Influence of Nonameric AU-rich Tristetraprolin-binding Sites on mRNA Deadenylation and Turnover*

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Tristetraprolin (TTP), a member of the tandem CCCH zinc finger protein family, promotes deadenylation of tumor necrosis factor-α and granulocyte-macrophage colony-stimulating factor mRNAs after binding to the AU-rich elements (ARE) in their 3′-untranslated regions. The high affinity TTP-ARE binding occurs between the tandem zinc finger domain and the preferred nonamer UUAUUUAUU. By mutating a well defined core sequence of 24 bases from the tumor necrosis factor-α ARE, we compared the influence of four possible nonameric TTP-binding sites in the wild-type ARE with that of a single binding site in the mutually probe on the binding of TTP to the RNA and the subsequent deadenylation of the poly(A) tail. By inserting this 24-base ARE into an otherwise stable transcript, we also attempted to determine the extent of the instability conferred by the presence of one or two TTP-binding sites. These sites were created or modified by mutating the As in the UUAUUUAUU nonamer or by changing the central U in the nonamer, in both cases to C residues. The results suggest that even a single nonamer TTP-binding site can confer at least partial sensitivity to the TTP-mediated mRNA turnover on an otherwise stable mRNA, but that two binding sites make the transcript much more unstable. Even though the central U of the nonamer binding site was predicted by structural studies possibly to permit base substitution, mutation of this U to C greatly inhibited the binding of TTP to the ARE, thus reducing the ability of the TTP to promote deadenylation and instability of the mRNA.

Tristetraprolin (TTP) is a member of a small family of three tandem CCCH zinc finger proteins in man that can bind to AU-rich elements in single-stranded mRNA and promote the successive deadenylation and degradation of those RNA species (for review see Ref. 1). Experiments in TTP knock-out mice and cells derived from them demonstrated that tumor necrosis factor-α (TNF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) transcripts were physiological targets for TTP-mediated destruction (2, 3). Both transcripts contain the so-called class 2 AU-rich elements (ARE) (4), and TTP was shown to bind to these elements through its tandem zinc finger (TZF) domain (2, 5). This binding is thought to be the first step in the process of subsequent transcript deadenylation and destruction.

More recently, details of the minimal ARE sequence required for TTP binding have emerged. For example, Worthington et al. (6) demonstrated through random selection procedures that the optimum minimum binding site was the nonamer UUAUUUAUU. By using a synthetic 73-amino acid peptide comprising the TTP TZF domain, we showed that the same nonamer was necessary for high affinity binding to the synthetic peptide, and that a 24-base RNA oligonucleotide derived from the TNF mRNA ARE could bind 2 mol of peptide/mol of RNA at two tandem nonamer binding sites (7). In the same paper, we showed by HSQC NMR analyses that the same nonamer represented the minimum ARE-binding site oligonucleotide required for a characteristic conformational shift of the TTP peptide, and that even minor shortening of the oligonucleotide resulted in changes in the peptide-RNA complex conformation. Similarly, by using a solution binding assay with the same synthetic 73-amino acid TTP TZF peptide, Brewer et al. (8) found that shortening the UUAUUUAUU nonamer to a UUAUUUAU heptamer increased the $K_d$ from 3.0 to 19 nM. On the other hand, lengthening the nonamer by adding two Us to each end did not affect the binding affinity.

Very recently, Hudson et al. (9) solved an NMR structure for the TZF domain of the TTP family member TIS11D (ZFP36L2), which is 73% identical to the TTP TZF domain over the 64 amino acids of the core TZF domain, in complex with the same UUAUUUAUU nonamer. They found that this nonamer bound to the TZF domain peptide in the absence of RNA secondary structure, suggesting that the RNA primary sequence is the critical determinant of effective binding. They also noted that the first U residue in the nonamer was unstructured in the complex, perhaps allowing for substitutions at that site in naturally occurring AREs. We used the coordinates of this complex to model the human TTP TZF domain in complex with the same oligonucleotide, and we found that the amino acid residues in contact with the RNA were identical in the two proteins, increasing the likelihood that this modeled TTP TZF domain-RNA complex is valid (10).

An important question that arises is: What is the relative contribution of each ARE nonameric TTP-binding site to TTP-mediated RNA turnover in naturally occurring mRNAs? In one naturally occurring target, the TNF mRNA, there are often five partly overlapping nonamers in the mammalian transcripts, with some variation among mammalian species (10). There are fewer nonamers in the GM-CSF mRNA, and in the most common mammalian pattern, three nonamers overlap significantly (10). Perhaps coincidentally, GM-CSF mRNA in bone marrow-derived stromal cells has a half-life of 99 min (3), whereas TNF in bone marrow-derived macrophages from normal mice has a half-life of 39 min (2).

The main objective of the present study was to determine whether an individual TTP-binding site conferred instability on an otherwise stable
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transcript, and whether two tandem binding sites conferred even greater instability. We used a well defined core sequence of 24 bases from the mouse TNF ARE, which in its unmutated state is able to bind two tandem molecules of TTP TZF peptide per molecule of RNA (7). This core sequence was then mutated at one or both of the As in the nonamer, or the central Us, to permit 2, 1, or 0 mol/mol of peptide binding, and the effects of these mutations were evaluated in cell co-transfection assays with full-length TTP and in cell-free deadenylation assays. The results suggest that even a single nonamer TTP-binding site can confer some instability on an otherwise stable mRNA, but that two makes the transcript much more unstable. In addition, they indicate the importance of the central U residues in binding of TTP to RNA. This binding and the ability of the TTP to degrade RNA were greatly decreased when these U residues were changed to Cs, even though the central U residue (U5), like the terminal U9, is thought to form only a single hydrogen bond with an amino acid in the protein (9), and thus might be assumed to be replaceable with minimal loss of activity.

