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Interplay of Two Functionally and Structurally Distinct Domains of the c-*fos* AU-Rich Element Specifies Its mRNA-Destabilizing Function

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AU-rich elements (ARE) in the 3′ untranslated region of many highly labile mRNAs for proto-oncogenes, lymphokines, and cytokines can act as an RNA-destabilizing element. The absence of a clear understanding of the key sequence and structural features of the ARE that are required for its destabilizing function has precluded the further elucidation of its mode of action and the basis of its specificity. Combining extensive mutagenesis of the c-*fos* ARE with in vivo analysis of mRNA stability, we were able to identify mutations that exhibited kinetic phenotypes consistent with the biphasic decay characteristic of a two-step mechanism: accelerated poly(A) shortening and subsequent decay of the transcribed portion of the mRNA. These mutations, which affected either an individual step or both steps, all changed the mRNA stability. Our experiments further revealed the existence of two structurally distinct and functionally interdependent domains that constitute the c-*fos* ARE. Domain I, which is located within the 5′ 49-nucleotide segment of the ARE and contains the three AUUUA motifs, can function as an RNA destabilizer by itself. It forms the essential core unit necessary for the ARE-destabilizing function. Domain II is a 20-nucleotide U-rich sequence which is located within the 3′ part of the c-*fos* ARE. Although it alone can not act as an RNA destabilizer, this domain serves two critical roles: (i) its presence enhances the destabilizing ability of domain I by accelerating the deadenylation step, and (ii) it has a novel capacity of buffering decay-impeding effects exerted by mutations introduced within domain I. A model is proposed to explain how these critical structural features may be involved in the c-*fos* ARE-directed mRNA decay pathway. These findings have important implications for furthering our understanding of the molecular basis of differential mRNA decay mediated by different AREs.

Modulation of gene expression by altering mRNA stability is generally recognized by now as a major control point. It provides a powerful means for controlling gene expression during cell growth and differentiation as well as other physiological transitions (for reviews, see references 4, 8, and 24). It is now well established that the metabolic lifetime of mRNA can be specified by specific *cis*-acting elements within mRNA sequences (for a review, see reference 27). One type of these RNA sequence elements, which has been receiving considerable interest recently, is an adenylate/uridylate-rich sequence of 50 to 100 nucleotides (nt) in length, termed the AU-rich element (ARE). It is usually found in the 3′ untranslated region (UTR) of many highly labile mammalian mRNAs for proto-oncogenes and for genes encoding cytokines, lymphokines, and some transcription factors (7, 31). Shaw and Kamen (31) were the first to show that a 51-nt ARE in the 3′ UTR of human granulocyte-monocyte colony-stimulating factor (GM-CSF) mRNA functions as a potent mRNA destabilizing sequence upon its insertion into a heterologous stable mRNA. More recent experiments have further demonstrated that AREs from the 3′ UTRs of c-*fos*, c-*myc*, and β-interferon also function as an RNA-destabilizing element (12, 19, 25, 33, 36, 37).

Studies of the decay of transcripts containing the c-*fos* or c-*myc* ARE and its variants have suggested that the ARE facilitates rapid removal of the poly(A) tail as a first critical step in mRNA decay and that mRNA deadenylation may be a general mechanism by which ARE-containing mRNAs are triggered for rapid degradation (5, 21, 32, 35, 37). In the case of the c-*fos* ARE, Shyu et al. (32) showed that insertion of the c-*fos* ARE into the 3′ UTR of β-globin mRNA (to generate BBB+ARE mRNA) markedly increases the rate and extent of poly(A) shortening of the β-globin mRNA and that the observed accelerated shortening of the poly(A) tail directed by the c-*fos* ARE precedes the decay of the transcribed portion of the message.

There are two common structural features among the different AREs: (i) the presence of various copies of an AUUUA pentanucleotide and (ii) a high content of adenylate and uridylate residues. Previously, Shyu et al. (32) found that while single point mutations in each of the three AUUUA motifs within the c-*fos* ARE (termed ARE3 mutant; see Fig. 1) slightly affected the ability of this element to facilitate rapid deadenylation of β-globin mRNA, the same mutations significantly increase the overall stability of the corresponding mRNA. This observation suggests that the AUUUA motifs may play a role in the decay of the mRNA body. However, the exact role of the AUUUA motifs and the importance of the general AU richness of the ARE remain elusive. The absence of a clear understanding of the key sequence and structural features of the ARE that allow it to function has precluded the further elucidation of its mode of action and the basis of its specificity.

