Interactions of CCCH Zinc Finger Proteins with mRNA

TRISTETRAPROLIN-MEDIATED AU-RICH ELEMENT-DEPENDENT mRNA DEGRADATION CAN OCCUR IN THE ABSENCE OF A POLY(A) TAIL*

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The CCCH family of tandem zinc finger proteins has recently been shown to promote the turnover of certain mRNAs containing class II AU-rich elements (AREs). In the case of one member of this family, tristetraprolin (TTP), absence of the protein in knockout mice leads to stabilization of two mRNAs containing AREs of this type, those encoding tumor necrosis factor α (TNFα) and granulocyte-macrophage colony-stimulating factor. To begin to decipher the mechanism by which these zinc finger proteins stimulate the breakdown of the CCCH proteins into 293 cells with vectors encoding full-length TNFα, granulocyte-macrophage colony-stimulating factor, and interleukin-3 mRNAs. Co-expression of the CCCH proteins caused the rapid turnover of these ARE-containing mRNAs and also promoted the accumulation of stable breakdown intermediates that were truncated at the 3′-end of the mRNA, even further 5′ than the 5′-end of the poly(A) tail. To determine whether an intact poly(A) tail was necessary for TTP to promote this type of mRNA degradation, we inserted the TNFα ARE into a nonpolyadenylated histone mRNA and also attached a histone 3′-end-processing sequence to the 3′-end of nonpolyadenylated interleukin-3 and TNFα mRNAs. In all three cases, TTP stimulated the turnover of the ARE-containing mRNAs, despite the demonstrated absence of a poly(A) tail. These studies indicate that members of this class of CCCH proteins can promote class II ARE-containing mRNA turnover even in the absence of a poly(A) tail, suggesting that the progressive removal of the poly(A) tail may not be required for this type of CCCH protein-stimulated mRNA turnover.

Tristetraprolin (TTP), also known as TIS11, Nup475, and G0S24 (1–5) is the prototype of a small family of zinc finger proteins characterized by a pair of closely spaced zinc fingers of the CCCH type. Although TTP was originally thought to be a probable transcription factor, we have recently shown that TTP and related proteins can promote the turnover of certain mRNAs containing AU-rich elements (AREs) in their 3′-untranslated regions (UTRs). Using cells derived from TTP-deficient mice, we showed that TTP is a physiological inhibitor of the secretion of tumor necrosis factor α (TNFα) (6) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (7), an effect mediated by TTP’s ability to destabilize the mRNAs encoding both proteins. TTP mediates this effect after initially binding to the ARE in the 3′-UTRs of these mRNAs (6–8). In transfection experiments, the known vertebrate TTP-related proteins had similar effects to promote the breakdown of these ARE-containing mRNAs (9).

TTP and its related proteins bind to the ARE of these mRNAs and then appear to promote their initial destruction from the 3′-end. This results in a decrease in total hybridizable mRNA levels as well as the formation of what appear to be mRNA bodies missing their poly(A) tails (9). However, it is unclear whether TTP achieves this effect by stimulating the initial 3′ to 5′ progressive deadenylation of these mRNAs, by stimulating another 3′-exonuclease, or by some other mechanism (e.g. an ARE-dependent endonuclease activity).

The current view of ARE-dependent destruction of mRNAs containing so-called class II AREs is that there is first progressive deadenylation of the poly(A) tail, presumably by one or more poly(A)-specific exonucleases, followed by further exonuclease and perhaps endonucleolytic degradation of the mRNA body after removal of the protective poly(A) tail (10). Many proteins have been found to be capable of forming protein-ARE complexes in cell-free assays (for reviews, see Refs. 11 and 12); however, few ARE-binding proteins have been shown to participate in the destabilization of class II ARE-containing mRNAs in intact cells. TTP and its related proteins cMG-1 and TIS11d can all stimulate the degradation of class II ARE-containing mRNAs in cell transfection studies (9).

In an attempt to further elucidate the mechanism by which the CCCH proteins destabilize ARE-containing mRNAs, we have tested the hypothesis that TTP can promote degradation of class II ARE-containing mRNAs that do not have a poly(A) tail. We show here that TTP can stimulate the degradation of a nonpolyadenylated histone mRNA when a class II ARE is inserted into this mRNA and that TTP can stimulate the breakdown of mRNAs that normally contain a class II ARE but in which a histone 3′-end-processing sequence has replaced the normal poly(A) tail.

The abbreviations used are: TTP, tristetraprolin; hTTP, human TTP; TNFα, tumor necrosis factor α; mTNFα, mouse TNFα; ARE, AU-rich element; GM-CSF, granulocyte-macrophage colony-stimulating factor; mGM-CSF, mouse GM-CSF; IL, interleukin; mIL, mouse interleukin; 3′-UTR, 3′-untranslated region; HGH, human growth hormone; PCR, polymerase chain reaction; RT, reverse transcription; bp, base pair(s); kb, kilobase pair(s); CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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1 The abbreviations used are: TTP, tristetraprolin; hTTP, human TTP; TNFα, tumor necrosis factor α; mTNFα, mouse TNFα; ARE, AU-rich element; GM-CSF, granulocyte-macrophage colony-stimulating factor; mGM-CSF, mouse GM-CSF; IL, interleukin; mIL, mouse interleukin; 3′-UTR, 3′-untranslated region; HGH, human growth hormone; PCR, polymerase chain reaction; RT, reverse transcription; bp, base pair(s); kb, kilobase pair(s); CMV, cytomegalovirus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
FIG. 1. Schematic representation of plasmid constructs. Constructs were made as described under “Experimental Procedures.” A, CMV.mTNFα-(1–1627) contains the full-length mouse TNFα cDNA. The ARE is located at bp 1302–1332 of GenBank accession number X02611 as indicated. B, CMV.mGM-CSF contains a 775-bp full-length mouse GM-CSF cDNA. The ARE is located at bp 668–722 of the cDNA. The numbers in parentheses are the corresponding bp numbers of the mouse GM-CSF gene (GenBank accession number X03020). C, CMV.mIL-3 contains an 851-bp full-length mouse IL-3 cDNA. The ARE is located at bp 700–732 of the cDNA. The numbers in parentheses are the corresponding bp numbers of the mouse IL-3 gene (GenBank accession number K03233). D, CMV.H2a contains a 549-bp mouse histone H2a sequence that corresponds to bp 814–1362 (in parentheses) of GenBank accession number X16148. An EcoRV site was created at bp 352 (bp 1145 in GenBank accession number X16148).