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Expression plasmids CMV-hTTPtag and its tandem zinc finger (TZF) domain alone (CMV-hTTP-(97–173)-tag) were made as described (11). The CMV-HuR-tag, which expresses the widely expressed human ELAV-like HuR protein (12), was constructed as follows. A cDNA coding for the open reading frame of HuR was made by PCR using a plasmid kindly provided by Dr. Jack Keene, Duke University (13), as a template. The 5’ primer for the PCR amplification was 5’-ACGTggtaccATGGAAACACAACTGTCTAATGG-3’, and the 3’ primer was 5’-AAGctcgagTTAACGCTATTCGGGACGC TTGATGTTTGGGACCTT-GTTGG-3’, where the lowercase letters indicate the restriction sites for Asp718 and Xhol, respectively. The underlined letters represent the initiator methionine (5’ primer) and the stop codon (3’ primer). The italic letters in the 3’ primer encode the hemagglutinin (HA) epitope. The resulting PCR product was digested with the restriction enzymes and ligated into the Asp718 and Xhol sites of the expression vector CMV-BHG3/BS+ (5). The sequence of the HuR insert corresponds to bp 116–1196, from bp 3 bp before the start codon to the last amino acid, of GenBankTM accession number U38175. CMV-HuB-FLAG, which expresses the human ELAV-like neuronal protein 1 (Hel-N1, HuB), was made by PCR using a plasmid kindly provided by Dr. Jack Keene, Duke University (13), as a template. The 5’ primer for the PCR amplification was 5’-ACGTggtaccTGGAAAAACAACCTGTCTAAATTG-3’, and the 3’ primer was 5’-AAGctcgagTTAACGCTATTCGGGACGCTTGGATGTTTGGGACCTTGTTGG-3’, where the lowercase letters indicate the restriction sites for Asp718 and Xhol, respectively. The underlined letters represent the initiator methionine codon (5’ primer) and the stop codon (3’ primer). The italic letters in the 3’ primer encode the FLAG epitope. The resulting PCR product was digested with the restriction enzymes and ligated into the Asp718 and Xhol sites of the expression vector CMV-BHG3/BS+. The insert sequence of HuB corresponds to bp 585–1661 (from the initiation codon to the last amino acid) of GenBankTM accession number U12431. The correct sequences of both the HuR-tag and HuB-FLAG DNA inserts were confirmed by dRhodamine terminator cycle sequencing (PerkinElmer Life Sciences).

Cell-free Assays

Preparation of RNA Probes—Plasmid pTNFα-(1309–1332) (bp 1309–1332 of GenBankTM accession number X02611) and plasmid TNFα-(1309–1332) (A)350/SK– were constructed as described (5, 14).

To make the mutant TNFα-(1309–1332) (A)350 probes, a series of double-stranded oligonucleotides that encoded the corresponding RNA sequences listed in Fig. 1A was made to substitute for the wild-type TNFα-(1309–1332) sequence in the EcoRⅤ and Xbal sites of pTNFα-(1309–1332) (A)350/SK–. The templates for probes were PCR-amplified (primers M13 forward and T50Xba) from the plasmids and were sequenced to verify the 3’ ends, as described (14). The template for probe ARE was prepared by linearizing plasmid pTNFα-(1309–1332) with Xbal, and for probe V by linearizing vector SK–.

The RNA probes were transcribed in the presence of [α-32P]UTP (800 Ci/mmol). Linearized plasmids or PCR amplification products were used as templates, and the Promega riboprobe in vitro transcription systems protocol was employed. The resulting probes were separated from the free nucleotides using G-50 columns for RNA gel shift assays; for cell-free deadenylation assays, the probes were purified from urea-TBE gels.

Transfection of HEK 293 Cells and Preparation of Cell Extracts—To each 100-mm plate of 293 cells was added 5 μg of vector DNA (BS+) alone, or 0.2 μg of CMV-hTTP-tag, or 2 μg of the TZF domain expression plasmid CMV-hTTP-(97–173)-tag (11), or 0.5 μg of CMV-HuB-FLAG, or 0.5 μg CMV-HuRtag. Vector DNA (BS+) was added to each plate to make the total amount of co-transfected DNA 5 μg per plate by using the calcium-phosphate precipitation method. Twenty-four h after the removal of the transfection mixture, the cell monolayers were rinsed with ice-cold Ca2+- and Mg2+-free phosphate-buffered saline and then scraped into phosphate-buffered saline. After centrifugation at 600 × g for 3 min at 4 °C, the cell pellet was gently rinsed in ice-cold diethyl pyrocarbonate-treated water containing 8 μg/ml leupeptin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml pepstatin A. The cells were lysed in a hypotonic buffer containing 10 mM HEPES (pH 7.6), 5 mM KCl, 5% (v/v) glycerol, 0.2% (v/v) Nonidet P-40, 1 μg/ml pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride, and 8 μg/ml leupeptin. After a 15-min centrifugation for 15,000 × g at 4 °C, KCl and glycerol were added to the supernatant to achieve final concentrations of 40 mM and 15% (v/v), respectively. The cell extracts were stored at −70 °C. The expression of proteins from these constructs was determined by Western blotting with an antibody directed at the HA epitope tag (1:2,000, Santa Cruz Biotechnology) or the FLAG epitope tag (1:10,000, Sigma), essentially as described (11).

RNA Electrophoretic Mobility Shift Assay—Extracts prepared from 293 cells as described above (5 μg of protein) were incubated with 2 × 105 cpm of RNA probe (RNase TI-treated, 10 units/reaction, Epicenter) in 25 μl of a buffer that contained 10 mM HEPES (pH 7.6), 40 mM KCl, 3 mM MgCl2, 50 μg of heparin, 1.2 μg of yeast tRNA, as described (5), and the protein-RNA complexes formed were resolved on 8% nondeaturing acrylamide (37.5:1) gels.

In Vitro Deadenylation Assay—Extracts prepared from 293 cells as described above (10 μg of protein) were incubated with 5 × 108 cpm of RNA probe as described (14). Aliquots of reaction products were analyzed on 6% acrylamide gels containing 7 M urea. The resulting gels were exposed to x-ray films and were analyzed by PhosphorImager to quantify the amount of intact probe remaining.

Co-transfection Assays

Plasmids—A series of plasmids was constructed with each containing the following components: a CMV promoter, the protein coding sequence of the mouse MARCKS-like protein (MLP), used because both the protein and mRNA are relatively stable in the 293 cell co-transfection system; an HA epitope tag attached in-frame to the MLP protein sequence; a 24-base RNA insertion corresponding to the core

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ARE of human and mouse TNF; and the 3'-UTR and polyadenylation signal sequence from the bovine growth hormone mRNA 3'-UTR (see Fig. 1B). This was the parent plasmid for subsequent mutants, and served as the "target" for TTP-mediated mRNA turnover in the cell transfection assays. The various mutant forms of this plasmid and their construction are described in detail below, but they basically represented a group of substitution mutants of the wild-type plasmid described above at the 24-base ARE region. They include a series of mutant ARE-containing plasmids based on the mutants of the TNF ARE described in Blackshear et al. (7), as well as two novel mutations of the middle U residue in some of the UUU triplets. A schematic representation of these target plasmids is shown in Fig. 1B.

For the parent MLP expression plasmid CMV-MLP-HA, the protein coding region of mouse MLP (GenBank™ accession number NM_010807) was fused at its carboxyl terminus in-frame with the HA epitope coding sequence and inserted into CMV-BGH3'/BS+ (5). To create the fusion insert, plasmid pF52.ab, consisting of the mouse Mlp cDNA (16), was used as a PCR template. The 5' primer for PCR amplification was 5'-AATGgacctCATCATGGCGAGCAG-3', and the 3' primer was 5'-TATGctcgAGTTAAACGCGGTGAGCGTCTAGT-GGGATACCTTACTGCTGAGCTGAG-3'. The lowercase letters in the primers indicate the restriction sites for Asp718 and XhoI, respectively; the sequences representing the initiator methionine and the stop codon are underlined. The uppercase letters contain the sequences bp 182–198 and bp 767–786 from GenBank™ accession number NM_010807, respectively. The italic sequence encodes the HA epitope. The resulting PCR product was an ~0.6-kb cDNA that was digested with Asp718 and XhoI.

