Mapping of a Minimal AU-rich Sequence Required for Lipopolysaccharide-induced Binding of a 55-kDa Protein on Tumor Necrosis Factor-α mRNA*

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In monocyte/macrophage cells, the translation of tumor necrosis factor-α (TNF-α) mRNA is tightly controlled. In unstimulated cells, TNF-α mRNA is translationally repressed. However, upon stimulation of the cells with various agents (e.g. lipopolysaccharides (LPS) and viruses), this repression is overcome and translation occurs. The key element in this regulation is the AU-rich sequence present in the 3′-untranslated region of TNF-α mRNA. Several groups have described the binding of proteins on AU-rich elements (AREs). We have previously reported the binding of two cytosolic binding of proteins on AU-rich elements (AREs). We have previously reported the binding of two cytosolic protein complexes (1 and 2) to the TNF-α mRNA, one of which (complex 2) is observed only following induction of TNF-α production by LPS. In this report, we have demonstrated that complex 1 involves a long fragment of the ARE, whereas the formation of the LPS-inducible complex 2 requires a minimal sequence which corresponds to the nonanucleotide UUAUUUAUU. Furthermore, we show that the RNA-binding protein involved in this regulation is the AUUUA pentanucleotide. Oncogene mRNAs generally fall into class 1, having one to three AUUUA sequences spaced throughout the 3′-untranslated region. The poly(A) tails of these mRNAs are degraded synchronously. Class 2 is mainly composed of cytokine mRNAs, and is defined by the presence of multiple clustered pentamers. This confers a progressive mechanism of poly(A) tail decay.

AREs in cytokine mRNAs are also implicated in translational control (3, 4, 9). Tumor necrosis factor-α (TNF-α) is not expressed in most cell types or in unstimulated monocyte-derived cell lines. Using a reporter gene linked to a series of deletions of the TNF-α 3′-UTR, the sequences mediating this repression were localized to an ARE. In macrophages, TNF-α production is rapidly induced following stimulation with lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria. Although part of this induction can be accounted for by an increase in both the transcription of the TNF-α gene and the stability of the mRNA, it has been shown that the most important event following LPS stimulation is a relief from the ARE-mediated translational blockade.

Recently, we and others have shown that several proteins can complex with the ARE of the TNF-α mRNA in unstimulated cells (10, 11). Furthermore, an additional protein complex is recruited to this region following cell activation, suggesting a role in TNF-α mRNA translational derepression (10). Here, we show that this inducible complex contains a previously undescribed protein of 55 kDa and demonstrate that it binds to a single UUAUUUAUU sequence, but not to the AUUUA pentanucleotide.

Translational control is now considered to be an important means of gene regulation. The expression of many mRNAs, including those encoding the p53 protein (1), lipoxygenase (2), and cytokines (3–5), has been shown to be controlled at a translational level. In each of these cases, the sequences responsible for this effect have been mapped to the 3′-untranslated regions (UTRs) of the mRNAs.

AU-rich elements (AREs) are found in the 3′-UTRs of many oncogene and cytokine mRNAs (6). Originally, these motifs, in particular the AUUUA pentanucleotide, were shown to destabilize messages when placed downstream of the coding sequence (7). ARE-containing mRNAs have been divided into two classes based on the number and distribution of the AUUUA pentamers. Oncogene mRNAs generally fall into class 1, having one to three AUUUA sequences spaced throughout the 3′-untranslated region. The poly(A) tails of these mRNAs are degraded synchronously. Class 2 is mainly composed of cytokine mRNAs, and is defined by the presence of multiple clustered pentamers. This confers a progressive mechanism of poly(A) tail decay.

Experimental Procedures

Materials—Enzymes used for construction of the different plasmids were purchased from Promega or Life Technologies, Inc. RNase T1 was purchased from Boehringer Mannheim. RNase A and LPS (Escherichia coli strain 0127:B8) were obtained from Sigma. Synthetic RNAs were purchased from Eurogentec (Belgium).

Cell Culture—RAW 264.7 mouse macrophages were maintained as described previously (10). LPS was added at a final concentration of 10 ng/ml for 2 h in all experiments.

DNA Constructs—The TNF-α ARE probes have been previously described (10). The GM-CSF ARE probe was synthesized by inserting the 227-base pair NotI-BglII fragment corresponding to the distal part of the GM-CSF mRNA 3′-UTR (3) into the SP64 plasmid previously cut by PstI, blunted, and cut by BamHI. The c-myc 3′-UTR construct was a kind gift of Dr. Blanchard, Montpellier, France.