EXPERIMENTAL PROCEDURES

Plasmid Constructs

Full-length TNFα, GM-CSF, and Interleukin (IL)-3 mRNAs (Fig. 1, A–C)—CMV.mTNFα-(127–1325), containing a NorI–XbaI fragment spanning bp 127–1325 of a mouse TNFα cDNA sequence (GenBank accession number X02611), was made as described (8). The mTNFα cDNA clone, provided by Dr. B. Beutler (University of Texas Southwest Medical Center, Dallas, TX), contained an incomplete 3′-UTR that ended at bp 1325 of GenBank accession number X02611, with a 3′-UTR (for mIL-3) and cloned into the NorI and XbaI sites of plasmid pSK (for mIL-3 and pSK). The correct sequences of the primers were verified by dRhodamine Terminator Cycle Sequencing.

Full-length and Modified Histone mRNA (Fig. 1D)—Plasmid H2a-614, which contains sequence corresponding to bp 1–1645 of GenBank accession number X16148 (mouse histone H2a gene; Ref. 13), was purchased from ATCC (Manassas, VA). To create plasmid CMV.H2a, plasmid H2a-614 was used as a template. A 549-bp fragment (bp 814–1362 of X16148) containing the H2a precursor RNA (bp 814–1328 of X16148) and the sequence of the histone downstream element (bp 1342–1355 of X16148) was PCR-amplified with the following primers: 5′-gtcgcgCCTTTTGGTGTGAGG-3′, 3′-primer contain the 3′-end stem-loop and histone downstream element (bp 1303–1362 of GenBank accession number X03020), and those in the 3′-end of exon V (bp 2869–2889 of GenBank accession number K03233). The lowercase letters in the primers indicate the restriction sites for SalI and XhoI, respectively. The resulting PCR product is a 851-bp mouse IL-3 cDNA.

In the mTNFα expression vector CMV.mTNFα-(127–1325), polyadenylation residues attached to the last T of bp 1325 of GenBank accession number X02611 of CMV.mTNFα were deleted using the PCR primer-overlapping mutagenesis technique. An XhoI restriction site was also created in the same process. The 60-bp double-stranded oligonucleotide coding for the histone H2a stem-loop and histone downstream element was inserted into this newly created XhoI site at the end of CMV.mTNFα-(127–1325). The correct orientation of the histone sequence was confirmed by dRhodamine terminator cycle sequencing.
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Transfection of HEK 293 Cells, Northern Analysis, and RNase H Assays

HEK 293 cells were maintained, and transient transfection of 1.2 x 10^6 cells with plasmid constructs in calcium-phosphate precipitates was performed as described (9). In some experiments, pXGH5 (Nichols Institute Diagnostics, San Juan Capistrano, CA) was co-transfected to monitor transfection efficiency. Assays of released human growth hormone (HGH) were performed as described (14). In most experiments, the cells were co-transfected with various CCCH protein-expressing plasmids. Details of the construction of these plasmids have been described (8, 9).

Twenty-four h after the removal of the transfection mixture, aliquots of cell culture medium were analyzed for HGH according to the manufacturer’s protocol. Total cellular RNA was then harvested from the HEG 293 cells using the RNeasy system (Qiagen, Valencia, CA). Northern blots were prepared as described (1). Blots were hybridized as indicated with random-primed, α-32P-labeled cDNA probes coding for various CCCH zinc finger proteins, including mouse TTP (1), Xenopus XCH3-3 (15), or rat cMG1 (16). Blots were also hybridized as indicated with a 1.1-kb NarI- BamII fragment of a mTNFα cDNA (8), a 422-bp SalI-EcoRV 5’ fragment of mouse GM-CSF cDNA (from plasmid CMV-chemokine-ΔPSC), and a 488-bp SalI-XbaI 5’ fragment of mouse IL-3 cDNA (from plasmid CMV.mIL-3). Some blots were also hybridized to an α-32P-labeled GAPDH cDNA probe (6) to monitor gel loading.

RNase H assays were performed by annealing total cellular RNA (5–10 μg) and the appropriate synthetic oligonucleotide (0.5–1 μg) in 10 μl of 50 mM KCl for 5 min at 50 °C, followed by an additional 10 min at 22 °C. The mixture was incubated further at 37 °C for 40 min in a buffer (4 mM Hepes-KOH (pH 8), 50 mM KCl, 2 mM MgCl2, 0.2 mM dithiothreitol, and 1 μg/ml bovine serum albumin) containing 1 unit of RNase H (Promega, Madison, WI) in a final volume of 25 μl. The reaction mixture was then precipitated with ethanol and acetone, and the resulting RNA was subjected to Northern analysis as described above.

RESULTS

The Effect of Co-transfection of TTP and Related CCCH Proteins on Naturally Occurring ARE-containing Full-length mRNAs—Our previous transfection experiments in 293 cells examined the effects of TTP and related proteins on the turnover of a synthetic TNFα mRNA construct, in which 33 A residues encoded by the vector were attached to the middle of the class II ARE. To confirm that the effect of the co-transfected CCCH proteins was to remove this 3’-end, several of the RNA samples shown in Fig. 2a were hybridized with a probe spanning bases 1370–1627 of accession number X02611 (Fig. 2b). This probe represents a sequence entirely 3’ of the ARE (which occurs between bases 1299 and 1332) and includes the polyadenylation signal (bases 1614–1619). As expected, this probe did not hybridize to any TNFα mRNA species when the 127–1325 TNFα clone was transfected, either in the absence (lane 1) or the presence (lane 2) of co-transfected TTP. However, the probe readily hybridized to the two bands representing the longer TNFα mRNA species, either 127–1627 (lanes 6–10) or 1–1627 (lanes 11–15). This result confirms that the shortest of the three major TNFα species generated in response to CCCH protein expression in Fig. 2a represents a 3’-truncated form of TNFα mRNA that is missing not only the poly(A) tail but also sequences between the poly(A) tail and the ARE.

