TNF may be key to the mechanism underlying TNF-mediated protection. Indeed, all TNF killing-resistant cell lines tested including HeLa, Jurkat, U937, Sw480, H9, NIH 3T3, and Hut78 cells express *IEX*-1L after TNF stimulation (*6, 8*). In contrast, a decrease or delay in TNF-induced expression of *IEX*-1L is likely to increase cell susceptibility to TNF-induced apoptosis, as was found in p65KO3T3, Jurkat-IκBαM, and Jurkat cells bearing an antisense *IEX*-1L (*4*). Our unpublished data also showed that *IEX*-1L was potentially regulated by the *RelA*/c-*rel* complex (*8*), in agreement with previous observations that overexpression of the c-*rel* gene protected cells from TNF-induced cell death (*2, 3*) and that *RelA* gene knockout mice died at 15 days of gestation (*14*). However, unlike RelA−/− mice, mice lacking the c-*rel* gene are developmentally healthy (*15*), which suggests that *IEX*-1L may be only one of the NF-κB/Rel protein-regulated survival genes.

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9. PCR products of the *IEX*-1 coding frame were in-frame inserted into the NH_2-terminus of GFP using the site Eco RI–Bam HI in a pEGFP-N1 plasmid (Clontech). The resulting pEGFP-*IEX*-1L and pEGFP-*IEX*-1S were digested and ligated to the site Hind III–Bam HI in a pcDNA plasmid (Invitrogen) containing HA epitope sequence at the COOH-terminus of expressing protein. *IEX*-1L excised from pBluescript II/KS(−)–*IEX*-1L plasmid was subcloned into the Sal I–Not I site in a hygromycin-resistant gene-containing plasmid BCMGS/Hyg. pRc-Fas plasmid was a kind gift from S. M. Lehar (ImmunoGen Inc. Cambridge, MA).
10. The Ab was produced in rabbit by injecting affinity-purified glutathione S-transferase–IEX-1L fusion protein and was purified on a protein A column followed by an IEX-1L protein column. It reacted with both IEX-1L and IEX-1S proteins.
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12. Jurkat cells or Jurkat IκBαM cells were transfected by electroporation (200 V, 960 μF) and p65KO3T3 cells were transfected by the lipofectamine protocol (Gibco/BRL). Two days after transfection with the indicated plasmids, the cells were selected for neomycin (1.5 mg/ml) or hygromycin (600 μg/ml for p65KO3T3 cells and 800 μg/ml for Jurkat IκBαM cells) resistance for 4 weeks and then their resistance to apoptosis induced by TNF or by mAb 7C11 to Fas [immunoglobulin M (IgM), 1:10,000 diluted ascites] was tested. In each transfection of these bulk cultures, we used 5×10^6 cells and the efficiency of transfection was 8 to 20% for Jurkat and Jurkat IκBαM cells and 15 to 25% for p65KO3T3 cells, as tested by the pEGFP-*IEX*-1L plasmid. Thus, the results obtained from each of these bulk cultures theoretically represent about 0.4 to 1.2×10^6 individual clones.
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Feedback Inhibition of Macrophage Tumor Necrosis Factor–α Production by Tristetraprolin

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Tumor necrosis factor–α (TNF-α) is a major mediator of both acute and chronic inflammatory responses in many diseases. Tristetraprolin (TTP), the prototype of a class of Cys-Cys-Cys-His (CCCH) zinc finger proteins, inhibited TNF-α production from macrophages by destabilizing its messenger RNA. This effect appeared to result from direct TTP binding to the AU-rich element of the TNF-α messenger RNA. TTP is a cytosolic protein in these cells, and its biosynthesis was induced by the same agents that stimulate TNF-α production, including TNF-α itself. These findings identify TTP as a component of a negative feedback loop that interferes with TNF-α production by destabilizing its messenger RNA. This pathway represents a potential target for anti–TNF-α therapies.

TNF-α is one of the principal mediators of the inflammatory response in mammals (*1*). In addition to its well-known role in acute septic shock, it has been implicated in the pathogenesis of chronic processes such as autoimmunity, graft-versus-host disease, rheumatoid arthritis, Crohn's disease, and the cachexia accompanying cancer and acquired immunodeficiency syndrome (*2*). Therapies such as neutralizing antibodies to TNF-α and chimeric soluble TNF-α receptors have demonstrated efficacy against some of these conditions in clinical trials (*3*).