To make CMV-MLP-HA (ARE), which contained the mouse TNF core ARE (bp 1309–1332 of GenBank™ accession number X02611, identical to the core ARE of human TNF, see bp 1341–1364 of GenBank™ accession number NM_000594.2), a double-stranded oligonucleotide TTATATTTATATTTATATTTAT, including the XhoI and XbaI sites at its 5’ or 3’ ends, respectively, was inserted between the XhoI and XbaI sites 3’ to the stop codon of CMV-MLP-HA. This is designated as plasmid AAAAIA, with each of the six As representing the consecutive As in the native ARE sequence (Fig. 1). The first set of mutant plasmids, representing derivatives of CMV-MLP-HA in which single or multiple As in the 24-base ARE (Fig. 1B) were mutated to Cs, was made in a similar fashion. In each case the base substitution is underlined. The last two mutant plasmids contained mutations to the same ARE in which some of the As and the middle U of one or more UUU motifs was replaced by C (Fig. 1, A and B) so that the ARE contained one or two UUAU/C/UUUA nonamers. Correct insertion of the inserts in the vector, and the correct sequences of the intended mutations, were confirmed by dRhodamine terminator cycle sequencing.

Cell Transfection Assays in 293 Cells—These were performed exactly as described (5). The cells were co-transfected with the vector alone plasmid (BS+), the wild-type human TTP-expressing plasmid CMV-hTTP-tag, the human HuB expression plasmid CMV-HuB-FLAG, and the various target MLP plasmids. The amount of transfected DNA was adjusted so that each plate was transfected with the same amount of DNA (5 μg); this was made up of 10 ng of CMV-hTTP-tag or 30 ng of CMV-HuB-FLAG and 0.5 μg (or otherwise indicated) of MLP plasmids, and the total of 5 μg was made up by BS+. The cells were harvested, and total cellular RNA was prepared using RNeasy (Qiagen). For Northern blotting, the probes consisted of either a mouse MLP cDNA probe consisting of bp 281–1429 of GenBank™ accession number NM_010807 or a mouse TTP cDNA probe (5), with or without the HuB DNA probe. Northern blotting was performed as described (5).

In some experiments, the total cellular RNA was used for transcript level determination by real time PCR, so that large numbers of experiments could be evaluated conveniently. For this purpose, RNA isolated from transfected 293 cells was treated with DNase to remove plasmid DNA. This was done by treating 1.2 μg of RNA with 5 μl of RQ1 DNase (1 unit/μl; Promega) in 3.35 μl of buffer (final concentration, 33.3 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2); the total reaction volume was 25 μl. The RNA and DNase mixture was incubated for 15 min at room temperature. After addition of 2.5 μl of 25 mM EDTA to terminate the reaction, the mixture was heated at 65 °C for 10 min to inactivate the DNase and then immediately placed on ice for 5 min and centrifuged, and the DNase-treated RNA was converted to cDNA using a high capacity cDNA archive kit (Applied Biosystems). 40 μl of cDNA was made by incubating 20 μl of DNase-treated RNA with 20 μl of reverse transcription mix according to the manufacturer’s instructions (per 40 μl of reaction: 4 μl of 10X buffer, 1.6 μl of 25X dNTPs, 4 μl of 10X random primers, and 0.5 μl of reverse transcriptase, 50 units/μl). The reaction was incubated at 25 °C for 10 min and then at 37 °C for 2 h.

Real Time PCR Analysis—Levels of the bovine growth hormone mRNA sequences expressed in the transfected cells were analyzed by quantitative real time PCR. Primers and the 6-carboxyfluorescein-labeled minor groove binder probe were designed using Primer Express software and synthesis (Applied Biosystems). The primer sequences were as follows: forward 5'-GGTGGCAGGCATCTGGTTGT-3'; reverse 5'-GACAGTGGAAGTGCCACCT-3'; probe 5'-CCTCCCGTTGCCTCCCTTA-3'; these corresponded to bp 716–736, 767–786, and 740–760, respectively, of the bovine growth hormone mRNA sequence (GenBank™ accession number NM_180996.1).

The Taqman reactions were as follows: 3 μl of cDNA, 12.5 μl of Taqman Universal PCR Master mix (Applied Biosystems), 1.25 μl of probe/primer mix (5 μM probe, 18 μl each primer), and 8.25 μl of H2O. The reactions were performed in 96-well plates in an Applied Biosystems Prism 7700 real time PCR instrument. The thermal cycle conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s each and 60 °C for 60 s. Each sample was analyzed in duplicate. A positive control cDNA was analyzed on each plate in order to minimize plate-to-plate variations. A negative control (293 cells transfected with a TNF plasmid alone) cDNA was also assayed on each plate in order to remove any contribution from nonspecific 293 cell background.

A modified version of the ΔΔCt method of relative quantification (17) was used to determine the fold change in expression in ARE and TTP-transfected cells versus ARE and empty vector control-transfected cells (BS). Briefly, this was done by normalizing the resulting threshold cycle (Ct) values of the samples to the Ct values obtained with the negative control (293 cells transfected with TNF plasmid) to account for 293 cell background (ΔΔCt = Ct sample – Ct293 control). The background-normalized Ct values were then calibrated to the Ct values from empty vector control (BS) and ARE-transfected cells that were treated at the same time as the TTP- and ARE-transfected cells; ΔΔCt = ΔCtARE sample – ΔCtBS sample. The fold change in expression was then obtained (2−ΔΔCt).

Statistical Analysis—The mean fold changes in expression in the six groups of ARE and TTP co-transfected cells (normalized to BS control transfected cells) were analyzed with SAS Enterprise Guide 2.0 software. Differences in real time PCR mRNA levels among all six groups of transfected cells were determined by analysis of variance followed by Tukey-Kramer post hoc analysis, using a level = 0.05. In some experiments, the expression of the MLP protein was determined by Western blotting with an antibody directed at the HA epitope tag.
RESULTS

TTP or TZF Domain Peptide Binding to Mutant ARE Probes—We showed previously by gel electrophoretic mobility shift assays that both full-length TTP and its TZF domain peptide expressed in 293 cells could bind to the ARE sequence of the TNFα-(1309–1332) (A)₅₀ probe (ARE-A₅₀) (7, 14). In the present study, a series of mutant probes was made in which the flanking As of the AUUUA pentamers were mutated to Cs (Fig. 1A); the number of nonamer(s) UUAUUUAUU found in these
probes is listed in Fig. 1A. A pair of mutant probes with central U mutations was also made (Fig. 1A); and the number(s) of UAUAUAUUAU nonamer(s) remaining in either probe (Fig. 1A). These probes were digested with RNase T1 to release the 24-base ARE from the vector sequence and the poly(A) tail, and were then used in gel shift analysis with extracts prepared from 293 cells transfected with TTP or its TZF domain expression constructs. Extracts from 293 cells transfected with constructs expressing members of the Hu family ARE-binding proteins, HuB and HuR (human members of the ELAV family; for review, see Ref. 18), were also used in the assays.