Similar experiments were performed with full-length cDNAs encoding GM-CSF and IL-3. In the case of GM-CSF, we have demonstrated in experiments with wild-type and TTP-deficient mice that turnover of this mRNA is regulated by TTP in bone marrow stromal cells (7); however, no comparable physiological function of TTP has been demonstrated to date in the regulation of IL-3 mRNA stability. In the case of GM-CSF mRNA, low concentrations of transfected TTP, cMG1, and XCH3-3 DNA (Fig. 3a, lanes 2, 5, and 8) all stimulated a decrease in mRNA steady state levels, whereas higher DNA concentrations again resulted in the accumulation of at least two smaller species of GM-CSF mRNA. Cleaner results were obtained when the cells were treated for 1–8 h with actinomycin D to inhibit ongoing transcription (Fig. 3b). This experiment compared the effects of
no TTP, the genomic TTP construct H6E (5 μg DNA), and low concentrations of the TTP expression construct CMV.hTTP.tag (0.1 μg of DNA) on the levels of GM-CSF mRNA species without (lanes 1–3) or with actinomycin D treatment for various times. The expression of GAPDH mRNA is shown in the middle panel, and the expression of TTP mRNA in the same samples is shown in the bottom panel. The S-h actinomycin D samples showed that, in the cells expressing vector alone (lane 13), the GM-CSF mRNA appeared as a large, diffuse band. The amount of this species was greatly decreased in cells expressing TTP (lanes 14 and 15). In cells expressing the genomic TTP clone, most of the hybridizing GM-CSF species appeared to be full-length, whose levels were markedly decreased compared with control (compare lane 14 with lane 13). Also detectable were low levels of two smaller forms of GM-CSF mRNA when this TTP construct was expressed (see lanes 2, 5, 8, 11, and 14).

However, when the TTP cDNA was expressed, there was a marked decrease in total hybridizable GM-CSF mRNA, and the remaining mRNA was in the form of the shortest of the three hybridizing bands. RNase H and oligo(dT) experiments demonstrated that the single band observed in lane 15 was considerably shorter than the completely deadenylated form of the full-length mRNA (not shown), again consistent with a GM-CSF mRNA that was truncated in a 3′ → 5′ direction to approximately the position of the ARE.

Parallel experiments were performed with full-length IL-3 mRNA. This mRNA is considerably smaller than the 18 S ribosomal RNA, making the results of Northern analysis easier to interpret. As shown in Fig. 4A (top panel), the IL-3 mRNA was expressed as a single diffuse band that appeared as a tightly spaced pair of bands following a shorter autoradiographic exposure (lane 1). Steady state levels of this mRNA
were markedly decreased by TTP co-expression, either from the genomic clone H6E (5 μg of DNA; lane 2) or from low concentrations of the expression construct CMV::hTTP::tag (0.1 μg of DNA; lane 3). Levels were also markedly reduced by co-expression of higher concentrations of either cMG1 (1 μg of DNA; lane 4) or XC3H-3 (1 μg of DNA; lane 5). In all cases in which the CCCH proteins were expressed, but most obviously with cMG1, there was a decrease in the intensity of the upper two bands and the appearance of a third band. These changes were more obvious after transcription was inhibited with actinomycin D. For example, the tightly spaced doublet of IL-3 mRNA from cells transfected with vector (lane 16) largely disappeared with expression of the CCCH proteins, with the appearance of a smaller stable intermediate being apparent in the cMG1 lanes (lanes 4, 9, 14, and 19).

To determine the polyadenylation status of these forms of the IL-3 mRNA, total cellular RNA samples from this experiment were incubated with an oligo(dT) primer and RNase H (Fig. 4B). The samples shown in lanes 1–4 correspond to lanes 16–20 of Fig. 4A; the samples shown in lanes 5–8 represent the same samples after oligo(dT) and RNase H treatment. These results indicate that the smaller of the two tightly spaced bands in the IL-3 doublet was the deadenylated form of the mRNA (da), while the upper of the two bands is the full-length polyadenylated form (FL; compare lane 1 with lane 5). However, the still smaller band that was most evident in the cMG1 samples (lane 3) was not further reduced in size by the oligo(dT)/RNase H treatment (P, lane 7) and represents a further 3′-truncated mRNA. That this was not a 5′ truncation of the mRNA was shown by similar RNase H experiments using an internal primer complementary to bp 868–889 of accession number K03233. In this case, identical 173-base mRNA fragments corresponding to the 5′-end of the IL-3 mRNA were found in both samples from control and TTP-expressing cells (data not shown). As with the TNFα and GM-CSF data, these results are compatible with a CCCH protein-mediated 3′-truncation of the mRNA that was greater than that seen with deadenylation alone. All three further truncations appeared to be approximately at the location of the ARE in these mRNAs.

To confirm the ARE-dependence of the TTP-stimulated degradation of the full-length IL-3 mRNA, we co-transfected cells with genomic and cDNA TTP vectors in the presence of IL-3 mRNA that contained (Fig. 4C, lanes 1–6) or did not contain (lanes 7–12) the ARE. As before, both TTP expression vectors resulted in a concentration-dependent decrease in IL-3 mRNA expression, with the higher concentrations of TTP achieved with the cDNA expression vector resulting in accumulation of the lowest of the three IL-3 mRNA bands. When these experiments were repeated with the IL-3 mRNA lacking its ARE, the co-expression of TTP had no effect on the total level of IL-3 mRNA and did not stimulate the formation of the lowest mRNA band. In this experiment, the corresponding amounts of TTP mRNA are shown in the bottom panel of Fig. 4C, and the levels of GAPDH mRNA in the same samples are shown in the middle panel.

These experiments indicated that TTP and its related proteins cMG1 and XC3H-3 could stimulate the breakdown of three naturally occurring class II ARE-containing mRNAs and cause the accumulation of an mRNA fragment that was truncated in a 3′ to 5′ direction past the 5′-end of the poly(A) tail to approximately the location of the ARE itself. However, they do not distinguish between a stimulated poly(A) or other 3′-exonuclease activity, with “protection” at the ARE, and a hypothetical CCCH protein-stimulated endonuclease activity.

