acknowledgments**—We thank Bruce Beutler (University of Texas Southwestern Medical Center, Dallas, TX) for the TNF-α-CAT construct and Christine Metz (Picower Institute for Medical Research) for advice on the TNF-α ELISA.

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