For the A to C mutant probes, we previously determined the apparent mole of synthetic TTP73 peptide that bound per mol of the respective ARE probe (Fig. 1A, right-hand column) (7). These results were confirmed in the present study by gel shift analysis, using a TZF domain peptide (77 amino acids) that was expressed in 293 cells, with the gel shifts being performed with cytosolic extracts from those cells (Fig. 2A).

The wild-type sequence is labeled AAAAAA to indicate the six A residues in the 24-base sequence; although it contains four possible nonamer binding sites, it was found previously to bind only 2 mol of TTP73 per mol in two nonamer UAUAUAUUAU sequences at each end of the 24-base ARE sequence (7). A similar result was obtained in the present experiment (Fig. 2A, lanes 1–6), in which lane 1 shows the migration position of the free probe in the gel shift assay; lane 2 shows the effect of 293 cell extract proteins after transfection with vector alone; lane 3 shows the migration position of full-length TTP bound to probe, and lane 4 shows the formation of two complexes with the TTP-(97–173) peptide (TZF domain peptide) expressed in the 293 cell extracts. In Fig. 2A, the lower band in lane 4 is thought to represent a complex formed of 1 mol of peptide/mmol of probe (presumably comprised of peptide binding to each of the two potential binding sites), and the upper band is thought to represent a complex consisting of 2 mol of peptide per mol of RNA probe, in which both binding sites are occupied (7). Binding of full-length TTP to the wild-type probe also resulted in the formation of two major complexes (Fig. 2A, lane 3). However, at this time we cannot conclude that the upper band represents a complex consisting of 2 mol of protein per mol of probe, and the exact relationship between the two complexes remains to be determined. In Fig. 2A, lanes 5 and 6 show the migration of RNA–HuB or RNA–HuR complexes, using extracts from 293 cells transfected with those expression constructs, respectively. The RNA–HuR complex migrated to the same position as complex II, which formed between an endogenous 293 cell...
protein and the probe. Complex II was as abundant as that shown in Fig. 2A, lane 6, when 50 μg of vector alone-transfected cell extract was used (not shown), suggesting that complex II possibly contained endogenous 293 cell HuR protein. In contrast, in the presence of large amounts of 293 cell protein, the intensity of complex I remained low with this probe (not shown).

The mutant probe ACCAAC, which also contained two normal nonamer binding sites at each end, could also bind 2 mol per mol of synthetic TTP73 peptide (Fig. 1A) and of the TZF domain peptide expressed in 293 cells (Fig. 2A, lane 9). With this probe, full-length TTP formed almost identical RNA-TTP complexes as those seen with probe AAAAAA (Fig. 2A, compare lane 8 to lane 3). Thus, despite the presence of four potential nonamer binding sites in the AAAAAA probe (Fig. 1A), binding appeared to occur only to two of them, presumably the two sites remaining in ACCAAC. The complexes formed with either HuB or HuR and this mutant probe were similar to those formed with the wild-type probe (Fig. 2A, lanes 10 and 11).

Three of the other A to C mutant ARE probes shown in Fig. 2A (AAAAAC, AAACCA, and ACCACA) could bind only 1 mol of synthetic TTP73 peptide per mol of oligonucleotide (7). This result was confirmed in the present studies with the TZF domain peptide expressed in 293 cells, with only a single complex being formed in each case (Fig. 2A, lanes 16, 21, and 28). Thus, in these three cases, the single site binding data from the transfected-expressed peptide agreed with those obtained with the TTP73 synthetic peptide (7). However, there was also a less abundant, slower migrating complex formed with each of these probes and the full-length TTP protein, at approximately the same position as the higher band seen with the wild-type probe (see below). Although there were two possible overlapping binding UUAUUUAUU nonamers in both probes AAAACC and AAACCA (Fig. 1A), there was only one apparent RNA-probe complex formed with the TZF domain peptide in each case (7) (Fig. 2A). The migration patterns of the single HuB- or HuR-RNA complexes with the three mutant probes were very similar to those seen with the wild-type probe (Fig. 2A, compare lanes 17 and 18, 22 and 23, and 29 and 30 to lanes 5 and 6).

Mutant probe ACAACA lacked the three internal U residues of the essential nonamer unit but contained an octamer UUAUUUAUU (Fig. 1A). This change to two internal U residues was found to decrease the affinity of the TTP73 peptide for the mutant probe by about 6-fold when compared with the nonamer (8). Under the present gel shift conditions, the expressed TZF peptide did not appear to form a complex with this probe (Fig. 2A, lane 33). However, a slower migrating, low intensity protein-RNA complex was formed between this probe and full-length TTP that was similar to those seen with the above-mentioned three mutant probes (Fig. 2A, lanes 15, 20, 27, and 32), even though the major, lower RNA-TTP complex did not form with this probe, because the intensity of the band in that location was no different from that seen with the vector alone-transfected cell extract (Fig. 2A, compare lane 32 to 31, complex I). The binding of HuB and HuR to this mutant probe was again similar to their binding to the wild-type probes (Fig. 2A, lanes 34 and 35).

As predicted from the data with the TTP73 synthetic peptide (7), there was essentially no binding of the expressed TZF domain peptide to the last two mutant probes (ACCACA and CCCCCC) (Fig. 2A, lanes 40 and 45). Again, full-length TTP appeared to form a smaller amount of the slower migrating complex with the first of these two probes (Fig. 2A, lane 38), and exhibited essentially no binding to the last probe (lane 44) under these conditions. The amount of protein-RNA complex formed between HuB and these probes was very similar to that seen with the wild-type probe (Fig. 2A, lanes 41 and 46), but the HuR-RNA complex formation was somewhat decreased with probe ACCACA (lane 42), and even more decreased with probe CCCCCC (lane 47).

As listed in Fig. 1A, two additional probes were made that contained one or two UUAUCUAUU nonamers in the 24-base ARE. Similar gel shift experiments using these mutant probes are shown in Fig. 2B. As in Fig. 2A, the wild-type AAAAAA probe shown in lanes 1–6 bound both full-length TTP (lane 3) and the expressed TZF domain peptide (lane 4), in each case with formation of the characteristic two bands. When the UUAUUUUUU motifs in the ARE sequence were mutated to UUAUUAUU, there was essentially no RNA-TZF peptide complex formation with either probe (AUCUA2), containing two UUAUCUAUU motifs, or probe (AUCUA1), containing one motif (Fig. 2B, lanes 9 and 16). As seen with the other mutant probes, a low intensity upper band formed between full-length TTP and both mutant probes (Fig. 2B, lanes 8 and 15); however, essentially no detectable lower complex formed with full-length TTP (Fig. 2B, compare lane 8 to 7 and 15 to 14). The preserved ability of full-length TTP to bind to these mutant probes (AUCUA2) and (AUCUA1), as well as ACAACA, to which the expressed TZF peptide was not able to bind, was somewhat surprising, because there were no intact nonamers in any of these mutants (Fig. 1A). The binding of HuB to these probes was fairly similar to that of the wild-type probe (Fig. 2B, lanes 10 and 17), but the binding of HuR was obviously decreased (lanes 11 and 18).