Effect of TTP on an ARE-containing Histone mRNA Lacking a Poly(A) Tail—To address the question of whether TTP could stimulate the breakdown of ARE-containing mRNAs that lacked poly(A) tails, we took two approaches: inserting an ARE into the sequence of a nonpolyadenylated histone mRNA and replacing the IL-3 and TNFα poly(A) tail with a histone 3′-end-processing sequence (for a review, see Ref. 17). In the first series of experiments, we used a histone H2a precursor RNA coding sequence from a mouse genomic clone for H2A and H3 (accession number X16148) driven by the CMV promoter and containing the normal histone 3′-processing sequences without...
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FIG. 4. Effect of TTP and related proteins on the stability of IL-3 mRNA. Cell transfection, total cellular RNA preparation, electrophoresis, and Northern hybridization were performed as described under "Experimental Procedures." Each lane was loaded with 10 μg of total cellular RNA. A, upper panel, relative stability of IL-3 mRNA in the presence of TTP, cMG1, or XC3H-3. CMV.mIL-3 (1 μg/plate) was co-transfected into 293 cells with CCCH protein expression constructs or vector alone. Lanes 1, 6, 11, and 16, 5 μg/plate of vector control were co-transfected (BS+). Lanes 2, 7, 12, and 17, 5 μg/plate of H6E.GHG3' was co-transfected. Lanes 3, 8, 13, and 18, 0.1 μg/plate of CMV.hTTP.tag was co-transfected with 4.9 μg/plate of vector BS+ was co-transfected. Lanes 4, 9, 14, and 19, 1 μg/plate of CMV.cMG1.tag was co-transfected. Lanes 5, 10, 15, and 20, 1 μg/plate of CMV.XC3H-3.tag was co-transfected. For plates co-transfected with cMG1 or XC3H-3 expression constructs, 4 μg/plate of BS+ was also added. Total cellular RNA was harvested after the addition of actinomycin D to a final concentration of 10 μg/ml (−ActD) for 1, 2, or 4 h or buffer alone (−ActD), as described under "Experimental Procedures." B, evidence that one of the smaller species of IL-3 mRNA formed in the presence of CCCH proteins is a deadenylated intermediate. Cells were co-transfected with CMV.mIL-3 (1 μg/plate) and either vector alone or zinc finger expression constructs as follows. Lanes 1 and 5, vector alone (5 μg/plate; BS+); lanes 2 and 6, CMV.hTTP.tag (0.1 μg/plate); lanes 3 and 7, CMV.cMG1.tag (1 μg/plate); lanes 4 and 8, CMV.XC3H-3.tag (1 μg/plate). 5 μg of RNA was loaded into each gel lane. Oligonucleotide (dT)12–18 (1 μg) was added to samples 5–8. As indicated, the RNA samples were treated with (+) or without (−) 1 unit of RNase H as described under "Experimental Procedures." The Northern blots were probed with a 32P-labeled mouse IL-3 cDNA. The position of the 18 S ribosomal RNA is indicated. The three arrows indicate IL-3 mRNA species that contained (FL) or did not contain (ΔA) their poly(A) tails or were further truncated in a 3'→5' direction (P). C, upper panel, effect of TTP on the stability of wild-type and ARE-deleted mIL-3 mRNA. 1 μg/plate of CMV.mIL-3 or CMV.mIL-3 (dARE) was co-transfected into 293 cells with TTP expression constructs or vector alone. Lanes 1–6, wild-type CMV.mIL-3; lanes 7–12, CMV.mIL-3 (dARE), in which ARE-containing sequence bp 2738–2771 was deleted. Lanes 1 and 5, 7, and 10, 5 μg/plate of vector (BS+) was co-transfected; lanes 2, 3, 8, and 9, H6E.GHG3' was co-transfected in the indicated amounts (μg/plate); lanes 4–6 and 10–12, CMV.hTTP.tag was co-transfected in the indicated amounts (μg/plate). For lanes 2, 4–6, 8, and 10–12, vector DNA was also added to make the total co-transfected plasmid DNA equal to 5 μg/plate. The Northern blots shown in the upper panels of both A and C were probed with a 32P-labeled mIL-3 cDNA. After autoradiography, these blots were then stripped and reprobed with a 32P-labeled GAPDH probe, and the results are shown in the middle panels of A and C. Identical RNA samples as in the upper panels were blotted and probed simultaneously with 32P-labeled mouse TTP, rat cMG1, and Xenopus XC3H-3 cDNAs (A) or the TTP cDNA alone (C), and the results are shown in the lower panels of A and C. The position of the 18 S ribosomal RNA is indicated. The three arrows in the upper panels of A and C indicate the three species of IL-3 mRNA discussed. The expressed GAPDH, TTP, or other CCCH zinc finger protein mRNAs are also indicated by arrows.

an added polyadenylation signal or poly(A) tail. As shown in Fig. 5A (lane 1), the mouse histone cDNA probe hybridized to an endogenous human histone transcript of ∼0.5 kb in mock-transfected 293 cells. When the histone genomic construct was co-transfected into cells with vector alone, a prominent 0.5-kb band of histone mRNA was detected whose levels were ∼3 times greater than that of the endogenous histone, as determined by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA) analysis (lane 2). Co-transfection with TTP had little or no effect on the histone mRNA expression at 5 and 10 ng of co-transfected DNA (lanes 3 and 4); however, at 100 ng of TTP DNA, a decrease in expression of histone mRNA was apparent (lane 5). The zinc finger TTP mutant C124R had no effect on histone mRNA expression at 10 and 100 ng of the mutant DNA (lanes 6 and 7). When an otherwise identical histone construct was expressed that contained the 70-bp TNFα ARE, the resulting steady-state level of mRNA was approximately the same as that seen with the wild-type histone (lane 8). In addition, the added sequence allowed the mRNA expressed from the transfected DNA to be separated from the endogenous histone mRNA on the blot (lane 8). However, when TTP was co-transfected at 5, 10, and 100 ng of DNA, there was a marked decrease in the accumulation of histone mRNA (lanes 9–11), with minimal effect on endogenous histone mRNA levels. The zinc finger mutant of TTP had little or no effect on the histone-ARE mRNA levels (lanes 12 and 13). Otherwise identical experiments were performed with the wild-type histone sequence into which a EcoRV restriction site had been made by mutating bp 1167 and 1168 of accession number X16148 from C to A, and G to T, respectively. Except at the highest concentration of transfected TTP (100 ng of DNA; lane 17), both wild-type and mutant TTP had little effect on the expression of the modified histone mRNA (lanes 14–19).