We developed mice deficient in TTP, the prototype of a family of CCCH zinc finger proteins whose members have been identified in organisms ranging from humans to yeast (*4–7*). Although the TTP-deficient mice appear normal at birth, they soon develop a complex syndrome of inflammatory arthritis, dermatitis, cachexia, autoimmunity, and myeloid hyperplasia. Essentially all aspects of this syndrome can be prevented by repeated injections of antibodies to TNF-α (*8*). Macrophages derived from fetal liver of TTP-deficient mice, or from bone marrow precursors or resident peritoneal macrophages from adult mice, exhibited increased production of TNF-α, as well as increased amounts of TNF-α mRNA, after stimulation with lipopolysaccharide (LPS) (*9*). For example, relative to control macrophages, bone marrow–derived macrophages from the knockout mice secreted about five times as much TNF-α after incubation with LPS (1 μg/ml for 4 hours), and amounts of TNF-α mRNA were about twice as large in the knockout cells as in the controls (*9*).

To investigate the mechanism of this effect, we evaluated the potential influence of TTP on TNF-α gene transcription. We transfected a human TTP genomic construct, in which the instability-inducing 3′-untranslated region (UTR) of the TTP mRNA (*10*) was replaced by the 3′-UTR from the human growth hormone mRNA (*11*), with a TNF-α promoter–chloramphenicol acetyltransferase (CAT) reporter construct (Pro-CAT). This construct contained 2.3 kb of the mouse TNF-α promoter linked to the CAT coding sequence and a 3′-UTR from a human growth hormone cDNA (*12*). Transfection of several cell types (chick embryo fibroblasts, NIH 3T3 mouse fibroblasts, and Rat-1 fibroblasts) led to nonspecific "squelching" of several

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cotransfected expression constructs, including Pro-CAT, SV2CAT (CAT driven by the SV40 promoter), and CAT driven by the *Xenopus* MARCKS gene promoter (*13*). In DNA dose-response studies in these cells, there was no evidence for preferential inhibition by the TTP constructs of Pro-CAT expression, relative to the expression of the other cotransfected constructs. In human 293 cells, which express little if any endogenous TTP (*11*), transfection of TTP constructs did not significantly inhibit the expression of cotransfected constructs (by paired *t* test with the Bonferroni correction for multiple comparisons) (*14*). In experiments ($n = 5$) in which TTP was transfected with SV2CAT, TTP DNA was also without effect on CAT expression. Thus, the apparent effect of TTP to decrease amounts of TNF-α mRNA and protein in macrophages (*9*) appears not to result from inhibited TNF-α gene expression.

We evaluated the effect of TTP deficiency on the stability of TNF-α mRNA, which has a half-life of 12 min in human monocytes (*15*) and 39 min in the murine macrophage cell line Raw 264.7 (*16*). In bone marrow–derived macrophages from wild-type ($n = 6$) and TTP-deficient mice ($n = 6$) that were stimulated with LPS (1 μg/ml; Sigma) for 4 hours and then treated with actinomycin D (5 μg/ml; Sigma), the half-life of TNF-α mRNA in the macrophages lacking TTP was significantly increased compared to that observed in the wild-type cells (Fig. 1). Northern (RNA) analysis showed no evidence of stable mRNA degradation products in either the control or TTP-deficient macrophages (Fig. 1A). Similar studies with TTP (+/–) macrophages showed that TNF-α mRNA decayed at the same rate as in wild-type cells (*17*), indicating that ~50% of normal cellular TTP concentration (*8*) is sufficient to confer normal lability to TNF-α mRNA under these experimental conditions.

These results suggest that TTP regulates amounts of TNF-α mRNA posttranscriptionally. This increased half-life of TNF-α mRNA in macrophages from the TTP-deficient mice is thus likely to be responsible for the increased secretion of TNF-α by macrophages derived from these mice (*9*) and for the syndrome of TNF-α excess that characterizes the TTP-deficient mice (*8, 9*).

TTP is localized in the nucleus of quiescent fibroblasts; the protein either remains in the nucleus (*7*) or rapidly (<5 min) translocates to the cytosol after cells are stimulated with mitogens (*18*). To determine the subcellular localization of TTP in normal macrophages under these conditions, we incubated cells treated with LPS or TNF-α with [^{35}S]cysteine, separated them into nuclear and cytosolic fractions, and immunoprecipitated proteins with an antibody to TTP (*18, 19*). Both LPS and TNF-α caused an increased incorporation of ^{35}S into cytosolic TTP 4 hours after stimulation (Fig. 2). Protein immunoblotting showed that increases in TTP protein were evident in the cytosol 30 min after treatment of cells with LPS, and that amount remained increased in the cytosol 2 to 4 hours after simulation (*11*). Nuclear TTP was not detectable in either the immunoprecipitation or the protein immunoblot experiments (Fig. 2).