When the number of Cs substituted for flanking As or center Us in the AUAUUU motif was increased to three or more, there was increased binding of an endogenous 293 cellular protein to the probes, shown in Fig. 2, A and B, as complex I (Fig. 2A, lanes 26, 39, and 43; Fig. 2B, lanes 7 and 14). The lower TTP-RNA complex co-migrated with this endogenous protein complex I (Fig. 2A, lanes 39 and 44), possibly masking the appearance of the lower TTP-RNA complex with probes ACCACA-A10 or CCCCCC-A10. However, significant binding of TTP to either of these probes seems unlikely, because there was no observable binding of the TZF domain peptide to these probes, and TTP did not induce deadenylation of these mutant probes, nor did it destabilize the hybrid mRNAs containing these mutated AREs in cell transfection studies (see below).

Another observation from these gel shift assays is that the formation of the TTP-RNA complex that migrated to the position of complex I required the presence of the perfect nonameric sequence UUAUUUUAUU in the RNA probe as seen in probes AAAAAA, ACCAA, AAACCC, AAACCA, and ACCACA (Fig. 2A, lanes 3, 8, 15, 20, and 27). This sequence was not found in probes AACAACA, ACCACA, CCCCCC, (AUCUA2), and (AUCUA1), and with these probes there was no formation of this faster migrating TTP-RNA complex (Fig. 2A, lanes 32, 38, and 44; Fig. 2B, lanes 8 and 15).

It is interesting to note that, although the binding of HuB did not seem to be affected by the mutated nucleotides in any of the mutant probes tested here, the binding of HuR seemed diminished somewhat with probes that did not contain any UUAUUU or UUAUUU motifs (Fig. 1A), such as in probes CCCCCC or (AUCUA2) (Fig. 2A, lane 47; Fig. 2B, lane 11), or probes containing only one of the pentamers UUAUUU or UUAUUU, as in probe (AUCUA1) (Fig. 2B, lane 18). Fig. 2C shows Western blots of 293 cytosolic extracts from cells transfected with expression constructs of these ARE-binding proteins to document their expression.

To assess whether the upper band formed with TTP-expressing extracts and mutant probes that did not contain a perfect UUAUUUUAUU motif and did not bind the expressed TZF peptide (probes AACAACA, ACCACA, (AUCUA2), and (AUCUA1)) actually contained TTP, we performed “supershift” assays with the anti-HA antibody (Fig. 3). Both of the upper RNA-protein complexes formed
with expressed full-length TTP, and probes AAAAAA or AACCAA (Fig. 3A, lanes 3 and 6) migrated slower and appeared in the gel as supershifted bands (lanes 4 and 7), indicating that protein component of the double band complexes formed with either probe was indeed TTP. The amount of RNA-TTP complex shifted to a slower migrating position (Fig. 3A, SS) was dependent upon the amount of antibody present (data not shown). No supershifted band was formed when the anti-HA antibody was incubated with 50 μg of protein/lane of vector alone-transfected 293 cells incubated with any probes (not shown).

As in Fig. 2A, TTP formed two complexes with probes AAAAAC, AAACCA, or AACCCA, one migrating as an upper band of low intensity and a slightly more intense lower band (Fig. 3A, lanes 10, 15, and 18). When the anti-HA antibody was included in the incubation, the upper RNA-TTP complex shifted to a slower migrating position (Fig. 3A, SS) was dependent upon the amount of antibody present (data not shown). No supershifted band was formed when the anti-HA antibody was incubated with 50 μg of extract protein (a 10-fold greater amount of protein than used for the usual assay) from vector alone-transfected 293 cells incubated with any probes (not shown).

As in Fig. 2A, TTP formed two complexes with probes AAAAAA, AACCAA, or AACCCA, one migrating as an upper band of low intensity and a slightly more intense lower band (Fig. 3A, lanes 10, 15, and 18). When the anti-HA antibody was included in the incubation, the upper RNA-TTP complex formed with any of these probes was supershifted (Fig. 3A, lanes 11, 16, and 19; indicated as SS). However, although the lower complexes formed with the AAAAAA and AACCAA probes were apparently supershifted in this assay (Fig. 3A, compare lanes 3 and 4 and lanes 6 and 7), the lower complexes formed with the other probes did not supershift (compare lanes 11 to 10, 16 to 15, and 19 to 18, indicated as I), even when the amount of antibody was increased in the assays (not shown). The identity of this faster migrating complex formed with these mutant probes in the presence of full-length TTP is unclear. As shown in both Fig. 2 and Fig. 3, this complex co-migrated with that formed between the probes and an endogenous 293 cell protein; it is conceivable that the presence of TTP could increase the binding of the endogenous 293 cell protein to these probes. Another possibility, suggested by the presence of lower Mr proteolytic fragments of TTP observed in the transfected cell extracts blotted with the antibody to the carboxyl-terminal HA epitope tag, is that an untagged amino-terminal fragment of TTP was present that contained the TZF domain and caused the gel shift. The presence of such a fragment has been noted in similar cell extracts probed with an antibody to the intact TTP protein rather than to the HA epitope tag. However, such a possibility seems highly unlikely, because both of the TTP-RNA complexes formed with probes AAAAAA or AACCAA were supershifted in the presence of the anti-HA antibody (Fig. 3A, lanes 4 and 7).

