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The tumor necrosis factor-α AU-rich element inhibits the stable association of the 40S ribosomal subunit with RNA transcripts

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Abstract

Tumor necrosis factor-alpha (TNF-α) is a potent cytokine that is central to normal immune responses as well as autoimmune inflammatory diseases. The production of TNF-α protein is thus tightly regulated at multiple levels. Translational control is one of the means by which TNF-α production is repressed in unstimulated cells. To examine the mechanism by which the translation of TNF-α mRNA transcripts is repressed, we have used an in vitro translation system. The AU-rich element (ARE) in the 3′ UTR of TNF-α transcripts was sufficient to confer translational repression. This effect was observed using transcripts containing a 5′ m^7G cap but not uncapped transcripts, and was independent of a poly(A) tail. Sucrose gradient analysis revealed that ARE-containing transcripts were present at relatively lower amounts in 80S-associated fractions and higher amounts in non-ribosome-bound RNA fractions, with no accumulation of 48S-associated transcripts. ARE-mediated translational repression was competitively inhibited by ARE-containing transcripts. These data indicate that a TNF-α ARE-binding trans-acting factor(s) inhibits the association of the 43S complex with RNA transcripts.

Keywords: Tumor necrosis factor-α; AU-rich element; In vitro translation; Reticulocyte; Sucrose gradient; Poly(A); m^7G cap; Ribosome

Tumor necrosis factor-alpha (TNF-α) is a major pro-inflammatory cytokine whose expression is critical during normal immune responses to pathogens, and whose overexpression is associated with autoimmune inflammatory disorders. Neutralizing anti-TNF-α antibodies and soluble TNF-α receptors provide significant reduction in the symptoms and signs of disease activity in patients with rheumatoid arthritis, inflammatory bowel disease, psoriatic arthritis, and ankylosing spondylitis [1]. TNF-α thus appears to be at the apex of an inflammatory cytokine cascade.

The importance of tightly regulating TNF-α levels is reflected in the number of steps at which its production is regulated. These include RNA transcription, splicing, transport, stability, and translation [2]. The signaling pathways controlling TNF-α mRNA stability and translation have been best characterized in the LPS response of monocyte/macrophage cells. LPS binding to Toll-like receptors (TLRs) leads to the sequential recruitment of MyD88, IL-1R-associated kinases (IRAKs), TRAF6, MKK3/6, and p38 MAP kinase [3]. Increases in TNF-α mRNA stability and translation following p38 activation is mediated, at least in part, by activation of MAPKAPK-2 [4].

Sequences within the TNF-α 3′ untranslated region (3′UTR) confer mRNA transcript instability, i.e. the constitutive decay element (CDE), or mRNA transcript translational repression and instability, i.e. the AU-rich

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element (ARE) as well as a second AU-rich sequence located at 3′ of the ARE [5]. The TNF-α ARE contains multiple and overlapping nonamer (UUAUUUAUU) and pentamer (AUUUA) sequences, which are critical for mediating interactions with ARE-binding proteins (ARE-bps). TTP is an ARE-bp, which promotes transcript decay, at least in part, by recruiting the transcript to the exosome for 3′ exonucleolytic degradation [6]. In contrast, HuR appears to promote transcript stability [7]. TNF-α mRNA translational repression has been found to be promoted by the ARE-bps TIA-1 and TIAR [8], as well as FXR1 [9] and VHL [10].

Translation initiation is the most common point of translational control in mammalian cells. Under normal conditions, the 5′ m^7G cap of the RNA is bound by eIF4E within the eIF4F complex, which also contains eIF4G and eIF4A. This process is likely promoted by poly(A) binding protein (PABP) via its association with eIF4G. The small (40S) ribosomal subunit is recruited to the mRNA as part of a 43S complex that includes the ternary complex (eIF2, GTP, and Met-$tRNA_i$), eIF1, eIF1A, and eIF3, which interacts with eIF4F via eIF4G. The 43S complex scans the 5′UTR in a 5′–3′ direction until an AUG within an appropriate sequence is reached. eIF2 hydrolyzes its GTP, leading to release of many of the initiation factors and binding of the large (60S) ribosomal subunit to form the 80S initiation complex. After hydrolysis of GTP by eIF5B, translational elongation commences. Translation termination is then followed by recycling of the initiation factors [11,12].

Although most cellular mRNA transcripts are subject to global translational repression in response to cell stress [12,13], only a minority of mRNA species is subject to transcript-specific translational control. For example, ferritin mRNA transcripts contain a 5′UTR stem–loop sequence, which binds the repressor proteins IRP-1 and IRP-2 and inhibits the association of the 40S subunit in a cap-dependent manner [14]. 15-Lipoxygenase contains a 3′UTR element, which binds hnRNP-K and hnRNP-E1 that block 60S subunit joining in a cap-independent manner [15]. In *Drosophila*, male-specific lethal 2 (msl-2) mRNA contains elements in the 5′ and 3′ UTRs, each of which binds Sex-lethal [16]. This likely leads to inhibition of 43S binding in a cap-dependent manner, and inhibition of 43S scanning in a cap-independent manner. Translational control of RNA transcripts during development as well as cell cycle progression is mediated by 3′UTR sequences in a poly(A) tail-dependent manner [17].