Thus, TTP might participate in a negative feedback loop, in which the amount of TTP in the cytosol is increased by the same stimuli that cause increased expression of TNF-α in macrophages, leading to instability of TNF-α mRNA and inhibition of TNF-α secretion. We therefore measured amounts of TTP mRNA in LPS-stimulated, bone marrow–derived macrophages from normal mice. In cells treated with LPS (1 μg/ml), TTP mRNA accumulated, peaking at 60 min (Fig. 3, A and B). The amount of TNF-α mRNA also increased, with a more prolonged time course (Fig. 3, C and D). Exposure of primary macrophages to recombinant murine TNF-α (10 ng/ml; R&D Systems, Minneapolis) also resulted in increased amounts of TTP mRNA, which peaked after 30 to 60 min (Fig. 4, A and B). Amounts of TNF-α mRNA increased as well, peaking after 60 to 120 min (Fig. 4, C and D).

TTP appears to regulate TNF-α synthesis

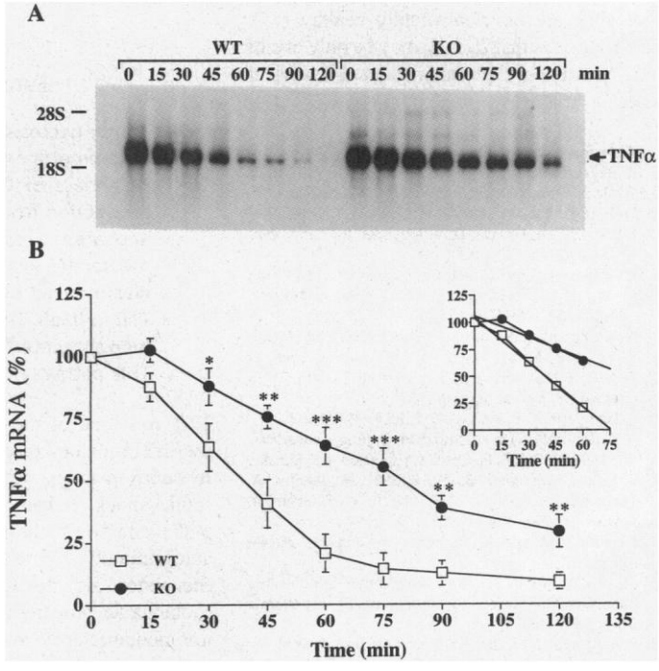

Fig. 1. Evaluation of TNF-α mRNA stability in macrophages from wild-type and TTP-deficient mice. Bone marrow–derived macrophages were prepared from six wild-type and six TTP-deficient mice (4 to 10 months old), as described (*9*). After washing twice with Dulbecco's modified Eagle's medium (DMEM) containing fetal calf serum (10%), the cells were stimulated with LPS (1 μg/ml) for 4 hours, and then actinomycin D (5 μg/ml) was added for the indicated times. Cell harvesting, total RNA extraction, and Northern analysis of TNF-α mRNA expression were done as described (*9*). The relative amounts of TNF-α mRNA were determined by PhosphorImager analysis (Molecular Dynamics), and the results were normalized by reprobing the same blots with a cDNA coding for rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (*27*). (**A**) Northern blot of TNF-α mRNA after addition of actinomycin D. WT, wild-type mice; KO, TTP-deficient mice. (**B**) Average values from the six experiments performed to assess TNF-α mRNA stability. Results are expressed as the mean relative amount of TNF-α mRNA ± SEM at each time point, after correction for the amount of GAPDH mRNA (*$P < 0.05$ when comparing the means by Student's *t* test; **$P < 0.01$; ***$P < 0.001$). The amount of mRNA at time 0 of actinomycin D treatment was taken as 100% in each experiment. The inset shows a linear regression analysis of the 0- to 60-min values. WT $t_{1/2} = 39$ min; KO $t_{1/2} = 85$ min. Animal care was in accordance with institutional guidelines.

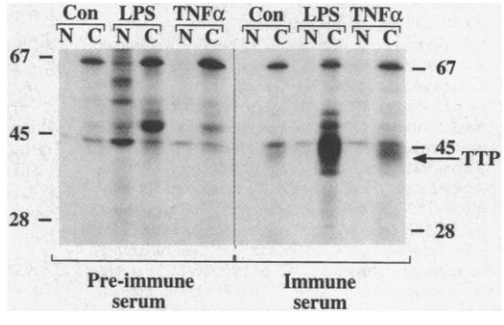

Fig. 2. Cellular localization of TTP in primary macrophages. Macrophages from wild-type mice were prepared as described (*9*) and grown to confluence in 100-mm dishes. After stimulation with either LPS (1 μg/ml) or TNF-α (10 ng/ml), cells were divided into nuclear (N) and cytosolic (C) fractions (*28*). Positions of molecular size standards are indicated (in kilodaltons); Con, control. The arrow indicates the position of TTP. The sharp band of M_r 44,000 present in all the fractions is an artifact from the serum, as described (*18*).

by promoting the turnover of TNF-α mRNA. TNF-α promotes rather than inhibits its own synthesis and secretion (*20*) (Fig. 4), but our data indicate that negative feedback also exists through TNF-α–induced or LPS-induced synthesis of TTP. When TTP is absent, as in the TTP-deficient mice, the self-stimulating property of TNF-α may lead to activation of inflammatory processes (*8, 9*).