Here, we report a mechanistic description of the critical structural characteristics of the c-*fos* ARE necessary for its destabilizing function. The identification of mutations that

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affect either the deadenylation step or the subsequent decay of the RNA body and the finding that all of these mutations affect the overall half-life of the corresponding mRNAs provide evidence to support a two-step mechanism for the c-*fos* ARE-mediated mRNA decay. Moreover, our data reveal the existence of two structurally distinct and functionally interdependent domains within the c-*fos* ARE. Interaction between the two domains specifies the ultimate destabilizing ability of the c-*fos* ARE. The results have important implications for furthering our understanding of the basis of differential mRNA decay mediated by different AREs.

MATERIALS AND METHODS

Cell culture and DNA transfection. Culturing, transient transfection, and serum stimulation of mouse NIH 3T3 cells were performed as described previously (33).

Analysis of mRNA decay and deadenylation. Total cytoplasmic RNA was isolated at various times after serum stimulation of transiently transfected NIH 3T3 cells and was analyzed by RNase protection for mRNA decay, as described previously (33). Decay of mRNA was quantitated directly by scanning gels on a Betascope 603 Blot Analyzer (Betagen). Human α1-globin mRNA was detected with a 272-nt RNA probe derived from *Eco*RI-linearized plasmid pT7α181 (32). BBB+ARE and its mutant derivative mRNAs were detected with a 238-nt RNA probe derived from *Bam*HI-linearized plasmid pT7BBF (32). Transcription reactions were performed according to Promega instructions. Labeled RNA transcripts were produced by inclusion of [α-^{32}P]UTP (800 Ci/mmol; Amersham or New England Nuclear).

Northern (RNA) blot analysis was performed to analyze the length of poly(A) tail as described previously (32). Electrophoresis was done on 1.4% formaldehyde agarose gels. A 123-nt single-stranded DNA probes were prepared by the method of random oligonucleotide priming (11). RNase H treatment of cytoplasmic mRNA was carried out as described previously (32).

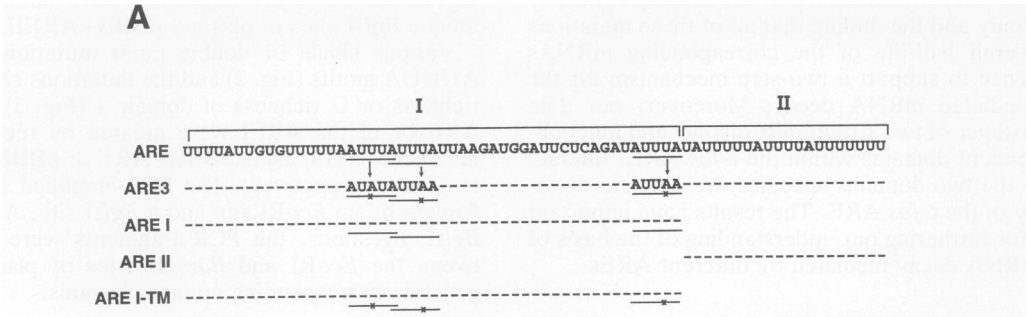
Plasmid constructions. To construct plasmid pBBB+AREI, a 150-bp region in the plasmid pBBB+ARE (33) spanning the last 80-bp carboxyl-terminal region of the rabbit β-globin gene and the first 49-bp region of the human c-*fos* ARE (Fig. 1A, region I) was amplified by standard PCR techniques (29). The PCR product was a 158-bp fragment flanked by an *Eco*RI site and a *Bgl*II site. After *Eco*RI and *Bgl*II digestion, the PCR fragments were subcloned between the *Eco*RI and *Bam*HI sites of plasmid pBBB (33). Plasmid pBBB+AREI-TM was constructed through the same procedure as for pBBB+AREI except that plasmid pBBB+ARE3 (32) was used as the template for PCR amplification of the desired region. Plasmids pBBB+AREII and pBBB+ARE (IIR) were generated in several steps as follows. A 45-bp fragment containing the last 20-bp sequence of the human c-*fos* ARE (Fig. 1A, region II) flanked by a *Bam*HI and a *Bgl*II site was prepared by annealing two complementary deoxyoligonucleotides followed by Klenow fill-in treatment. After *Bam*HI and *Bgl*II digestions, a desired 33-bp fragment was then subcloned into the unique *Bgl*II site of plasmid pBBB to generate pBBB+AREII. The same fragment was introduced into the unique *Bgl*II site of plasmid pBBB+AREI, and reverse orientation of the insert was selected to generate pBBB+ARE(IIR). To construct plasmid pBBB+ARE(II/I), region I in the plasmid pBBB+ARE (Fig. 1A) was amplified by standard PCR techniques and was flanked by a *Bam*HI site and a *Bgl*II site. After *Bam*HI and *Bgl*II digestions, the PCR fragments were subcloned into the unique *Bgl*II sites of plasmid pBBB+AREII.