To construct the 3′-PCG-generated deletions of the TNF-α ARE, the primer 5′-CGTACAGGATCCCTCGTCGACTCCATAC-3′ was used with the following primers: 5′-CCACTAGTGATCGATTATAATAATTAATATCAGGC-3′ (3′-d1), 5′-CCACTAGTATCGATAAATAATATAATGTCG-3′ (3′-d2), and 5′-CCACTAGTGATCGATTGCAAAT-
ATAAAT-3' (3'-d3). To construct the 5'-d1 construct the following primers were used: 5'-CCACTAGTTCAGCTCCGTTTTCACAG-3' and 5'-CGGTACCATCGATTATTTATTTGCTTATGA-3'. The TNF-α ARE was used as a template. The resulting DNA fragments were subcloned into the KpnI and SpeI sites of the pBluescript plasmid (Stratagene). All plasmids were sequenced.

In Vitro Transcription—To synthesize riboprobes, the pBluescript derived plasmids were linearized with XbaI and transcribed using T7 RNA polymerase (Life Technologies, Inc.); the TNF-α ARE and GM-CSF plasmids were linearized with EcoRI and transcribed using SP6 polymerase (Life Technologies, Inc.), and the c-myc plasmid was linearized with XhoI and transcribed using T3 polymerase (Life Technologies, Inc.). All reactions were carried out as described previously (10) for electrophoretic mobility shift assay (EMSA) probes, and with [α-32P]UTP (100 mCi; 3000 Ci/mmol) for the UV cross-linking probes. Unlabeled RNAs were synthesized using 0.5 mM amounts of each nucleotide.

Treatment of TNF-α ARE with RNase T1—TNF-α ARE probe was digested with RNase T1, which cleaves at the 3' side of G residues, yielding three major fragments of 39, 20, and 10 nucleotides (nt), respectively (Fig. 1a). The fragments of 39 and 20 nucleotides were purified by denaturing gel electrophoresis. We confirmed the identity of the two fragments by demonstrating that neither was susceptible to further RNase T1 digestion (data not shown).

EMSA, Gel Elution, and Denaturing PAGE—S100 macrophage extracts and EMSA were carried out exactly as described previously (10), except that migration was performed at 220 V for 150 min. Unless specified, 10 fmol of labeled probe (approximately 30,000 cpm) was added to each reaction. To elute RNA, bands were excised from the wet gel and placed into oligonucleotide elution buffer (2 M ammonium acetate, 1% SDS, 25 mg/ml tRNA) for 2 h at 37 °C. RNA was precipitated and, following resuspension in water, was analyzed on 6 M urea, 20% acrylamide gels. To elute UV cross-linked RNA-protein complexes, dried gel slices were rehydrated in 4% ammonium bicarbonate, 1% β-mercaptoethanol, 0.1% SDS and boiled for 5 min. Samples were left overnight on a rotating wheel and then centrifuged to remove the debris. The supernatants were concentrated using Ultrafree-MC columns (Millipore) and then subjected to SDS-PAGE.

UV Cross-linking and SDS-PAGE—Samples were prepared exactly as for EMSA, except that 500,000 cpm of probe and 20 μg of protein was used and 10 μg of tRNA was included in the binding reaction. Following RNase T1 digestion (60 units) and addition of heparin, samples were placed on ice at a distance of 4 cm from a UV light source (Stratalinker) for 10 min. Samples were then treated with RNase A (0.1 mg/ml) for 10 min at room temperature, before loading onto either native gels for EMSA analysis or 9% SDS-polyacrylamide gels (37.5:1 cross-linking ratio).

RESULTS
Both Inducible and Constitutive Complexes Bind to a 39-nt Fragment of the TNF-α ARE—We have shown previously that in S100 extracts of unstimulated RAW 264.7 murine macrophages, a single protein complex (complex 1) binding a 70-nucleotide ARE in the 3'-UTR of the TNF-α mRNA can be detected by EMSA. In similar experiments using extracts prepared from LPS-stimulated macrophages, an additional complex (complex 2) was observed that runs with a higher mobility than complex 1 (10). To further delineate the exact sequence requirements for complex 1 and 2 formation, we digested the ARE probe with T1 ribonuclease, yielding three major fragments of 39, 20, and 10 nucleotides (Fig. 1a). The purified 39-nt...
and 20-nt T1 products were used in an EMSA with S100 macro-
phage extracts as described previously (10). The 39-nucleotide
fragment supports the formation of the same complexes as
those observed previously using the complete TNF-α ARE (Fig.
1b). Binding to the 20-nt fragment is also detectable but at a
much reduced level. These results show that both complexes 1
and 2 can form with a RNA fragment containing clustered
AUUUA pentamers. However, neither complex forms strongly
with a RNA fragment containing a single AUUUA pentamer.