The mechanism by which TNF-α mRNA translation is inhibited has not been determined. Using an in vitro translation system, we have found that the TNF-α ARE inhibits the stable association of the small ribosomal subunit with associated RNA transcripts.

Materials and methods

Generation of plasmids and RNA transcripts. Starting with pbluescript, annealed oligonucleotides were used to sequentially insert 90 adenine residues between the *Xho*I and *Kpn*I sites, the MiniTNF sequence (ACACCATGAGCACAGAAAGCATGATCCGCTGAAG GGAATGGGCTGCAGAGAGActcgag) between the *Sac*I and *Xho*I sites, and a stem–loop sequence (AATTCTAGAAGCCTTTCTAGA ACAACAA) [18] into the *Sac*I site (blunted with T4 DNA polymerase) to create MiniTNF. The 70 nucleotide TNF-α ARE (TATTTAT ATTTGCACTTATTATTTATTATTTATTTATTATTTATTTATT TGCTTATGAATGTATTTATTT) or a mutated ARE sequence (TA GGTATAGGTGCACTTAGTAGGTAGTAGGTAGGTAGTAGG TAGGTAGGTGCTTATGAATGTAGGTATTG) was then inserted between the *Pst*I and *Xho*I sites to create MiniTNF.ARE and MiniTNF.Mtare, respectively.

These plasmids were linearized with *Xho*I before generation of RNA transcripts using reagents from Ambion, Inc., according to the manufacturer's protocols. In vitro transcription was performed using the T3 Megascript kit. Radiolabeled poly(A) tails were added using poly(A) polymerase and [^{32}P]ATP. 5′ caps (m^7G) were added using guanylyltransferase with GTP and *S*-adenosyl methionine (SAM). Radiolabeled caps were added using [^{32}P]GTP in place of GTP. Transcripts were purified with Megaclear columns.

The TNF-α cDNA was used for PCR amplification with oligonucleotide 5′-AAAAGCGGCCGCAGGGACTAGCCAGGAGGGAG and either oligonucleotide 5′-CACACTC GAGCTTTTCCAAGCGA TCTTTATTTC or 5′-AGGCTCCAGTGAATTCGGAAAGC. The product was digested with *Not*I and *Xho*I, and ligated into pbluescript. The stem–loop sequence (see above) was inserted between the *Sac*II and *Nar*I sites within the 5′UTR sequences to create plasmids TNF and TNF + 3′UTR, respectively. Plasmids TNF + ARE, TNF + CDE, TNF + 2nd, and TNF + 3′terminus contain the stem–loop and TNF-α cDNA up to the start of the ARE (base 1283 of the GenBank sequence NM_013693) followed by one of the following: the 70 nucleotide ARE, the 80 nucleotide CDE, the sequence 3′ of the CDE (after nucleotide 1433 of the GenBank sequence NM_013693), or only the 3′ terminal region after the *Bst*EII site (this region is present in all the constructs), respectively. These plasmids were linearized with *Xho*I or *Kpn*I in preparation for in vitro transcription of RNA transcripts, which either lacked or contained a poly(A) tail, respectively, using the T3 Megascript kit. Transcripts were capped and purified as above.

In vitro translation. Micrococcal nuclease-treated reticulocyte lysates were purchased from Ambion, Inc. Twenty-five microliter reactions containing [^{35}S]methionine were performed according to the manufacturer's protocol using 0.3 pmol of RNA for 15 min at 30 °C. A volume of 15 μl from each reaction was added to 60 μl of loading buffer (2.5% SDS, 75 mM Tris, pH 6.8, 12.5% glycerol, 0.1% bromphenol blue, and 200 mM DTT), and boiled for 10 min. Aliquots (15 μl) were loaded onto a lane of SDS–PAGE, transferred to nitrocellulose, and exposed to film for autoradiography.

Northern blot analysis. A volume of 10 μl of each in vitro translation reaction was diluted with 115 μl of 100 mM KAc/10 mM Tris 7.5, and then 375 μl Trizol LS (Gibco). RNA was purified according to the manufacturer's protocol, loaded into a well of a denaturing agarose gel, and transferred to Nytran (Schleicher and Schuell). Reagents for non-isotopic Northern blotting were obtained from Roche. A digoxigenin-labeled TNF-α-specific probe was generated by PCR amplification of mouse TNF-α cDNA between the *Spe*I site and the stop codon (bases 524–783 of the GenBank sequence NM_013693) using the PCR DIG labeling mix. This probe was hybridized to the RNA using DIG Easy Hyb Granules and detected using anti-digoxigenin-AP Fab fragments and CDP-Star according to the manufacturer's protocol, with the addition of filtering the antibody solution through a 0.45-μm filter before applying it onto the Nytran membrane.