To control for the possibility that insertion of 70 bp of any random DNA sequence into the histone mRNA might affect its susceptibility to TTP-induced mRNA turnover and to shift the resulting hybrid transcript away from the endogenous histone mRNA on the gels, a 75-bp multiple cloning site fragment from the plasmid cloning vector Bluescript pSK− (Stratagene; bp 653–731) was inserted into the EcoRV site of the modified
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The effect of TTP on the accumulation of this hybrid mRNA was assessed along with its effect on the ARE-containing histone mRNA. This experiment used lower concentrations of TTP DNA (1.0–10 ng) to prevent the apparent nonspecific effects of higher concentrations of TTP on histone mRNA levels. As shown in Fig. 5B (upper panel), TTP (5 and 10 ng of DNA) again appeared to decrease the accumulation of histone-ARE mRNA at (lanes 1–4). We also tested whether the AREs from GM-CSF and IL-3, as well as TNFα, could make the histone mRNA susceptible to TTP-induced degradation. As shown in Fig. 5B (upper panel), insertion of the AREs from IL-3 (lanes 5–8) and GM-CSF (lanes 9–12) all conferred TTP sensitivity on the histone mRNA, at similar TTP concentrations. However, the control histone sequence that contained the insert of similar size from pSκ– was unaffected by the increasing amounts of co-transfected TTP (lanes 13–16). The low concentrations of TTP DNA used in this experiment had minimal nonspecific effects on histone gene transcription, as confirmed by the lack of effect on the pSκ–containing histone (lanes 13–16). The low concentration of TTP required for mRNA degradation is illustrated by a comparison of the upper and lower panels of Fig. 5B. Identical cpm of the histone probe (upper panel) and TTP probe (lower panel) were added to two identical blots in Fig. 5B; however, the autoradiographic exposure of the blot shown in the upper panel was 45 min at –70 °C, whereas the exposure of the blot shown in the lower panel was 16 h at the same temperature.

These studies confirmed that the ability of TTP to decrease histone-ARE expression was not just a function of disrupting the normal histone mRNA with 70 bp of foreign sequence: In addition, the effect of TTP was more obvious when the expressed histone transcripts were physically separated from the endogenous histone mRNA bands.

To confirm that the transfected histone gene was appropriately processed and was not polyadenylated in this transient expression system, RNA samples were digested with RNase H in the presence of oligo(dT). Incubation with RNase H and oligo(dT) did not affect the levels or size of endogenous histone mRNA (Fig. 5C, lanes 1 and 2). Furthermore, this treatment did not result in the disappearance or shortening of a significant amount of histone mRNA expressed by the transfected constructs, whether this was wild-type histone mRNA (lanes 3–6) in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of TTP (5 µg of DNA); TNFα ARE-containing histone mRNA (lanes 7–10) in the absence (lanes 7 and 8) or presence (lanes 9 and 10) of TTP; or the histone mRNA modified with the 75-bp pSκ– insert (lanes 11–14) in the absence (lanes 11 and 12) or presence (lanes 13 and 14) of TTP. These data confirm that the transfected histone pre-mRNA was appropriately 3′-processed and that spurious poly(A) tail addition did not take place in the presence or absence of TTP.

Effect of TTP on IL-3 and TNFα mRNAs Containing Histone 3′-End-processing Sequences—To further test the hypothesis that a poly(A) tail was not necessary for TTP activity, we replaced the naturally occurring polyadenylation signal in the IL-3 mRNA and the 33 adenine residues in the TNFα expression construct with the histone 3′-end-processing sequence (see CMV.H2a histone sequence. The effect of TTP on the accumulation of this hybrid mRNA was assessed along with its effect on the ARE-containing histone mRNA. This experiment used lower concentrations of TTP DNA (1.0–10 ng) to prevent the apparent nonspecific effects of higher concentrations of TTP on histone mRNA levels. As shown in Fig. 5B (upper panel), TTP (5 and 10 ng of DNA) again appeared to decrease the accumulation of histone-ARE mRNA at (lanes 1–4). We also tested whether the AREs from GM-CSF and IL-3, as well as TNFα, could make the histone mRNA susceptible to TTP-induced degradation. As shown in Fig. 5B (upper panel), insertion of the AREs from IL-3 (lanes 5–8) and GM-CSF (lanes 9–12) all conferred TTP sensitivity on the histone mRNA, at similar TTP concentrations. However, the control histone sequence that contained the insert of similar size from pSκ– was unaffected by the increasing amounts of co-transfected TTP (lanes 13–16). The low concentrations of TTP DNA used in this experiment had minimal nonspecific effects on histone gene transcription, as confirmed by the lack of effect on the pSκ–containing histone (lanes 13–16). The low concentration of TTP required for mRNA degradation is illustrated by a comparison of the upper and lower panels of Fig. 5B. Identical cpm of the histone probe (upper panel) and TTP probe (lower panel) were added to two identical blots in Fig. 5B; however, the autoradiographic exposure of the blot shown in the upper panel was 45 min at –70 °C, whereas the exposure of the blot shown in the lower panel was 16 h at the same temperature.

These studies confirmed that the ability of TTP to decrease histone-ARE expression was not just a function of disrupting the normal histone mRNA with 70 bp of foreign sequence: In addition, the effect of TTP was more obvious when the expressed histone transcripts were physically separated from the endogenous histone mRNA bands.

To confirm that the transfected histone gene was appropriately processed and was not polyadenylated in this transient expression system, RNA samples were digested with RNase H in the presence of oligo(dT). Incubation with RNase H and oligo(dT) did not affect the levels or size of endogenous histone mRNA (Fig. 5C, lanes 1 and 2). Furthermore, this treatment did not result in the disappearance or shortening of a significant amount of histone mRNA expressed by the transfected constructs, whether this was wild-type histone mRNA (lanes 3–6) in the absence (lanes 3 and 4) or presence (lanes 5 and 6) of TTP (5 µg of DNA); TNFα ARE-containing histone mRNA (lanes 7–10) in the absence (lanes 7 and 8) or presence (lanes 9 and 10) of TTP; or the histone mRNA modified with the 75-bp pSκ– insert (lanes 11–14) in the absence (lanes 11 and 12) or presence (lanes 13 and 14) of TTP. These data confirm that the transfected histone pre-mRNA was appropriately 3′-processed and that spurious poly(A) tail addition did not take place in the presence or absence of TTP.