Identification of the RNA Sequence Involved in Formation of
Complexes 1 and 2—To gain information on the RNA species
present in complexes 1 and 2, we performed a binding reaction
with the TNF-α ARE probe and protein S100 extract. The
samples were subsequently digested with RNase T1 and loaded
on a non-denaturing gel. After migration, the RNA-protein
complexes were eluted from the gel and the purified RNA
fragments were analyzed by denaturing polyacrylamide gel
electrophoresis (Fig. 2). Complex 1 mostly bound to the 39-nt
fragment (see Fig. 1a). Complex 2, however, bound a number of
fragments, the strongest band corresponding to a 10-nt diges-
tion product. This could not be further digested with RNase T1
following elution and purification, identifying it as the
UAUUUAUUUG fragment found in the 3’ end of the TNF-α ARE. Surprisingly, only a small amount of the 39-nt fragment
was eluted. Presumably, in the conditions used for the EMSA
experiment, complex 1 has a higher affinity for this sequence or
is more abundant and efficiently competes for binding. In ad-
dition, the 20-nt fragment was clearly present, together with a

![Graphical representation](image-url)

**Fig. 3. Identification by deletion analysis of the sequence required for complexes 1 and 2 formation.** a, sequence of the deletion
constructs used for EMSA. b, EMSA performed with S100 extract from unstimulated (−) and LPS-stimulated (+) cells and the indicated RNA
probes. c, competition experiment in which S100 extract from LPS-stimulated cells (+) were incubated with TNF-α ARE probe in the absence or
the presence of increasing ratio of unlabeled TNF-α ARE or 5’-d1 RNAs.
Characterization of an LPS-induced AU-rich Binding Protein

In order to test whether this sequence is sufficient for complex 2 formation, we synthesized an artificial RNA consisting of this nonamer motif. This oligoribonucleotide was used in competition experiments with a labeled TNF-α ARE probe. We observed a concentration-dependent inhibition of labeled complex 2 formation (Fig. 4, upper panel). As expected, there was no change in complex 1 binding. Further synthetic RNA nonamers were synthesized with mutations in the sequences flanking the central AUUUA pentamer. Again, these oligoribonucleotides were used in competition experiments with a labeled TNF-α ARE probe. Mutation of the two outer U residues to C residues significantly reduced the ability of the RNA to compete for complex 2 binding (Fig. 4, upper panel). Changing all four of the flanking U residues to C completely abolished the competition. In addition, no competition was observed with a nonamer consisting only of U residues (Fig. 4, lower panel). These observations further indicate that the protein(s) forming complex 2 require(s) UUAUUUAUU sequences for high affinity binding and that the pentameric AUUUA motif is not sufficient. Moreover, the reduced affinity observed for the CUAUUUAUC sequence explains why complex 2 can form on the 20-nt T1 digestion product (Fig. 1), albeit with a lower affinity.

A 55-kDa Protein Is a Component of Complex 2—We have used the specificity of the 5′-d1 probe for complex 2 as a means of determining the molecular size of its protein constituents. S100 extract from either unstimulated or stimulated macrophages was incubated with 5′-d1 probe labeled to a high specific activity. Binding was carried out in the presence of tRNA to reduce nonspecific binding (data not shown). Following T1 digestion and addition of heparin to reduce nonspecific electrostatic interactions, the samples were exposed to short-wavelength UV irradiation. Proteins were separated by SDS-PAGE, and the dried gels were autoradiographed to visualize proteins that had become cross-linked to the radiolabeled 5′-d1 probe. A single band of approximately 55 kDa was observed with stimulated but not with unstimulated extracts (Fig. 5, left panel). This 55-kDa band was not detected in the absence of UV irradiation (data not shown). The same experiments were carried out using a radiolabeled TNF-α ARE probe. Again, an inducible protein of approximately 55 kDa was observed. In addition, proteins of 48 kDa and greater than 200 kDa cross-linked to the TNF-α ARE in a constitutive manner.