Various single or double point mutations affecting the AUUUA motifs (Fig. 2) and the mutations changing the AU richness or U richness of domain I (Fig. 3) of intact c-*fos* ARE or of the AREI were created by recombinant PCR mutagenesis (15) using pBBB+ARE or pBBB+AREI as the template, respectively. The PCR-amplified fragments were flanked by an *Eco*RI site and a *Bgl*II site. After *Eco*RI and *Bgl*II digestions, the PCR fragments were subcloned between the *Eco*RI and *Bam*HI sites of plasmid pBBB to generate corresponding mutant plasmids.

RESULTS

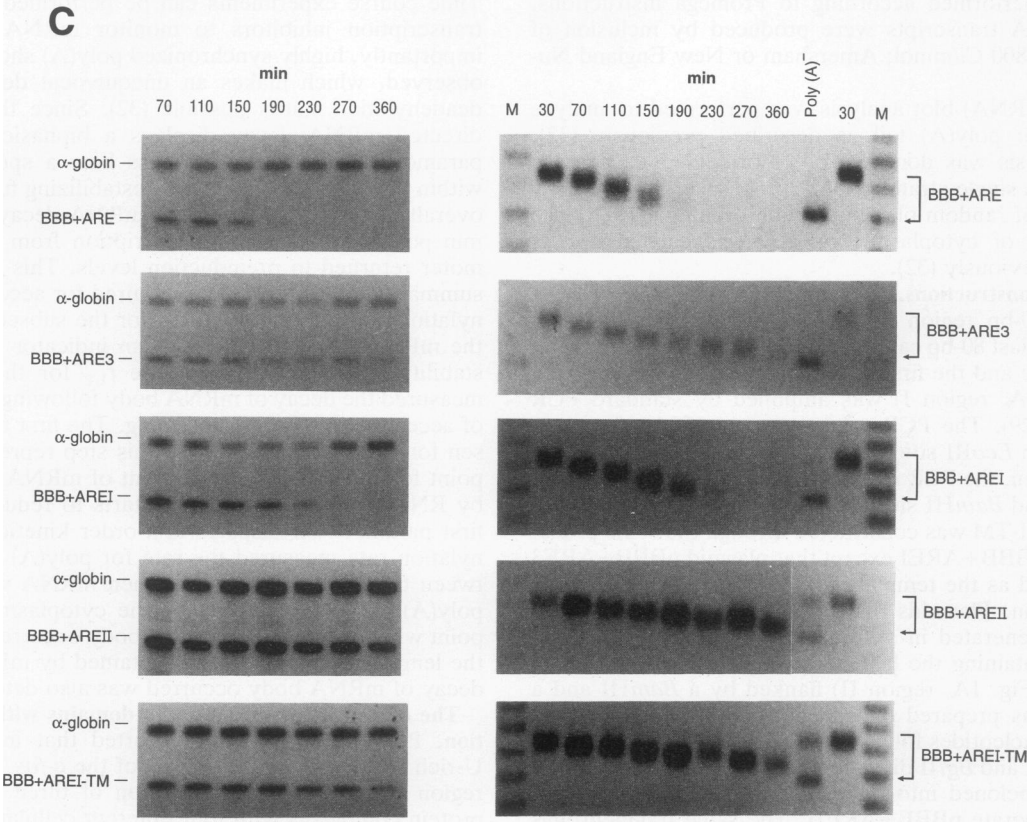
Experimental approach. To elucidate critical structural features of the c-*fos* ARE that are responsible for directing different steps of the ARE-mediated mRNA decay, our approach has been to introduce mutant ARE derivatives into the 3′ UTR of the stable β-globin mRNA (BBB mRNA [33]), whose transcription is driven by the serum-inducible c-*fos* promoter. The mRNA decay rate and the poly(A) shortening status, including the rate and extent of deadenylation of β-globin mRNAs carrying various mutant AREs, were then determined by RNase protection and Northern blot analyses (32). The use of the c-*fos* promoter to drive transcription allows transient synthesis of mRNA to be induced by treatment of transfected NIH 3T3 cells with serum (14). Time course experiments can be performed without using transcription inhibitors to monitor mRNA decay. More importantly, highly synchronized poly(A) shortening can be observed, which makes an unequivocal determination of deadenylation status possible (32). Since the c-*fos* ARE-directed mRNA decay displays a biphasic pattern, four parameters were used to assess how a specific mutation within the c-*fos* ARE affect its destabilizing function. (i) The overall half-life ($t_{1/2}$) measured mRNA decay starting at 70 min postinduction when transcription from the c-*fos* promoter returned to preinduction levels. This parameter is a summation of both the time required for accelerated deadenylation and the time required for the subsequent decay of the mRNA body. It served as an indicator for the overall stability of an mRNA. (ii) The $t_{1/2}$ for the second step measured the decay of mRNA body following the first phase of accelerated poly(A) shortening. The first time point chosen for calculating the $t_{1/2}$ for this step represents the time point following which the amount of mRNA as determined by RNase protection analysis starts to reduce. Unlike the first parameter, it displays first-order kinetics. (iii) Deadenylation rate measured the rate for poly(A) shortening between the 30-min time point when mRNA with full-length poly(A) tails just appeared in the cytoplasm and the time point when the decay of mRNA body occurred. Finally, (iv) the length of poly(A) tail still retained by mRNA when the decay of mRNA body occurred was also determined.