TNF-α mRNA contains several AU-rich elements (AREs) as well as considerable predicted secondary structure, both of which can influence mRNA stability (*21*). To investigate whether the TNF-α mRNA ARE was involved in the TTP effect, we cotransfected 293 cells with constructs expressing TTP (*22*) and constructs in which the AREs from TNF-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin 3 (IL-3) mRNAs were placed 3′ of the c-*fos* promoter and the β-globin protein coding sequence (*23*). Coexpression of human TTP (*6, 10*), either with a genomic construct driven by the native human TTP promoter (5 μg) or with a human TTP cDNA driven by the CMV promoter (5 μg), markedly inhibited mRNA accumulation from all three constructs (Fig. 5A). Cotransfection with 0.01 μg of the CMV-TTP construct or an unrelated CMV-MLP construct (*24*) had little effect (Fig. 5A). TTP mRNA was highly expressed in the cells transfected with 5 μg of the CMV-driven TTP, was expressed to an intermediate extent after 5 μg of the TTP genomic construct, and was barely detectable after the 0.01-μg concentration of CMV-TTP (Fig. 5B). Parallel experiments in which a c-*fos* promoter–CAT construct (*25*) was cotransfected with the TTP expression vectors revealed that the c-*fos* promoter was not inhibited by the expression of the genomic TTP construct (5 μg), 5 μg of the CMV-MLP construct, or 0.01 μg of the CMV-TTP construct, whereas it was inhibited by about 30% by 5 μg of the CMV-TTP construct (*11*).

These results pointed to the ARE as the common element in these constructs responsible for TTP-induced mRNA lability. To test whether TTP affected binding of cellular proteins to this region of the TNF-α mRNA, we transfected 293 cells with a construct expressing epitope-tagged human TTP (*22*) and attempted to cross-link proteins in a cytosolic extract from these cells to a 153-bp probe from the mouse TNF-α 3′-UTR (*26*) that spanned the ARE. In untransfected cells, the radiolabeled mRNA probe was cross-linked to a major protein species of ~85 kD (Fig. 6, lane 3). When extracts from TTP-expressing cells were used in a similar experiment, labeling of the 85-kD protein decreased while a new binding protein of ~40 kD appeared (Fig. 6, lane 4). Immunoprecipitation with an antibody specific to the epitope tag revealed that the 40-kD protein was TTP itself (Fig. 6, lane 8). Expression of the tagged protein in these cytosolic extracts was confirmed by protein immunoblotting (Fig. 6, lane 10); the protein that reacted with the epitope tag antibody also reacted with TTP antibodies (*11*). Essentially identical results were obtained when the probe was a 70-bp fragment consisting only of the TNF-α ARE (*11, 26*).

These data show that TTP binds directly to the ARE contained within the 3′-UTR of TNF-α mRNA—and probably of other labile, ARE-containing mRNAs—and suggest that this binding destabilizes these mRNAs. Because the TTP knockout mice display evidence of chronic TNF-α excess, and their macrophages hypersecrete TNF-α, this interaction is likely to be of physiological importance. The data also suggest that the CCCH proteins as a class may be RNA-binding proteins; this should help in the elucidation of functions and binding partners for the other members of this protein family. Finally, this demonstration of direct binding of TTP to TNF-α mRNA, an interaction that presumably occurs in the cytosol, should permit the development of screening assays for compounds that potentiate, mimic, or increase the specificity of this reaction, and may ultimately lead to drugs capable of inhibiting TNF-α biosynthesis.