The above results suggest that the 55-kDa protein may be a constituent of complex 2. To confirm this, we ran the UV-irradiated samples on native gels and excised complex 2. Protein and RNA was eluted and subjected to SDS-PAGE as be-
fore. The same 55-kDa protein eluted from complex 2 formed with both the 5'-d1 and TNF-α ARE probes (Fig. 5, right panel).

The 55-kDa Protein of Complex 2 Binds to the GM-CSF ARE, but Not to the c-myc ARE—From the results shown in Fig. 3, we predicted that complex 2 should form on the GM-CSF ARE, which contains several nonamer motifs, but not on the c-myc ARE, which contains only AUUUA pentameric motifs (Fig. 6a). To test this hypothesis, we used riboprobes containing the AREs of the GM-CSF and c-myc mRNAs in EMSA experiments. It can be seen in Fig. 6a that the GM-CSF probe supports formation of an inducible complex. No complex formation was observed using the c-myc riboprobe. In addition, a 55-kDa protein could be cross-linked to the GM-CSF probe, but only in LPS-induced extracts (Fig. 6c).

DISCUSSION

TNF-α mRNA translational regulation is governed by the AU-rich sequence present in its 3'-untranslated region. In a previous report, we showed by gel retardation experiments that this AU-rich sequence is able to form two different complexes (1 and 2) with proteins present in the cytosolic fraction of macrophage cell extracts. Complex 1 is characterized by a low electrophoretic mobility, is constitutively formed, is present in both nuclear and cytosolic fractions, and probably corresponds to previously reported complex A (11) and AU-A (13). Complex 2 has a higher electrophoretic mobility and is only detected with cytosolic extracts obtained from cells stimulated to produce TNF-α. In this study, we characterized the sequence requirements for the formation of these two complexes.

Consistent with data concerning the specificity of complex A (11), we showed that complex 1 is able to form with the 39-nt fragment generated by RNase T1 digestion of the TNF-α ARE probe but not with a 20-nt fragment (Figs. 1 and 2). Interestingly, the 39-nt fragment contains five overlapping AUUUA pentamer motifs, whereas only one pentamer is present in the 20-nt fragment. A progressive deletion of the TNF-α ARE within the 39-mer region rapidly abolishes the formation of complex 1 (Fig. 3b). Furthermore, complex 1 could not bind to a synthetic UUAUUUAUU sequence. Altogether, these results suggest that complex 1 formation requires a large fragment of the TNF-α ARE containing clustered AUUUA pentamers. The functional significance of complex 1 remains to be elucidated. TNF-α mRNA is both unstable and translationally repressed in quiescent macrophages (14). As complex 1 is constitutively formed with cytosolic macrophage extract, this complex could mediate one of these effects. AU-rich regions, but not single AUUUA pentamers, are associated with mRNA instability (12). Hence, there is a correlation between the sequence requirements for mRNA degradation and those required for complex 1 formation. However, Han and co-workers (4) demonstrated that a UUAUUUAUU sequence alone is a poor mediator of translational repression. This is consistent with the translational repression being mediated by complex 1. Further experiments will be required to elucidate the exact relationship between complex 1, translational repression, and mRNA destabilization.

Several lines of evidence point to the target RNA sequence involved in the formation of the LPS-inducible complex 2 being composed of a single (U)UAUUUAUU(U) motif, but not an AUUUA pentanucleotide. This is, to our knowledge, the first demonstration that this nonamer is by itself a recognizable sequence motif. First, the purification of the RNA product present in complex 2 in a gel shift experiment leads to the isolation of a major product identified as a UAUUUAUUUG. Second, gel shift experiments using different probes derived from the TNF-α ARE demonstrate that RNA sequences containing the UUAUUUAUU motif can form an LPS-inducible
complex (probes 3′-d2 and 5′-d1; Fig. 3). Complex 2 formation is poorly supported by a probe containing only a UUAUUUAU motif (probe 3′-d3). Third, competition experiments between an RNA nonanucleotide and the complete TNF-α ARE show that a UUAUUUAU motif is more efficient than a CUAUUUAUC motif at competing for the formation of the complex 2, whereas the CCAUUUACC nonamer does not compete at the concentrations used (Fig. 4).