The c-*fos* ARE consists of two domains with distinct function. Previously, we have reported that in vitro a 20-nt U-rich region within the 3′ part of the c-*fos* ARE (Fig. 1A, region II) supports the formation of three specific RNA-protein complexes with four different cellular proteins (38). Furthermore, this region can be distinguished structurally from the 5′ 49-nt region containing the three AUUUA motifs (Fig. 1A, region I). As an initial attempt to characterize the c-*fos* ARE, our first set of experiments were to test whether these two regions may represent two functionally distinct domains of the c-*fos* ARE.



B

mRNA	Overall $t_{1/2}$ (min)	$t_{1/2}$ for 2nd step (min)	Deadenylation rate (nt/min)	Poly (A) size (nt)[a] / time point
BBB+ARE	35 ± 8	21 ± 1	1.2	60 (110')
BBB+ARE3	198 ± 27	107 ± 7	0.79	38 (230')
BBB+ARE I	60 ± 26	20 ± 5	0.88	35 (190')
BBB+ARE II	378 ± 56	–[b]	0.33	23 (360')[c]
BBB+AREI-TM	320 ± 23	–[b]	0.31	56 (360')[c]



To test the destabilization ability of region I, region II was deleted from the c-*fos* ARE of the BBB+ARE mRNA (Fig. 1A, BBB+AREI RNA). As shown in Fig. 1, this alteration produced an approximately twofold stabilizing effect and increased the overall half-life of the BBB+ARE from ~35 min to ~60 min, indicating that region II enhances the destabilizing ability of region I. Nevertheless, the results show that region I alone is sufficient to function as an RNA

FIG. 1. The c-*fos* ARE consists of two structurally and functionally distinct domains. (A) The 69-nt human c-*fos* ARE sequence is shown on the top with AUUUA motifs underlined and domain I (I) and domain II (II) bracketed. Asterisks mark the three nucleotides that were mutated from U to A in the ARE3 mutant (32). Dashed lines indicate the portions of the ARE or the ARE3 which were introduced into the β-globin mRNA. (B) Summary of mRNA decay rates and deadenylation for the mutant AREs. Quantitation of data was obtained by scanning the radioactive gels or blots (shown in panel C) with a Betascope blot analyzer (the Betagen). (a) This column shows the size of the poly(A) tail that still remained in the corresponding mRNA when decay of the RNA body occurred at the time point given in parentheses. (b) Not detectable during the time course experiments. (c) 360 min was the last time point of the time course experiment. (C) NIH 3T3 cells were transiently cotransfected with the control plasmid pSVα1 (33) and one of the test plasmids. Total cytoplasmic mRNA was isolated at various time intervals after serum stimulation and analyzed by RNase protection (left panel) and Northern blotting (right panel). α-Globin mRNA serves as an internal standard for RNase protection experiments. The times given on the top correspond to minutes after serum stimulation. Poly(A)$^-$ RNA was prepared in vitro by treating RNA samples from 30-min time points with oligo(dT) and RNase H. M, molecular size standards.

destabilizer albeit less potently than the wild-type (wt) ARE. In contrast, when region I was removed from the ARE (Fig. 1A, BBB+AREII RNA), region II alone had no destabilizing effect at all (Fig. 1). The BBB+AREII hybrid message ($t_{1/2}$ > 6 h) was nearly as stable as wt β-globin mRNA ($t_{1/2}$ > 8 h [33]).