FIGURE 3. Analyzing TTP binding to mutant ARE probes in supershift assays. Cytosolic extracts of 293 cells transfected with either vector (BS/H11001) alone, or the expression construct CMV-hTTP-tag, as described in the legend to Fig. 1, were used in the electrophoretic mobility shift assays. A and B, cell extracts (5 μg of protein/lane) without (−) or with a monoclonal antibody to HA (+; 1 μl/lane of a 1:6 dilution of HA-probe (F-7), Santa Cruz Biotechnology) were incubated on ice for 20 min followed by an additional 30 min of incubation at 20 °C with 2 × 105 cpm (digested with RNase T1) of either the wild-type TNF ARE-A50 probe (AAAAAA-A50), or other ARE-A50 mutant probes, as indicated. The migration positions of the TTP-RNA complexes (TTP) are indicated at left. The supershifted (SS) RNA-TTP complexes seen in the presence of the anti-HA antibody, and the RNA-protein complexes I and II formed with endogenous 293 cell proteins, are shown at right. For each probe, a lane was loaded with a probe sample that was incubated under the same conditions but without the added cell extract (P). The gels were run at 250 V for 115 min to allow the supershifted complexes to run into the gels. FP, free probe.
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Effects of ARE Mutations on TTP-stimulated Transcript Deadenylation in a Cell-free Assay—We then tested the same extracts from 293 cells transfected with either vector alone (BS+) or TTP in a cell-free deadenylation assay. In this experiment, each target RNA consisted of 58 bases transcribed from the SK− vector, 24 bases of the same wild-type or mutant ARE sequences used in the gel shift reactions, and 50 bases of a poly(A) tail (the ARE sequence of each probe is listed in Fig. 1A). Each RNA target was incubated with 293 cell extracts for 60 min at 37 °C in the presence or absence of 20 mM EDTA. At this concentration of EDTA, the RNA probes were stable in extracts prepared from cells transfected with vector alone or with other constructs that expressed a variety of different proteins, including TTP (not shown). In the absence of EDTA, the disappearance of the RNA target and the accumulation of the deadenylated RNA were monitored by electrophoresis and autoradiography, as described previously (14). The amount of target RNA remaining was quantified by PhosphorImager analysis, and the probe radioactivity in the absence of EDTA was compared with that measured in the presence of EDTA for each individual RNA probe. The migration positions of the ARE probe that did not have a poly(A) tail (Fig. 1A, lanes 1–3 in A and B) as well as the major deadenylated product of ARE-A50, A, shown is the effect of TTP on the deadenylation of the wild-type (AAAAAA-A50) probe or two ARE-A50 probes in which various center Us in the AUUUA motif were substituted with Cs or probe ACAACA-A50.

In probes (AUCUA)2 and (AUCUA)1, the ARE had been changed to contain two or one mutant nonamer UUAUCUAUU (Fig. 1A), with no intact wild-type nonamers. As in Fig. 2B, these formed low intensity RNA-TTP complexes that migrated to the same position as the upper band when wild-type probe AAAAAA was used (Fig. 3B, lanes 6 and 10, compared with lane 3). The upper complex formed with these probes also supershifted with the anti-HA antibody (Fig. 3A, lanes 7 and 11, indicated as SS) indicating that the upper band contained TTP, whereas the intensity of the lower band was unchanged.

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1–3), and the deadenylated, ARE-containing RNA, are indicated by the arrow in Fig. 4. The 58-base RNA-containing vector was also shown (Fig. 4A, lanes 25–27).

The effect of TTP on the wild-type AAAAAA-A50 probe in this assay is shown in Fig. 4A, lanes 4–6. When extracts from 293 cells transfected with vector alone (−) were incubated with the AAAAAA-A50 probe for 1 h in the absence of EDTA, there was a slight decrease in the amount of full-length probe and no detectable increase in the accumulation of the deadenylated species (Fig. 4A, compare lane 5 to lane 4). In four individual experiments, this decrease averaged 22.6 ± 1.9% (S.D.) when compared with the identical incubation in the presence of EDTA. However, when identical amounts of 293 cell protein from cells transfected with TTP (+) were used, there was an obvious decrease in the amount of the remaining full-length probe, as well as the accumulation of the deadenylated RNA, indicated by the arrow (Fig. 4A, lane 6). This decrease averaged 62.1 ± 1.6% (n = 4 experiments) compared with the sample in the presence of EDTA (TABLE ONE). When the decreased amount of full-length probe in extracts from cells transfected with TTP was compared with that seen in extracts from cells transfected with vector alone (both in the absence of EDTA), the average decrease was 2.74-fold greater in the presence of TTP (TABLE ONE). Virtually identical results were observed when the mutant ARE probe was used that contained two tandem nonamer TTP73-binding sites (AACCAA-A50) (Fig. 4A, lanes 7–9; see also TABLE ONE).

In contrast, when mutant AREs were used that contained a single binding site for TTP73, TTP caused less disappearance of the full-length probe and less accumulation of the deadenylated RNA when compared with the probes containing two TTP73-binding sites (AACCAA-A50) (Fig. 4A, lanes 10–12 for AAAACC-A50, lanes 13–15 for AAACCA-A50, lanes 16–18 for AACCCA-A50). When the two TTP73 nonbinding mutants were

### TABLE ONE

| RNA probe name | Extract | % probe decreased (mean ± S.D.) | Average fold decrease with TTP vs. BS+ |
|---------------|---------|---------------------------------|--------------------------------------|
| Wild-type     |         |                                 |                                      |
| AAAAAA-A50 BS+ | 22.6 ± 1.9 | 2.74:1                          |
| hTTP          | 62.1 ± 1.6 |                                  |
| Mutants       |         |                                 |                                      |
| AACCAA-A50 BS+ | 19.6 ± 7.0 | 2.77:1                          |
| hTTP          | 54.4 ± 5.1 |                                  |
| AAAACC-A50 BS+ | 26.6 ± 5.8 | 1.96:1                          |
| hTTP          | 52.3 ± 10.9 |                                 |
| AAACCA-A50 BS+ | 31.0 ± 9.5 | 1.38:1                          |
| hTTP          | 42.8 ± 7.5 |                                  |
| ACCACA-A50 BS+ | 22.8 ± 5.7 | 1.17:1                          |
| hTTP          | 26.8 ± 5.1 |                                  |
| CCCCCC-A50 BS+ | 27.8 ± 1.8 | 0.85:1                          |
| hTTP          | 23.5 ± 3.4 |                                  |
| Wild-type     |         |                                 |                                      |
| AAAAAA-A50 BS+ | 30.3 ± 4.5 | 1.18:1                          |
| hTTP          | 70.0 ± 2.5 |                                  |
| (AUCUA)1-A50 BS+ | 34.3 ± 8.3 | 1.28:1                          |
| hTTP          | 43.8 ± 5.4 |                                  |
| (AUCUA)2-A50 BS+ | 34.6 ± 5.1 | 0.99:1                          |
| hTTP          | 34.6 ± 5.1 |                                  |
| Mutants       |         |                                 |                                      |
| HuR           | 35.6 ± 7.5 | 1.02:1                          |
| HuR           | 35.6 ± 7.5 |                                  |
| HuR           | 35.6 ± 7.5 |                                  |
| HuR           | 35.6 ± 7.5 |                                  |
| AACAACA-A50 BS+ | 27.6 ± 5.1 | 1.49:1                          |
| hTTP          | 41.1 ± 4.6 |                                  |
| Tzf           | 27.5 ± 8.4 | 1.00:1                          |
| HuR           | 29.1 ± 11.3 |                                 |
| HuR           | 30.5 ± 8.4 | 1.11:1                          |
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used, ACCACA-A50 (Fig. 4A, lanes 19–21) and CCCCCC-A50 (lanes 22–24), there was no apparent effect of TTP to decrease the probe levels or increase the accumulation of the deadenylated probe (see also TABLE ONE). The average fold changes in probe in the presence of TTP compared with vector alone (BS+) were 1.17- and 0.85-fold for ACCACA-A50 and CCCCCC-A50, respectively (BS+ = 1; TABLE ONE).