We have shown that the class II GM-CSF ARE also binds complex 2. Most Class II AREs derived from cytokine mRNA 3′-UTRs contain at least one copy of the nonanucleotide UUAUUUAUU (8). Class I AREs, which are found in several oncogene mRNAs, do not contain nonamer motifs. It is therefore unlikely that these could support complex 2 formation. Indeed, we have failed to observe an LPS-inducible complex forming on the c-myc ARE in gel retardation experiments (Fig. 6b).

From our data, it is clear that the presence of the nonamer UUAUUUAUU motif could be a key factor in the differential recognition of class I and II AREs by some proteins. The stabilities of class I and II ARE-containing mRNAs are differentially regulated (8, 15). A mutational analysis has suggested that the key feature of class II AREs that leads to this physiological difference is reiterated AUUUA motifs, and not UUAUUUAUU sequences. As our data show that complex 2 forms readily on this nonamer sequence, it suggests that complex 2 is not involved in the regulation of TNF-α mRNA stability. Other class II-specific binding activities may be responsible for this differential regulation of mRNA stability (13). Since formation of complex 2 tightly correlates with translational derepression of the TNF-α mRNA, the nonamer motif may be a key element for translational control. It may be that the exact physiological function of class II AREs arises from a complex interplay between the number and positioning of reiterated pentamers and individual nonamer motifs.

UV cross-linking experiments using the 5′-d1 probe specific for complex 2 clearly demonstrate that this complex contains a 55-kDa protein. Using the same technical approach, several groups have described proteins that bind to the AU-rich element of cytokine mRNAs in different cellular systems. With the exception of the work of Bohjanen et al., all these data concerned constitutive ARE binding activities. Bohjanen et al. (13) described 30-kDa (AU-B) and 43-kDa (AU-C) class II ARE-specific binding activities that were only present following activation of T cells. Unlike complex 2, these activities co-migrated with a constitutive ARE binding activity in gel retardation experiments. On these bases, we believe that complex 2 contains a previously undescribed inducible 55-kDa ARE-binding protein.

This work forms the basis of an analysis into the relationship between translational control and mRNA stability in macrophages, and demonstrates further differences between cytokine and oncogene AREs. Isolation of the 55-kDa protein by using the specific RNA probes described in this report should provide further information as to its role in the translational control of TNF-α mRNA.

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REFERENCES
1. Fu, L., and Benchimol, S. (1997) EMBO J. 16, 4117–4125
2. Ostareck, D. H., Ostareck Lederer, A., Wilm, M., Thiele, B. J., Mann, M., and Hetz, M. W. (1997) Cell 89, 597–606
3. Kruys, V., Marinx, O., Shaw, G., Deschamps, J., and Huez, G. (1989) Science 245, 852–855
4. Han, J., Brown, T., and Beutler, B. (1990) J. Exp. Med. 171, 465–475
5. Kern, J. A., Warnock, L. J., and McCafferty, J. D. (1997) J. Immunol. 158, 1187–1193
6. Caput, D., Beutler, B., Hartog, K., Thayer, R., Brown Shimer, S., and Cerami, A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1670–1674
7. Shaw, G., and Kamen, R. (1986) Cell 46, 659–667
8. Xu, N., Chen, C. Y., and Shyu, A. B. (1997) Mol. Cell. Biol. 17, 4611–4621
9. Espel, E., Garcia Sanz, J. A., Aubert, V., Menoud, V., Sperisen, P., Fernandez, N., and Spertini, F. (1996) Eur. J. Immunol. 26, 2417–2424
10. Guesdon, C., Houzet, L., Marchant, A., Sels, A., Huez, G., and Kruys, V. (1996) Mol. Med. 2, 479–488; Correction (1996) Mol. Med. 2, 786
11. Hel, Z., Skamene, R., and Radzioch, D. (1996) Mol. Cell. Biol. 16, 5579–5590
12. Lagnado, C. A., Brown, C. Y., and Goodall, G. J. (1994) Mol. Cell. Biol. 14, 7984–7995
13. Bohjanen, P. R., Petryniak, B., June, C. H., Thompson, C. B., and Lindsten, T. (1994) Mol. Cell. Biol. 11, 3288–3295
14. Han, J. H., Beutler, B., and Huez, G. (1991) Biochim. Biophys. Acta 1090, 22–28
15. Schuler, G. D., and Cole, M. D. (1988) Cell 55, 1115–1122