To further determine how region I alone may exert its destabilization function and in which step(s) region II may participate to enhance the destabilization function of region I, the poly(A) shortening status of the two mutant chimeras was examined (Fig. 1B and 1C). The poly(A) shortening of BBB+AREI message exhibited a somewhat reduced rate of 0.88 nt/min versus 1.2 nt/min of BBB+ARE mRNA (Fig. 1B), which resulted in an approximately 80-min temporal lag (from 110 min as in BBB+ARE mRNA to 190 min as in BBB+AREI mRNA) after transcriptional repression before onset of decay of the mRNA body (Fig. 1C). Remarkably, when the decay rate for the second step of the BBB+AREI RNA was determined, there was no apparent change of this decay rate with respect to that of the BBB+ARE RNA (Fig. 1B). The results clearly show that deletion of region II from the c-*fos* ARE affects only the first step, i.e., accelerated poly(A) shortening. In contrast, the BBB+AREII mRNA underwent slow and highly synchronized poly(A) shortening with a rate (0.33 nt/min) even slightly slower than that of wt β-globin mRNA (0.47 nt/min; [32]). When the process of deadenylation stopped at 360 min after serum induction, no apparent decay of the RNA body was detected. Together, these data indicate that region I is involved in both steps of c-*fos* ARE-mediated decay pathway, whereas region II only participates in the first step.

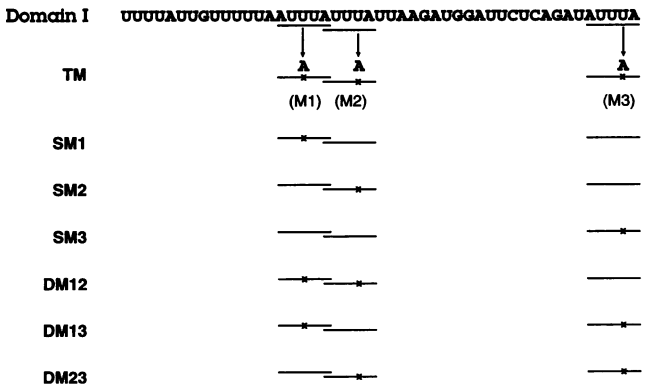


FIG. 2. Sequence and schematic diagram of the c-*fos* AREI showing the point mutations in the AUUUA motifs. The three AUUUA motifs are underlined. Asterisks mark the nucleotides that were mutated from U to A. SM, single point mutation; DM, double point mutation; TM, triple point mutation.

To substantiate the above conclusion, we further examined how deletion of region II from the mutant ARE of BBB+ARE3 mRNA (to generate BBB+AREI-TM RNA, Fig. 1A) might affect the poly(A) shortening of this hybrid message. As described above, ARE3 is a triple point mutation which has only slight effect on the rate of deadenylation but significantly impedes the decay of RNA body (Fig. 1) (32). As shown in Fig. 1 (B and C), deletion of region II from ARE3 dramatically reduced the poly(A) shortening rate of BBB+ARE3 from 0.79 nt/min down to 0.31 nt/min. The decay of BBB+AREI-TM RNA was very much like that of wt β-globin mRNA. We conclude that the two regions of c-*fos* ARE represent two distinct domains, domain I and domain II, that are required to specify the full destabilizing effect of the c-*fos* ARE. Domain I by itself can serve as a destabilizing element but is somewhat less potent than the intact ARE, whereas domain II cannot and, instead, it exerts its destabilizing function by enhancing the deadenylation process mediated by domain I.

Each of the three AUUUA motifs within the c-*fos* ARE is functionally equivalent and together they give an additive effect. Having defined two domains with distinct function within the c-*fos* ARE, we then further characterized the unique structural features of domain I: the AUUUA motifs and the general AU richness. To determine how each individual AUUUA motif may contribute to the destabilization function of domain I, various point mutations affecting AUUUA motifs were created by recombinant PCR mutagenesis (15) using BBB+AREI as a template (Fig. 2). Effects of these mutations on the destabilizing function of domain I (AREI) were then determined. As summarized in Table 1, all the single point mutations displayed a very similar decay-impeding effect. Each of them caused an increase in the half-life of BBB+AREI by approximately twofold. Similarly, each of the three double point mutations also had a stabilizing effect analogous to yet more profound than that of the single point mutations. They caused a ~fourfold increase in the half-life of BBB+AREI mRNA. Furthermore, the triple point mutation caused an increase in the half-life of BBB+AREI by more than fivefold. These data indicate that the three AUUUA motifs are functionally equivalent in terms of their RNA destabilization.