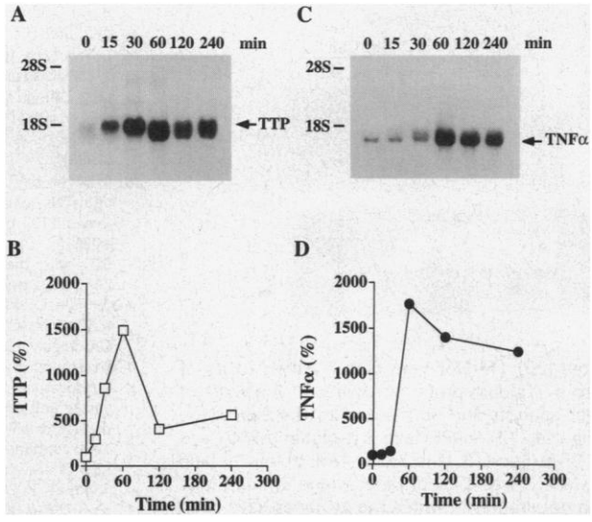

Fig. 3. TTP expression induced by LPS in macrophages. Primary macrophages were prepared from bone marrow of wild-type mice (*9*). Confluent 100-mm dishes were washed and exposed to LPS (1 μg/ml). RNA was obtained with an RNeasy kit (Qiagen) as directed by the manufacturer. (**A**) RNA from macrophages stimulated with LPS for the indicated times was analyzed by Northern blot with a mouse TTP cDNA (*4*). The blot shown is representative of three independent experiments. (**B**) Average values from three independent experiments, showing the amounts of TTP mRNA after LPS stimulation. Values were obtained with a PhosphorImager and normalized for GAPDH mRNA expression, and final values were expressed as the percentage of the value at time 0. (**C**) The same blot shown in (A) was stripped and reprobed with a mouse TNF-α cDNA. (**D**) Average values from three independent experiments, showing amounts of TNF-α mRNA after LPS stimulation.

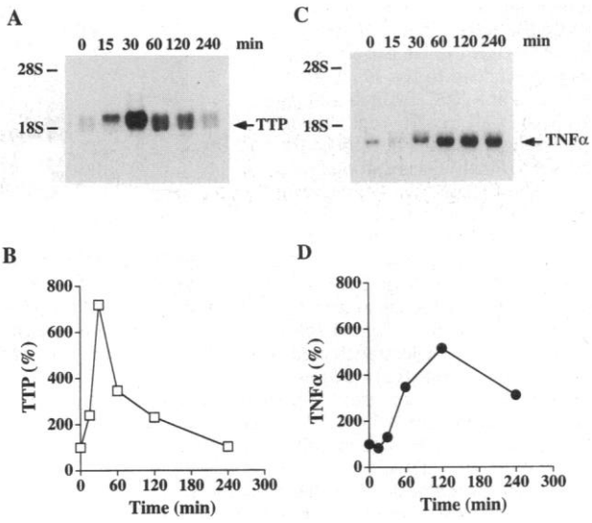

Fig. 4. TNF-α–induced expression of TTP in macrophages. Primary macrophages were prepared from bone marrow of wild-type mice as described (*9*). Confluent cells on 100-mm dishes were washed, exposed to TNF-α (10 ng/ml), and processed for Northern blotting with the Qiagen RNeasy kit. (**A**) RNA from macrophages stimulated with TNF-α for the indicated times was analyzed by Northern blot with a mouse TTP cDNA (*4*). The blot shown is representative of three independent experiments. (**B**) Averages from three independent experiments showing amounts of TTP mRNA after TNF-α stimulation. (**C**) The blot shown in (A) was stripped and reprobed with a mouse TNF-α cDNA. (**D**) Average values from three independent experiments, showing amounts of TNF-α mRNA after TNF-α stimulation.

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22. Plasmid H6E was first made by inserting a 3.7-kb Eco RI–Xba I fragment from the human genomic TTP clone (*10*) into the plasmid vector pBS+ (Stratagene). This insert contained ~1 kb of promoter, the first exon, the intron, the second exon, and 30 bp of 3′-flanking region. For H6E.HGH3′, a 597-bp Nsi I–Xba I fragment in the 3′-UTR of the human TTP gene that contained five rapid degradation signal sequences was replaced by the entire 110-bp human growth hormone (HGH) 3′-UTR. The polymerase chain reaction (PCR) primers used to amplify this fragment were 5′-GTGGCTTCTAGatgcatGGGTGGC-ATC-3′ (5′ primer) and 5′-GAAGGACACCtctagaGA-CAAAATGATGC-3′ (3′ primer), where capital letters represent the HGH sequences and lowercase letters represent the recognition sites for Nsi I (5′ primer) and Xba I (3′ primer). For CMV.TTP.tag, the influenza hemagglutinin (HA) epitope tag [P. A. Kolodziej and R. A. Young, *Methods Enzymol.* **194**, 508 (1991)] was attached to the last amino acid of the human TTP cDNA (*6*) by the PCR primer-overlapping mutagenesis technique [W. S. Lai, M. J. Thompson, P. J. Blackshear, *J. Biol. Chem.* **273**, 506 (1998)]. The fusion insert, containing the entire human TTP protein coding region and the HA epitope, was then cloned into the Hind III site of the vector CMV.BGH3′/BS+. This vector was created by blunt-ligating a Nru I–Pvu II fragment from pRc/CMV2 (Invitrogen), which contains the hCMV promoter/enhancer and the bovine growth hormone polyadenylation signal, into the Eco RI and Hind III sites of pBS+ (Stratagene).
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26. RNA probes were prepared as follows: Plasmid p3′mTNF-α, containing the mouse TNF-α 3′-UTR (bases 1110 to 1627 of GenBank accession number X02611), was created by reverse transcription (RT) PCR, with the use of total cellular RNA from Raw 264.7 cells treated for 4 hours with LPS (1 μg/ml) as templates for RT. The 5′ primer was 5′-CTTTCCgaat-tcACTGGAGCCTC-3′, and the 3′ primer was 5′-TA-GAtctagaAGCGATCTTTATTTCTCTC-3′ (lowercase letters indicate the restriction sites for Eco RI and Xba I, respectively). The resulting PCR fragment was digested and cloned into the Eco RI and Xba I sites of the vector pSK– (Stratagene). Plasmid pTNF-α1197-1350 contained a 153-bp fragment that included the ARE of the mouse TNF-α 3′-UTR (bases 1197 to 1350 of X02611); this was made using plasmid p3′mTNF-α as the template. The 5′ primer was 5′-GATAagatctCAGGCCTTCC-3′, and the 3′ primer was 5′-GCCTctagaTAAATACATTCATAAGC-3′. The resulting PCR product was digested with Bgl II and