A different series of deadenylation assays was performed using mutant probes that contained either two nonamers or one nonamer in which the center U in the AUUUA motif was substituted by C or a probe in which there was one UUAUUAUU octamer (Fig. 1A). In addition to extracts from 293 cells transfected with vector alone or TTP, extracts from 293 cells transfected with the CMV-hTTP-1(97–173)tag, expressing the 77-amino acid TZF peptide, and constructs expressing the Hu family proteins HuB or HuR, were used. The results with these expressed proteins using the wild-type probe AAAAAA-A50 are shown in Fig. 4B, lanes 4–9. The ability of TTP to increase AAAAAA-A50 probe degradation, and cause the accumulation of the deadenylated RNA, was similar to the series shown in Fig. 4A (Fig. 4B, lanes 4–6; TABLE ONE). As we have shown previously (14), the presence of the TZF domain peptide did not induce more probe degradation when compared with vector alone (Fig. 4B, compare lane 7 to 5), with the fold change in the presence of this peptide averaging 1.14-fold of control (TABLE ONE). When compared with extracts from cells transfected with vector alone, there was no change in probe stability in the presence of HuB or HuR (Fig. 4B, lanes 8 and 9 compared with lane 5), with no decreases in probe observed compared with control (TABLE ONE). This result is in line with a previous report that HuR had little effect on the rate of deadenylation of mRNAs containing AREs (19).

The three mutant probes in this series did not bind the expressed TZF peptide, but their binding to HuB or HuR (probe ACAACA) was similar to that seen with the wild-type probe. The gel shift assays had demonstrated weak binding to full-length TTP. In the presence of full-length TTP, their degradation was slightly increased when compared with that seen with the vector alone-transfected cell extract (Fig. 4B, lanes 12, 18, and 24 compared with lanes 11, 17 and 23; TABLE ONE). The expressed TZF peptide and the Hu proteins did not stimulate the degradation of these probes (Fig. 4B, lanes 13–15, 19–21, and 25–27; TABLE ONE).

Effect of ARE Mutations on MLP-HA mRNA Stability in the Presence and Absence of TTP in Co-transfection Experiments—As shown in Fig. 1B, the TTP target constructs for co-transfection in 293 cells consisted of a CMV promoter, the protein coding region of mouse MLP, an HA epitope tag in-frame with the carboxyl-terminal amino acid of MLP, a 24-bp sequence encoding the wild-type or a mutant ARE, and the bovine growth hormone 3′-UTR and polyadenylation signal. The ARE consisted of the same 24 bases of core TNF sequence whose binding to the TTP73 synthetic TZF domain of human TTP was described (7), and which was present in the probe used in the gel shift and deadenylation assays.

These constructs were co-transfected into 293 cells in the presence or absence of TTP, and the effect of TTP on deadenylation of the target mRNA was assessed by Northern blotting and real time PCR. A control used the same construct in which the 24-base ARE sequence was omitted altogether. As shown in Fig. 5, co-transfection of TTP with the MLP construct containing the AAAAAA probe sequence (wild-type ARE) resulted in a marked decrease in MLP mRNA accumulation in 293 cells (Fig. 5, compare lane 2 to lane 1, top panel). The MLP artificial construct was used in preference to the previously used TNF, GM-CSF, and interleukin-3 constructs because there was no detectable accumulation of a deadenylated mRNA fragment, which can complicate PhosphorImager analysis of the mRNA levels. PhosphorImager analysis of this experiment indicated a 66% decrease in steady-state MLP mRNA levels in the presence of TTP when the mRNA contained two TTP73 nonamer binding sites. There was a commensurate decrease in expressed MLP protein (Fig. 5, bottom panel, lanes 1 and 2) in the presence of TTP, as assayed by immunoblotting with an antibody to the HA epitope. The expression level of TTP mRNA is shown in the middle panel of Fig. 5.

Co-transfection and expression of TTP caused a similar decrease (68%) in steady-state MLP mRNA levels when the AACCAA mutant was present, which also contains two TTP73-binding sites (Fig. 5, top panel, lanes 3 and 4). This also resulted in marked decreases in the expression of MLP protein (Fig. 5, bottom panel), whereas the expression of TTP mRNA is shown in lane 4 of the middle panel. These data suggested that the presence of two nonamer TTP-binding sites in the ARE were capable of mediating similar decreases in mRNA stability, whether or not the intervening sequence contained two Cs instead of two As.

When one of the mutant AREs containing a single intact nonamer TTP-binding site (AACCCA) was tested in the same experiment, there was very little decrease in steady-state MLP mRNA levels (Fig. 5, lanes 5 and 6). PhosphorImager analysis of this experiment revealed that the MLP mRNA in the presence of TTP was decreased by only 12%, as compared with the presence of co-transfected vector alone. Similarly, there was little detectable decrease in MLP protein in the presence of TTP (Fig. 5, lower panel, lanes 5 and 6). TTP mRNA levels were similar in all three groups (Fig. 5, middle panel). These data suggest that the presence of a single TTP73-binding site contributed only slightly to the increased turnover of the ARE-containing MLP mRNA, whereas the presence of two TTP73-binding sites had a marked effect to decrease
FIGURE 6. Effect of ARE mutations on MLP-HA mRNA stability in the presence of TTP. Expression constructs CMV-MLP-HA (0.5 μg/plate), with or without the wild-type ARE, or with mutant AREs in which various flanking-As were substituted by Cs as indicated, were co-transfected into 293 cells with vector alone (lanes 1, 3, 5, 7, 9, 11, or 13) or with CMV-hTTP-tag (+ hTTP, lanes 2, 4, 6, 8, 10, 12, or 14), as described in the legend to Fig. 5. Each gel lane was loaded with 10 μg of total cellular RNA. A, electrophoresis and Northern hybridization were performed as described in the legend to Fig. 5. B, statistical analysis of real-time PCR using RNA samples described in A from two (1st bar) and five (2nd to 6th bars) co-transfection experiments.

In order to determine the average effect of having zero, one, or two TTP73-binding sites in the ARE with susceptibility to TTP-induced degradation, we developed a real-time PCR assay for MLP transcripts; this involved developing a procedure for removing plasmid DNA, as well as normalization for the presence of 18 S RNA. This type of assay was conducted on several separate transfection experiments, as shown in Fig. 6B. In Fig. 6B, the 1st bar in the histogram, labeled with a minus sign, indicates that the presence of co-transfected TTP caused an average increase of about 50% in the levels of an MLP mRNA containing no ARE when compared with co-transfected vector alone (n = two experiments). Similarly, when the mutant ARE containing no TTP73-binding sites was present (ACCACA), TTP caused an increase in mean MLP mRNA levels of about 35% compared with vector alone (Fig. 6B, last bar on the histogram). When two binding sites were present, as in the case of theAAAAAA and ACCCAA AREs (Fig. 6B, 2nd and 3rd bars on the histogram), TTP co-expression caused ~5-fold decreases in mean MLP mRNA levels in each case, when compared with the construct lacking an ARE, and ~5-fold decreases when compared with the ACCACA mutant; these changes were highly statistically different, as determined by one-way analysis of variance followed by Tukey-Kramer correction (p < 0.01).