Effect of TTP on IL-3 and TNFα mRNAs Containing Histone 3′-End-processing Sequences—To further test the hypothesis that a poly(A) tail was not necessary for TTP activity, we replaced the naturally occurring polyadenylation signal in the IL-3 mRNA and the 33 adenine residues in the TNFα expression construct with the histone 3′-end-processing sequence (see
which expressed IL-3 as a broad band consisting of two major bands, whose size was approximately the same as the shortest transcript seen after the expression of full-length chimeric IL-3 mRNA. At the highest TTP concentration used (100 ng/plate), a truncated transcript accumulated (lane 10), whose size was approximately the same as the shortest transcript seen after the expression of full-length IL-3 mRNA and the same concentration of TTP (lane 5). The expression of TTP mRNA in this experiment is shown in the lower panel of Fig. 6A. Similar results were obtained from several experiments, which also revealed that the concentration dependence of the TTP effect was similar with both the native IL-3 mRNA and the chimeric IL-3-histone transcript.

To confirm that the hybrid IL-3-histone mRNA was not polyadenylated in these experiments, the same RNA samples were digested with RNase H in the presence of oligo(dT). As indicated in Fig. 6B, lanes 1–4, this experiment confirmed that the upper of the two normal IL-3 transcript bands was the fully polyadenylated species, whereas the lower of the two bands was the deadenylated species. When the hybrid mRNA formed by the transfected IL-3-histone plasmid was treated in this way, its migration was unaffected, confirming that no significant poly(A) tail was present (lanes 5–8). These data indicate that the primary transcript formed by the expression of the IL-3-histone cDNA was completely resistant to the effects of RNase H and oligo(dT), confirming that it was not polyadenylated under the conditions of these experiments.

Similar experiments were performed using a chimeric TNFα-histone construct (Fig. 7C). In this experiment, the co-expression of two concentrations of TTP DNA (5 and 10 ng/plate) with the TNFα expression plasmid CMV.TNFα-(127–1325) resulted in the consistently observed decrease in total transcript amount as well as the formation of the deadenylated species (Fig. 7A, upper panel, lanes 1–3). When the 33 adenylate residues in the CMV.TNFα-(127–1325) construct were replaced with the histone 3′-processing sequence and then co-transfected with low concentrations of TTP vector, there was a decrease in total hybridizable IL-3 transcripts and the appearance of two concentration-dependent bands in the Northern blot, with the upper of the two normal IL-3 transcript bands being the fully polyadenylated species and the lower of the two bands being the deadenylated species. When the hybrid mRNA formed by the transfected IL-3-histone plasmid was treated in this way, its migration was unaffected, confirming that no significant poly(A) tail was present (lanes 5–8). These data indicate that the primary transcript formed by the expression of the IL-3-histone cDNA was completely resistant to the effects of RNase H and oligo(dT), confirming that it was not polyadenylated under the conditions of these experiments.

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FIG. 7. Effect of TTP on a TNFα mRNA containing a histone 3'end-processing sequence. Cell transfection, total cellular RNA preparation, electrophoresis, and Northern hybridization were performed as described under "Experimental Procedures." A, each lane was loaded with 10 μg of total cellular RNA. Upper panel, stability of TNFα mRNA expressed with or without a poly(A) tail in the presence of TTP. TNFα expression constructs (1 μg/plate) were co-transfected into 293 cells with CMV.TTP.tag or vector alone. Lanes 1–3 (Control), CMV.TNFα-(127–1325); lanes 4–6 (H2aSL), CMV.TNFα-(127–1325 H2aSL), in which the 33 3'-terminal adenylate residues were replaced with the histone stem loop and histone downstream element as described under "Experimental Procedures." Lane 7 (dA), CMV.TNFα-(127–1325), in which the 33 adenylate residues attached to the 3'-most bp of the TNFα sequence were deleted. Lanes 1, 4, and 7, 4 μg/plate of vector (BS+) was co-transfected; lanes 2, 3, 5, and 6, CMV.TTP.tag was co-transfected in the indicated amount (ng/plate). For lanes 2, 3, 5, and 6, vector DNA was also added to make the total transfected plasmid DNA 5 μg/plate. The Northern blot was probed with a 32P-labeled TNFα cDNA. Identical RNA samples as in the upper panel of A were blotted and probed simultaneously with 32P-labeled mouse TTP cDNA, and the results are shown in the lower panels of A. The position of the 18S ribosomal RNA is indicated. The two arrows indicate the two species of TNFα mRNA discussed. The expressed TTP mRNA is also indicated by an arrow in the lower panel. B, evidence that the species of TNFα mRNA expressed from transfected CMV.TNFα-(127–1325 H2aSL) did not contain a poly(A) tail. Lanes 1–3 (Control), cells were transfected with CMV.TNFα-(127–1325) (1 μg/plate). Lanes 4–6 (H2aSL), Cells were transfected with CMV.TNFα-(127–1325 H2aSL) (1 μg/plate). The cells were co-transfected with vector BS+ (4 μg/plate). 5 μg of RNA was loaded into each gel lane. Oligonucleotide (dT)12–18 (T; 1 μg) was added to samples 2 and 5. An oligonucleotide that was complementary to the stem-loop sequence of histone H2a (1 μg) was added to lanes 3 and 6. All RNA samples were treated with 1 unit of RNase H as described under "Experimental Procedures." The Northern blot was probed with a 32P-labeled TNFα cDNA. C, schematic representation of the CMV.TNFα-(127–1325) constructs. Control, CMV.TNFα-(127–1325) in which the 33 adenylic residues were directly attached to the 3'-most bp of the TNFα sequence. H2aSL, CMV.TNFα-(127–1325 H2aSL) in which the 33 adenylic residues were deleted and a 3' portion of the mouse histone H2a sequence containing the stem-loop (capital letters, underlined) and histone downstream element (HDE; in capital letters) was inserted immediately after the TNFα sequence. The 3'-end of the H2a mRNA (C) is indicated by italic type.

decrease in the levels of the expressed hybrid TNFα-H2a mRNA (Fig. 7A, upper panel, lanes 4–6). In contrast to the effect of TTP co-transfection on the TNFα mRNA expressed from construct CMV.TNFα-(127–1325), which resulted in two mRNA species that were different in size (Fig. 7A, upper panel, lanes 2 and 3), the presence of TTP altered the amount but not the size of the hybrid TNFα-H2a mRNA. The virtual absence of expression of a TNFα construct that contained neither the poly(A) tail nor the histone 3'-end-processing sequence is shown in Fig. 7A (lane 7, upper panel).