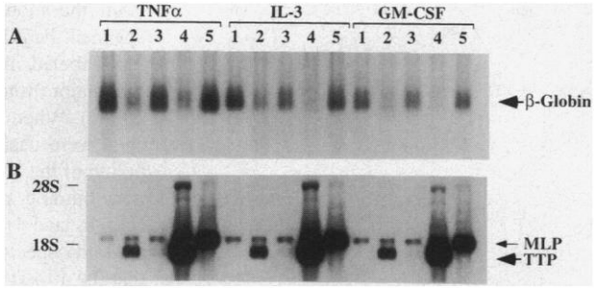

Fig. 5. Effect of TTP expression on accumulation of ARE-containing mRNAs. Constructs consisting of vector alone (5 μg, lanes 1), H6E.HGH3′ (5 μg, lanes 2), CMV.TTP.tag (0.01 μg, lanes 3; 5 μg, lanes 4), and a CMV-MLP construct (5 μg, lanes 5) were cotransfected into 293 cells with constructs consisting of the c-*fos* promoter, the β-globin coding region, and 3′-UTR into which were inserted AREs from TNF-α, IL-3, or GM-CSF, as indicated (*23*). (**A**) Northern blot in which 10 μg of total cellular RNA from these cells was hybridized to a β-globin probe (arrowhead). Amounts of β-globin mRNA were quantitated by PhosphorImager analysis and normalized to the expression of GAPDH in the same lane. For constructs containing the TNF-α ARE, lane 2 β-globin mRNA was 42% of lane 1; lane 3, 90%; lane 4, 26%; and lane 5, 86%. For IL-3, lane 2 was 43% of lane 1; lane 3, 66%; lane 4, 29%; and lane 5, 87%. For GM-CSF, lane 2 was 44% of lane 1; lane 3, 74%; lane 4, 24%; and lane 5, 103%. (**B**) Northern blot of the same samples in which the gel lanes have been aligned with the lanes in (A), hybridized to a mouse TTP probe (arrowhead) or to a mouse MLP probe (arrow). The faint bands at the position of the arrow represent endogenous 293 cell MLP. The positions of the major species of ribosomal RNAs are shown.