When the poly(A) shortening status was examined (Table 1), the single point mutations had little effect on the deadenylation rate while the double point mutations caused a modest reduction in deadenylation rate. Triple point mutation essentially abolished the ability of AREI to direct rapid deadenylation. When the decay rates of the second step for these messages were determined, it is clear that these AUUUA mutations had a major effect on the second step. All single mutations caused a similar three- to fourfold stabilizing effect, whereas double mutations gave a four- to

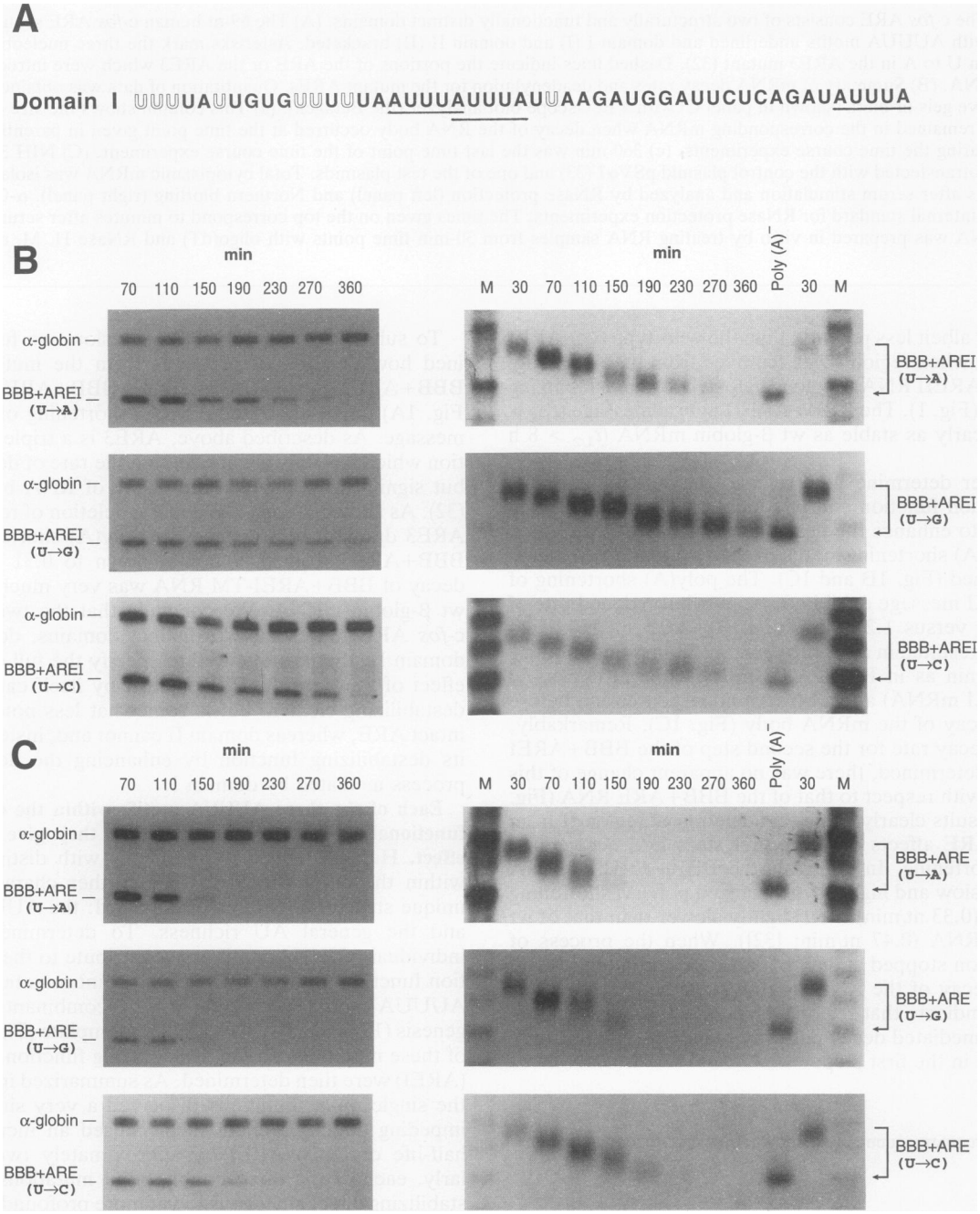


FIG. 3. Decay-impeding effect of mutations altering the general AU richness of domain I can be rescued by domain II. (A) The 49-nt domain I sequence of c-*fos* ARE. The 8 U residues that were changed to A, G, or C residues are shown with outlined characters. The AUUUA motifs are underlined. (B) Effect of AU richness alterations on the destabilizing function of AREI. (C) Effect of AU richness alterations on the destabilizing function of ARE. RNA isolation and time course experiments were carried out as described in the legend to Fig. 1. Left panel, results of RNase protection analyses. Right panel, results of Northern blot analyses. The times given on the top correspond to minutes after serum stimulation. α-Globin mRNA serves as an internal standard for RNase protection experiments. Poly(A)$^-$ RNA was prepared in vitro by treating RNA samples from 30-min time points with oligo(dT) and RNase H. M, molecular size standards.

fivefold decay-impeding effect. The most severe effect was caused by the triple point mutation, in which no apparent decay was detected for the second step. These results indicate that AUUUA motifs play multiple roles and are involved in both steps of domain I-mediated decay pathway. More importantly, the three AUUUA motifs appears to work in an independent manner to give an additive effect on mRNA destabilization.