To confirm that the chimeric TNFα-H2a mRNA was not polyadenylated in these experiments, additional RNase H studies were performed on the RNA samples shown in Fig. 7A (upper panel, lanes 1 and 4). When RNA from 293 cells transfected with the control plasmid CMV.TNFα-(127–1325) was treated with oligo(dT)12–18 and RNase H, TNFα mRNA decreased in size (Fig. 7B, lanes 1 and 2). However, when RNA from 293 cells transfected with plasmid CMV.TNFα-(127–1325 H2aSL) was treated with RNase H, there was no apparent difference in the size of hybrid TNFα-H2a mRNA in the presence or absence of oligo(dT)12–18 (Fig. 7B, lanes 4 and 5). This experiment confirmed that the TNFα-H2a hybrid mRNA had not undergone polyadenylation under these experimental conditions. When an oligonucleotide complementary to the stem-loop sequence was used, the presence of this oligonucleotide slightly decreased the size of the hybrid TNFα-H2a mRNA (Fig. 7B, lane 6) while having no effect on the size of the TNFα mRNA expressed from the control plasmid CMV.TNFα-(127–1325) (Fig. 7B, lane 3).

**DISCUSSION**

Previous experiments in TTP knockout mice and cells derived from them have shown that TTP is a physiologically important regulator of the stability of mRNAs encoding two important cytokines, TNFα and GM-CSF. In cultured bone marrow-derived macrophages from normal mice, the normal half-life of TNFα mRNA was about 40 min following the addition of actinomycin D to the cells; in this situation, the mRNA disappeared without the appearance of detectable degradation intermediates in Northern blots. Intermediate products of GM-CSF mRNA disappearance following lipopolysaccharide or TNFα stimulation of bone marrow-derived stromal cells revealed the presence of a degradation intermediate (7). Specifically, following the induction of GM-CSF gene transcription by either agent, the first detectable transcript seen on Northern blots was approximately 1 kb in length, corresponding to the full-length mouse transcript containing a poly(A) tail of ~200 residues. This full-length mRNA species was first visible approximately 1 h after stimulation of the cells. However, this was rapidly...
followed by the appearance of a smaller band of ~0.8 kb, which thereafter accounted for approximately half of the total hybridizable GM-CSF mRNA. Both species then disappeared in a longer time course or following actinomycin D treatment of the cells. Importantly, the smaller band was demonstrated by RNase H analysis to be the essentially completely deadenylated form of the mRNA, and in the Northern blots from wild-type cells, a smear of mRNAs of intermediate sizes could be detected between the two major transcripts (7). The two major mRNA species have been seen before in Northern blots of GM-CSF mRNA (18, 19); our studies firmly established that these bands represent the fully adenylated and deadenylated forms of the GM-CSF mRNA. These data fit the current model of class II ARE-dependent mRNA degradation, in which progressive deadenylation leads to the usually transient accumulation of deadenylated mRNA bodies (10).

However, parallel experiments conducted with bone marrow-derived stromal cells from the TTP-deficient mice revealed a strikingly different pattern (7). First, GM-CSF mRNA stability was increased to the point that no half-life could be calculated. In addition, there was a greatly increased accumulation of the fully polyadenylated GM-CSF mRNA relative to the deadenylated species, so that the latter was virtually undetectable in most Northern blots. In other words, TTP deficiency not only led to stabilization of the full-length GM-CSF mRNA but also greatly inhibited the formation of the deadenylated mRNA body from the polyadenylated full-length mRNA.

These data strongly support a model in which TTP deficiency inhibits the first important step in class II ARE-containing mRNA degradation, the progressive removal of the poly(A) tail by 3′ to 5′ exonuclease activity. Conversely, they also support a model in which TTP binding to the ARE in some way increases the rate of deadenylation of the full-length GM-CSF mRNA.

In the current studies, we attempted to gain further insight into the importance of the poly(A) tail in CCCH protein-stimulated turnover of mRNAs containing class II AREs. To do this, we performed three types of experiments: 1) co-expression of full-length transcripts for TNFα, GM-CSF, and IL-3 with TTP and its related CCCH proteins cMG1 and XC3H-3; 2) insertion of class II ARE sequences into a normally nonpolyadenylated histone transcript, followed by co-expression with TTP; and 3) co-expression with TTP of two chimeric class II ARE-containing transcripts that lacked a poly(A) tail but instead terminated in a histone 3′ stem-loop sequence.

The first group of studies, using full-length mRNAs for TNFα, GM-CSF, and IL-3, demonstrated that co-transfection of normal TTP and its related CCCH proteins, but not of a non-binding mutant form of TTP, could stimulate the breakdown of these class II ARE-containing mRNAs in this 293 cell transfection system. The effect of TTP to stimulate the breakdown of the mRNAs is in keeping with our previous data concerning TNFα and GM-CSF mRNAs in cells derived from the TTP knockout mice (6, 7). This suggests that, at low concentrations of transfected TTP DNA, TTP is stimulating the breakdown of these ARE-containing mRNAs in 293 cells in a manner that is directly related to its behavior in the normal physiology of macrophages and bone marrow-derived stromal cells. We have not yet shown that TTP deficiency results in stabilization of the IL-3 mRNA in cells derived from TTP knockout mice, but the present results suggest that this might be the case in an appropriate cell type. In support of this idea is a recent report demonstrating that co-expression of TTP destabilizes IL-3 mRNA in HT1080 cells (20).