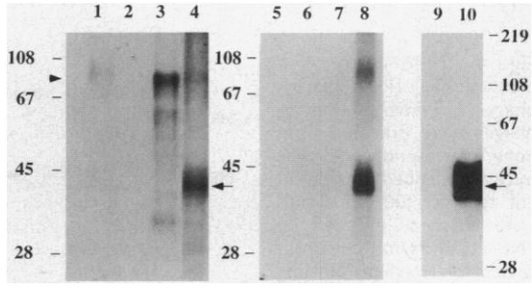

Fig. 6. Cross-linking of cellular proteins to a TNF-α mRNA ARE probe. Cytosolic extracts were prepared from 293 cells 24 hours after they had been transfected with CMV.TTP.tag (5 μg) or an equivalent amount of vector alone. Cells were lysed on ice for 20 min in 10 mM Hepes (pH 7.6), 3 mM $MgCl_2$, 40 mM KCl, 5% (v/v) glycerol, 0.5% (v/v) NP-40, 2 mM dithiothreitol, 0.5 mM PMSF, and leupeptin (8 μg/ml). The extracts were then centrifuged at 16,000*g* at 4°C for 15 min. Glycerol was added to the supernatant to 20% (v/v), and the resulting cytosolic extract was stored at –70°C. Extracts (20 μg of protein) were incubated with 2×10^6 cpm of RNA probe (*26*) in a 96-well plate at room temperature for 20 min in 0.02 ml of the lysis buffer described above without the protease inhibitors. Heparin and yeast tRNA were added to final concentrations of 5 mg/ml and 50 μg/ml, respectively, for an additional 10 min of incubation. The 96-well plate was then placed on ice and irradiated with 254-nm ultraviolet light in a Stratalinker (Stratagene) for 30 min at a distance of 5 cm from the light source. RNA was then digested with 100 units of ribonuclease (RNase) T1 (Life Technologies, Gaithersburg, Maryland) for 20 min at room temperature, and further digested with RNase A (1 mg/ml; Pharmacia) at 37°C for 15 min. The RNase-resistant RNA-protein complexes were then analyzed by SDS-PAGE followed by autoradiography. In the left panel, the arrowhead indicates undigested probe in lane 1, which has been digested in lane 2. Lane 3 indicates cross-linking to a protein of ~85 kD in extracts of cells transfected with vector alone; lane 4 shows cross-linking of RNA to a protein of ~40 kD (arrow). In the center panel, identical RNase-resistant RNA-protein complexes from cells transfected with vector alone (lanes 5 and 6) or CMV.TTP.tag (lanes 7 and 8) were diluted to 0.5 ml in a buffer consisting of 50 mM tris-HCl (pH 8.3), 150 mM NaCl, 5 mM EDTA, and 1% (v/v) NP-40, then precleared with nonimmune rabbit serum (1:100 dilution, 1 hour at 4°C) and protein A–Sepharose (1 hour at 4°C), and then incubated overnight at 4°C in the presence of either preimmune serum (1:100; lanes 5 and 7) or a 1:100 dilution of HA.11, a polyclonal rabbit antibody to the HA epitope tag (BAbCO, Richmond, California) (lanes 6 and 8). Immune complexes were recovered by centrifugation after addition of protein A–Sepharose, washed three times with a buffer consisting of 50 mM tris-HCl (pH 8.3), 150 mM NaCl, 1 mM EDTA, and 0.5% (v/v) NP-40, resuspended in 0.1 ml of SDS sample buffer, and subjected to SDS-PAGE and autoradiography. The arrow indicates the position of the immunoprecipitated ^{32}P-labeled RNA-TTP complex. In the right panel, cytosolic extracts (7 μg of protein) from cells transfected with 5 μg of vector alone (lane 9) or 5 μg of CMV.TTP.tag (lane 10) were separated on an SDS gel and subjected to immunoblotting with a rabbit antiserum to the HA epitope. The blot was visualized with chemiluminescence; the arrow indicates immunoreactive TTP. Positions of molecular size standards are indicated (in kilodaltons).

Xba I (sites indicated by lowercase letters in the primers) and cloned into the Bam HI and Xba I sites of the vector pSK–. Plasmid pTNF-α1281-1350 contained the seven AUUUA motifs of the TNF-α ARE (bases 1281 to 1350 of X02611). This was constructed using similar methods. Correct sequences of these plasmids were confirmed by dideoxy sequencing (Amersham). For radiolabeling of the RNA transcripts with [α-^{32}P]uridine triphosphate (800 Ci/mmol), plasmid TNF-α1197-1350 was linearized with Xba I and used as the template in the Riboprobe in vitro transcription system (Promega) protocol. The resulting product was precipitated with ammonium acetate and ethanol.

27. J. Y. Tso, X. H. Sun, T. H. Kao, K. S. Reece, R. Wu, *Nucleic Acids Res.* **13**, 2485 (1985).

28. Confluent dishes were washed three times with cysteine-free medium supplemented with fetal calf serum (10%). Cells were stimulated for 4 hours in the same medium with control conditions, LPS (1 μg/ml), or TNF-α (10 ng/ml). For the last 3 hours of the incubation, [^{35}S]cysteine (200 μCi/ml; NEN Life Sciences, Boston) was added to the cultures. Cells were washed twice with ice-cold PBS, scraped into 10 ml of PBS, and pelleted by centrifugation (1000*g* for 5 min at 4°C). Cells were then resuspended in 600 μl of lysis buffer [50 mM tris-HCl (pH 7.5), 50 mM NaCl, 3 mM $MgCl_2$, 5% (v/v) glycerol, 0.5% (v/v) NP-40, 0.02% (w/v) sodium azide, 5 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor (20 μg/ml), and leupeptin (8 μg/ml)], incubated on ice for 20 min, and lysed by passage five times through a 28-gauge needle attached to a 1-ml syringe with no dead space (Becton Dickinson). The nuclear pellet (after centrifugation at 1000*g* for 5 min at 4°C) was washed once in ice-cold wash buffer [10 mM tris-HCl (pH 7.5), 15 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM PMSF, and 5% glycerol], centrifuged at 1000*g* for 5 min at 4°C, and then resuspended and sonicated in the same volume of lysis buffer used initially to lyse the cells. The cytosolic fraction (supernatant) was clarified by centrifugation at 45,000*g* for 30 min at 4°C, using a tabletop ultracentrifuge (Beckman TL-100, rotor TLA.45). This method results in separation of cytosol and nuclear fractions, as assessed by protein immunoblotting with an antibody to SP1 as described (*18*). Cytosolic extracts matched by trichloroacetic acid–precipitable radioactivity and equivalent volumes of nuclear extracts were incubated with preimmune rabbit serum (1:100 dilution, 1 hour at 4°C) and protein A–Sepharose (1 hour at 4°C), and then incubated overnight at 4°C in the presence of either preimmune serum (1:100) or a 1:100 dilution of a polyclonal rabbit antibody to mouse TTP (*18, 19*). Immune complexes were recovered by centrifugation after the addition of protein A–Sepharose, washed three times with wash buffer [50 mM tris-HCl (pH 8.3), 150 mM NaCl, 1 mM EDTA, and 0.5% NP-40], resuspended in 100 μl of SDS sample buffer [P. J. Blackshear, *Methods Enzymol.* **104**, 237 (1984)], and subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (9% gel). For autoradiography, gels were fixed and treated with Autofluor (National Diagnostics, Atlanta).