The AU richness of domain I is required to maintain its overall destabilizing function exerted through AUUUA motifs. The lack of sequence homology and the presence of a high general AU content among different AREs led us to characterize this second unique feature of domain I. Two questions were addressed: how important the AU richness is in determining destabilizing function of domain I, and if it is important, which step(s) this property is required for. To answer

TABLE 1. mRNA decay rates and deadenylation for the AUUUA mutants

Mutation[a]	$t_{1/2}$ (min)		Deadenylation rate (nt/min)	Poly(A) size (nt) (time point)[b]
	Overall	For 2nd step		
AREI				
wt	60 ± 26	20 ± 5	0.88	35 (190)
SM1	123 ± 26	72 ± 9	0.86	40 (190)
SM2	103 ± 23	59 ± 10	0.83	50 (190)
SM3	121 ± 20	76 ± 5	0.79	50 (190)
DM12	239 ± 47	97 ± 0	0.49	64 (270)
DM13	212 ± 62	86 ± 9	0.59	45 (230)
DM23	205 ± 42	104 ± 20	0.58	40 (230)
TM[c]	320 ± 23	—[d]	0.31	56 (360)[e]
ARE				
wt	35 ± 8	21 ± 1	1.2	60 (110)
SM1	38 ± 9	29 ± 5	1.0	56 (150)
SM2	44 ± 6	33 ± 2	1.0	33 (150)
DM13	77 ± 8	61 ± 4	0.83	32 (190)
DM23	79 ± 12	58 ± 7	0.88	44 (190)
TM[c]	198 ± 27	107 ± 7	0.79	38 (230)

[a] SM, single point mutation; DM, double point mutation; TM, triple point mutation.
[b] Number in parentheses refers to the time point (in minutes) when decay of the mRNA body occurred. The size of the poly(A) tail at this time point is given in the same column.
[c] ARE-TM is equivalent to ARE3.
[d] —, not detectable during the time course experiment.
[e] 360 was the last time point of the time course experiment.

these questions, we randomly picked eight U residues within AREI (Fig. 3A) and changed them to A residues [Fig. 3B, BBB+AREI(U→A) RNA]. Notably, this manipulation significantly changes both the A to U ratio from 0.46 to 1.55 and the exact AU sequence of AREI but maintains the AU% and the three AUUUA motifs as well as their relative spacing. Remarkably, β-globin mRNA containing this mutant decayed slightly faster than the BBB+AREI RNA did (Fig. 3B and Table 2). This result suggests two possibilities. First, a general AU richness without a specific sequence context is sufficient for the three AUUUA motifs within domain I to exert their destabilizing function. Alternatively, the context in which AUUUA motifs reside is not necessary to be AU rich.

To address these possibilities, the same set of 8 uridylate residues were then changed either to G or to C residues [Fig. 3B, BBB+AREI(U→G) or BBB+AREI(U→C) RNA]. Both alterations changed the AU% from 85% down to 67.3% as well as the sequence identity of domain I. As shown in Fig. 3B and summarized in Table 2, U to G and U to C changes had nearly 3-fold and 1.6-fold decay-impeding effects, respectively. Thus, these data indicate that the general AU richness is required by domain I for its destabilizing function.

TABLE 2. mRNA decay rates and deadenylation for the AU richness mutants

Mutations	$t_{1/2}$ (min)		Deadenylation rate (nt/min)	Poly(A) size (nt) (time point)[a]
	Overall	For 2nd step		
AREI				
wt	60 ± 26	20 ± 5	0.88	35 (190)
(U→A)	48 ± 9	36 ± 7	1.1	54 (150)
(U→G)	182 ± 32	104 ± 20	0.62	42 (230)
(U→C)	97 ± 13	73 ± 12	0.78	44 (190)
ARE				
wt	35 ± 8	21 ± 1	1.2	60 (110)
(U→A)	21 ± 5	21 ± 5	1.9	45 (70)
(U→G)	24 ± 7	19 ± 3	1.7	32 (110)
(U→C)	43 ± 11	24 ± 1	1.4	25 (150)

[a] Number in parentheses refers to the time point (in minutes) when decay of the mRNA body occurred. The size of the poly(A) tail at this time point is given in the same column.