In the experiments in which the CCCH proteins stimulated the breakdown of full-length TNFα, GM-CSF, and IL-3 mRNAs, the destabilization involves the 3′-end. This can be concluded, because in all three cases, relatively high level CCCH protein expression resulted in the formation of stable breakdown products that were shown by RNase H experiments to have normal 5′-ends but shortened 3′-ends. However, in the cases of all three class II ARE-containing transcripts, at high concentrations of transfected TTP, cMG1, and XC3H-3 DNA, stable mRNA intermediates were formed that were even shorter than the completely deadenylated mRNAs. These smaller 5′ to 3′ truncated fragments appeared to terminate at approximately the site of the ARE in all three naturally occurring mRNAs. This phenomenon was also noted in previous experiments using an artificially truncated TNFα mRNA in which a 33-residue poly(A) “tail” was directly attached to the middle of the ARE; in these studies, high concentrations of expressed TTP, cMG1, and XC3H-3 resulted in the increased accumulation of a deadenylated intermediate (8, 9). Although we speculated that at high TTP concentrations the binding of TTP to the ARE might prevent further 3′ to 5′ exonuclease activity, the mechanism of this apparent “protective” effect is not known; possibilities include physical interference with a 3′ to 5′ exonuclease, sequestering an unknown mRNA-stabilizing protein or factor from the ARE-associated complex, and others.

Whatever the mechanism of this effect, several factors suggest that this accumulation of a 3′-truncated intermediate that is even shorter than the deadenylated mRNA body is an artifactual consequence of the expression of high concentrations of the CCCH proteins. First, as noted above, this only occurs at concentrations of expressed CCCH proteins that are at least 10-fold greater than the concentration required to promote maximum disappearance of the ARE-containing mRNAs (8). Even when the concentration dependence of the TTP effect was assessed in the presence of actinomycin D, there was a striking effect of TTP to decrease native IL-3 mRNA accumulation at 5 ng of DNA/plate, whereas the first accumulation of the further 3′–5′ truncated intermediate did not occur until ~100 ng of TTP DNA. Second, when even higher concentrations of non-binding tandem zinc finger domain mutants have been co-transfected with ARE-containing mRNAs, we have never seen accumulation of the 3′–5′-most truncated intermediate (8). Third, when only the tandem zinc finger domain of TTP was transfected, the 3′–5′-most truncated intermediate was readily seen, although the overall decrease in full-length ARE-containing mRNA was less than that seen with full-length TTP (9). Fourth, although we have looked hard for the formation of such an intermediate derived from TNFα, GM-CSF, and IL-3 mRNAs in Northern blots of macrophages, stromal cells, mast cells, and fibroblasts from wild-type and TTP-deficient mice, we have never seen them (6, 7). This is despite the fact that the AREs in these three mRNAs are located considerably 5′ from the 3′-end of the mRNA body, which should make a significant accumulation of these intermediates readily apparent on Northern blots. Finally, as shown in the present study, even high concentrations of TTP had no effect on the formation of either type of 3′–5′ truncated mRNA when the “target” mRNA was an IL-3 mRNA that lacked an ARE. For all these reasons, we believe that the 3′–5′-most truncated mRNA intermediate seen in these experiments is likely to be an artifact seen only with high concentrations of transfected CCCH protein DNA.

However, an interesting study by Yang et al. (21) detected a degradation intermediate in IL-11 mRNA in PU-34 cells, a primate bone marrow stromal cell line. The IL-11 mRNA contains several class II ARE-like sequences in its 3′-UTR (see

\[ W. S. Lai and P. J. Blackshear, unpublished data. \]

\[ E. Carballo and P. J. Blackshear, unpublished data. \]
GenBank™ accession number M57766.1). These authors established that the intermediate identified in these cells was truncated from the 3′-end to approximately base 790 (corresponding to approximately base 700 in accession number M57766.1), a position within 7–24 bases of the longest ARE-like sequence (UUAAUUAAUUAAUU; bases 707–724 in M57766.1). They showed that deletion of this sequence did not affect the stability of the IL-11 mRNA in transfection studies in this cell type, and they were unable to determine whether the intermediate was formed by an endonuclease or a 3′–5′-exonuclease activity. It will be of interest to determine whether this ARE sequence can bind CCCH proteins and, more importantly, whether it contributes to TTP-mediated IL-11 mRNA instability in bone marrow-derived stromal cells or other cell types derived from wild-type and TTP-deficient mice.

To evaluate the requirement for a poly(A) tail in TTP activity, we took advantage of the fact that some mammalian histone mRNAs do not contain poly(A) tails. Instead, they rely on the processing of a characteristic 3′ stem-loop-containing sequence to permit export of the mature mRNA from the nucleus (17, 22). We used this information to construct two types of expressed mRNAs. In the first type, we inserted class II ARE sequences from the TNFα, GM-CSF, and IL-3 mRNAs into a wild-type histone gene. In the second type, we used the native mouse IL-3 mRNA and a truncated TNFα mRNA, in which the polyadenylation signals had been mutated or the poly(A) tail removed and the genomic histone 3′-processing sequence had been attached to the 3′-end of the mRNA. This should ensure that histone-like cleavage of this 3′-element would be required for the mRNA to be processed and released from the nucleus, but polyadenylation would not occur. In both types of experiment, the hybrid mRNAs were expressed with CCCH proteins in co-transfection studies in 293 cells, and the absence of polyadenylation in each case was confirmed by RNase H analysis with an oligo(dT) primer.

These experiments showed that, in all cases, the co-expression of wild-type TTP, but not of a nonbinding mutant, led to the increased destruction of the ARE-containing mRNAs, although no poly(A) tails were present in these mRNAs. In the case of the IL-3/histone chimeric mRNA, there was accumulation of a stable intermediate in the TTP-treated samples, in which the mRNA was truncated at the 3′-end; as seen with the wild-type IL-3 transcript, the 3′-end of the stable intermediate appeared to correspond approximately to the site of the naturally occurring IL-3 ARE and was only seen at the highest concentration of transfected TTP DNA used. In the case of the ARE-containing histone mRNA and the TNFα-histone chimeric mRNA, no stable intermediates were evident after TTP-stimulated breakdown of the mRNA. Thus, it appears that TTP can stimulate the breakdown of ARE-containing mRNAs even in the absence of poly(A) tails.

How can we explain this effect of TTP and its related CCCH proteins, in light of the data described above, using cells derived from TTP knockout mice, in which the predominant initial effect of TTP appears to be stimulation of deadenylation?
Interactions of CCCH Zinc Finger Proteins with mRNA:
TRISTETRAPROLIN-MEDIATED AU-RICH ELEMENT-DEPENDENT mRNA
DEGRADATION CAN OCCUR IN THE ABSENCE OF A POLY(A) TAIL
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