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Genetic Dissection of a Mammalian Replicator in the Human β-Globin Locus

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The timing and localization of DNA replication initiation in mammalian cells are heritable traits, but it is not known whether initiation requires specific DNA sequences. A site-specific recombination strategy was used to show that DNA sequences previously identified as replication initiation sites could initiate replication when transferred to new chromosomal locations. An 8-kilobase DNA sequence encompassing the origin of DNA replication in the human β-globin locus initiated replication in the simian genome. Specific deletions within the globin origin did not initiate replication in these chromosomal sites. These data suggest that initiation of DNA replication in mammalian cells requires specific sequence information and extend the replicon hypothesis to higher eukaryotes.

The decision to initiate DNA replication is crucial to normal progression through the cell cycle (*1*). The replicon model (*2*) proposed that cells regulate DNA replication by means of a bipartite control mechanism comprising a trans-acting "initiator" that interacts with a cis-acting DNA element called the replicator. The replicon model has been validated, with some modifications, in bacteria (*3*), prokaryotic and eukaryotic DNA viruses (*4*), and yeast (*5*). Identification of replicators in these systems was expedited by their ability to function in extrachromosomal plasmids (*6*). In mammalian cells, initiation can be localized to specific chromosomal regions by biochemical methods, but the chromosomal sites identified in this manner as initiation regions (IRs), origins or origins of bidirectional replication (OBRs), could not, by themselves, support replication of plasmids (*7*). This may be due to elimination of acentric extrachromosomal DNA (*8*), failure to license transfected DNA for initiation before it integrates (*9*), or greater complexity of mammalian replicators, exceeding the cloning capacity of the vectors used (*7*). Alternatively, replication initiation may be determined by a specific nuclear structure established within a defined cell cycle interval rather than by DNA sequence (*10*).

We developed a genetic system to identify mammalian replicators on the basis of intrachromosomal initiation. Candidate replication origins that are included within lambda phage and cosmid clones can initiate DNA replication when transfected into mammalian cells and integrated into random sites (*7*), indicating that some specific DNA sequences or structures may act as replicators in ectopic chromosomal locations. If sequence-specific replicators exist, such sequences should initiate DNA replication when transferred to defined chromosomal sites that do not otherwise exhibit origin activity. Furthermore, if short, specific sequences direct initiation, then it should be possible to produce nonfunctional replicators by mutation or deletion. Because chromosome structure is an important component of replicator activity (*10–12*), wild-type and variant replicators must be analyzed at the same chromosomal location.

Our approach (Fig. 1A) uses the site-specific recombinases FLP from yeast and Cre from bacteriophage P1 to target replicators to unique chromosomal locations containing the small target sequences recognized by these proteins (*13*). We analyzed the human β-globin (hβG) IR using this approach because previous studies showed that cells with a naturally occurring deletion of the IR do not initiate replication within the >70-kb globin locus (*14*). Because initiation also requires the locus control region (LCR), located ~50 kb from the IR (*11*), we used the dual recombination system to create isogenic variants containing the IR with or without a 5′ mini-LCR (Fig. 1A).

We placed an 8-kb region from the hβG locus that encompasses the IR in ectopic sites in the simian (*Cercopithecus aethiops*) genome (Fig. 1A). A polymerase chain reaction (PCR)–based nascent strand abundance assay (Fig. 1B) (*15*) revealed that short, newly replicated nascent strands were generated from the β-globin IR in both sites (Figs. 2A and 3) (*16*). Such short strands are produced from regions within which DNA replication initiates (*11*). By contrast, short nascent strands were not generated at detectable levels from the β-galactosidase (β-Gal) gene sequences located 5 kb 3′ of the IR (Figs. 2A and 3). Globin sequences were also present in preparations of small nascent strands ob-

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