The mean values for the constructs containing a single TTP73-binding site were decreased by an average of 55% in the case of the ACCACA construct, and 38% in the case of the ACCCAA construct, when compared with the mean values determined with the construct containing no ARE (in Fig. 6B compare the 4th and 5th bars in the with the 1st bar). They were decreased on average by 47 and 29% when compared with the values obtained from the construct containing the mutant ARE (Fig. 6B, compare the 4th and 5th bars to the last bar). Although these decreases

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in steady-state mRNA levels were substantial compared with the expression of the MLP mRNA containing either no ARE (Fig. 6B, 1st bar) or the mutant ARE lacking any TTP-binding sites (last bar), none of these decreases was statistically significant.

These experiments demonstrated that the presence of two TTP73 binding nonamers in the MLP transcript conferred significant TTP susceptibility on the MLP transcript, resulting in 4–5-fold decreases in steady-state mRNA levels. Although the situation with the single binding site constructs was less obvious, they were clearly less sensitive to TTP-mediated mRNA destruction than the transcripts containing two TTP73-binding sites.

MLP expression constructs containing the center U to C (UUAUC-UAUU) mutations in the ARE insert, and a flanking A mutant that contained an octamer UUAUUAUU in the ARE insert, were also tested in similar co-transfection experiments, followed by Northern blotting and PhosphorImager analysis. ARE mutant probes from this group did not bind the expressed TGFβ peptide but showed weak binding to full-length TTP (Figs. 2 and 3), and their binding to HuB was not different from that of the wild-type ARE probe. A Northern blot from a typical co-transfection experiment (n = 4) is shown in Fig. 7. The co-transfection of the TTP expression construct with the MLP construct lacking an ARE caused a slight increase in steady-state MLP mRNA levels to 107.8 ± 19.4% (mean ± S.D.) of control cells that were co-transfected with vector alone (Fig. 7, lanes 1 and 2). The resulting steady-state MLP mRNA level was similarly unchanged when this construct was co-transfected with the HuB expression construct (119.1 ± 8.7%, lane 3). The presence of the 24-base wild-type ARE insert, AAAAAA, resulted in an average 77.2 ± 3.0% TTP-induced decrease in MLP mRNA levels (Fig. 7, lanes 4 and 5). The co-expression of HuB did not decrease the steady-state MLP mRNA levels of this construct, with mRNA levels 122.8 ± 14.7% of control (Fig. 7, lane 6). For the two center U to C mutant ARE inserts (AUCUA2 and (AUCUA)1), the presence of TTP induced a decrease in steady-state MLP mRNA levels by 30.2 ± 6.2 and 31.7 ± 15.5%, respectively (Fig. 7, lanes 7 and 8, 10, and 11). The presence of HuB did not decrease the MLP mRNA levels expressed from either construct (116.2 ± 27.0 and 141.7 ± 62.2% of control, respectively, Fig. 7, lanes 9 and 12, respectively). Although the mutant ARE insert ACAACA contained no apparent nonamer binding sites for TTP, there was still a modest decrease in steady-state MLP mRNA levels in the presence of TTP (by 30.8 ± 13.5%; Fig. 7, lanes 13 and 14), whereas HuB expression did not affect the steady-state MLP mRNA levels (105.4 ± 13.1%; Fig. 7, lane 15). The expression of TTP and HuB mRNAs in this experiment is shown in the lower panel of Fig. 7.

**DISCUSSION**

It has become clear from several lines of evidence that the minimum TTP-binding site, and presumably that for its other family members, is well represented by the single-stranded RNA nonamer UUAUUAUUU. This binding site was identified independently by random selection techniques (6) and by the successive truncation of the larger TNF ARE (7); in the latter case, the nonamer was optimal for changing the NMR conformation of a 73-amino acid synthetic peptide comprising the TZF domain of human TTP, whereas even slightly shorter RNA oligonucleotides produced more degenerate conformations. This conclusion was further supported by the data of Brewer et al. (8), who showed that shortening the nonamer to a heptamer by removing the two outer Us decreased the binding affinity for the TTP73 peptide by ~6-fold. These data were recently extended by the determination of the three-dimensional structure of the analogous TZF from the TTP relative ZFP36L2 (TIS11D) in complex with the same nonamer (9). Theoretical modeling suggested strongly that the RNA contact amino acids were identical when comparing the TTP TZF domain to that of ZFP36L2, supporting the idea that the structure of the TTP TZF domain complex with the nonamer is virtually identical to that of the ZFP36L2 TZF domain (10).

The study of Hudson et al. (9) also supported the results of our previous study in which we showed that mutations of any of the CCCH residues, or any of the aromatic residues located within the Cx5C and Cx3C subdomains of the zinc fingers, resulted in the loss of TTP binding to the ARE (20). These zinc-binding CCCH residues, and the aromatic residues involved in base-stacking interactions with RNA (9), are identical between TTP and TIS11D.

The primary goals of the present study were to determine whether the presence of a single nonameric TTP-binding site in the 3’-UTR of an otherwise stable mRNA would confer TTP-dependent instability on that mRNA, and whether the presence of a second binding site would further increase the TTP susceptibility of the mRNA. This information is important for our ongoing attempts to elucidate the mechanism of action of TTP, as well as to inform bioinformatics and experimental approaches to determining bona fide physiological mRNA targets for TTP and its related proteins.

We attempted to address these goals by using several experimental approaches, including analysis of RNA binding by gel shift assays, analysis of TTP-stimulated target mRNA deadenylation in a cell-free assay, and an assessment of the ability of the TTP to promote the deadenylation and instability of mRNA targets in a 293 cell co-transfection assay. For the last experiments, we devised a target molecule based on the

![Image](https://example.com/image.png)
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It is interesting to compare these results to the naturally occurring AREs present in the TNF and GM-CSF mRNAs, still the only demonstrated physiological targets for TTP (as determined by the rather strict criterion of mRNA stabilization in cells derived from TTP knock-out mice). Both of these AREs contain multiple copies of the optimum binding nonamer, although there are species differences in how the nonamers are arranged (10). Although the current study cannot answer the question of why the half-life of GM-CSF mRNA was more than twice as long as the half-life of TNF mRNA in cells derived from the TTP knock-out mice, it should be possible, using the assays described here, to determine the stoichiometry of TTP peptide binding to the naturally occurring TNF and GM-CSF AREs from various animal species and, by extension, the corresponding TTP sensitivity of those AREs in the intact mRNAs. Obviously, many other factors influence whether these interactions will occur in vivo, including the presence of TTP (or related protein) in the same cells as the mRNA target, the presence of adequate concentrations of TTP, the occurrence in the same part of the cell at the critical times, and others.

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