To further address the role of AU richness in determining the destabilizing function of domain I, the poly(A) shortening status of these three mutant transcripts were examined and the decay rates for the second step were then determined. As shown in Fig. 3B and summarized in Table 2, all three alterations exerted a more severe effect on the second step than on the first step. Even the U to A change that appeared to result in somewhat faster decay of the corresponding message showed a ~1.8-fold reduction of the decay rate for the second step. Its poly(A) shortening rate, however, showed a moderate increase from 0.88 nt/min to 1.1 nt/min, which accelerated the first step and may counteract the slight impeding effect on the second step. This may explain the somewhat faster overall decay of the BBB+AREI(U→A) mRNA than of BBB+AREI mRNA. The decay rate for the U to G or U to C change reduced approximately 5-fold or 3.5-fold, respectively, while there was only a slight change in the deadenylation rate. Therefore, we conclude that the general AU richness of domain I appears to be necessary for maintaining its overall destabilizing function, whereas the U richness is specifically required by domain I to direct the second step of mRNA decay.

Domain II has a novel property of buffering against the effects of mutations within domain I. Since domain II can enhance the mRNA destabilizing ability of domain I (Fig. 1), we then tested how the decay-impeding effects of those AREI mutations described above may be affected by domain II. Thus, various mutations affecting domain I as described above were also introduced into the intact c-*fos* ARE. These manipulations were essentially equivalent to additions of domain II immediately downstream of the AREI mutations. As shown in Table 1, additions of domain II had a general destabilizing effect (ca. two- to threefold) on the BBB+

AREI mRNAs carrying a single point mutation. Decay of BBB+ARE-SM mRNAs proceeded with a rate which was analogous to that of mRNA carrying wt ARE. Similarly, adding domain II back to the mutants carrying double point mutations also caused a general ca. threefold destabilizing effect. Finally, addition of domain II back to AREI-TM also gave a 1.6-fold destabilizing effect. Therefore, in the presence of domain II all the point mutations showed a diminished ability to impede the ARE destabilization function.

To find out on which step(s) domain II may exert its stimulating effect, the poly(A) shortening status of these mutants and their decay rates for the second step were determined. As summarized in Table 1, upon the addition of domain II, all the AUUUA point mutants displayed an accelerated poly(A) shortening rate. Moreover, an accelerated decay rate for the second step were detected for all the mutant mRNAs as well. Therefore, we conclude that domain II enhances decay of AREI mutant messages by accelerating rates for both steps of the AREI-directed decay.

In previous sections, we have shown that changes of AU-rich environment of domain I affected its destabilization function (Fig. 3B). To test whether domain II may have a general buffering capacity to rescue the effects caused by these mutations as well, decay of these mutant mRNAs in the presence of domain II [Fig. 3C, ARE(U→A), ARE (U→G), or ARE(U→C)] were also examined. Remarkably, domain II was also able to enhance the destabilizing function of all three mutant AREI as demonstrated by a two- to eightfold reduction of the overall half-lives (Fig. 3C and Table 2). Interestingly, we noticed that both BBB+ARE (U→A) and (U→G) mRNAs decayed with a rate that was faster than that of the BBB+ARE mRNA (Table 2).

To learn more about how this dramatic decay-enhancing effect by domain II may be achieved, we further examined the poly(A) shortening status of the three mutants and determined their decay rates for the second step (Fig. 3C and Table 2). In all three cases, we observed a significant increase in both the poly(A) shortening rate and the decay rate for the second step. Both rates were either similar to or faster than those of BBB+ARE mRNA and were correlated well with the increased overall half-life caused by domain II. Together, the data led to a novel finding that domain II has a general buffering capability to rescue several different kinds of mutations within domain I by facilitating both steps of the AREI-mediated mRNA decay.

Domain II can function when placed upstream of domain I but not when the sequence encoding domain II is inverted. With the finding that interaction between domain I and domain II determines the final destabilizing ability of the c-*fos* ARE, we then tested whether these two domains can be interchanged or whether the sequence encoding domain II can be inverted without losing its enhancing ability. As shown in Fig. 4, placing domain II immediately upstream of domain I [ARE(II/I)] had no effect on its destabilization function at all ($t_{1/2}$ = ~33 min). In contrast, inversion of the sequence encoding domain II [ARE(IIR)] which changed its extraordinary U richness into A richness had a significant decay-impeding effect. The BBB+ARE(IIR) message decayed with a half-life of ~123 min (Fig. 4) which was even more stable than the message without domain II (Fig. 1, BBB+AREI, $t_{1/2}$ = ~60 min).

Further analyses of poly(A) shortening status of these messages (Fig. 4B) showed that neither poly(A) shortening status nor subsequent decay of the RNA body was affected when domain I and domain II were interchanged. Conversely, in the case of the mutant message with the sequence encoding domain II inverted, both the first and second steps of ARE-directed decay were affected. The rate of poly(A) shortening was slightly reduced from 1.2 nt/min to 0.81 nt/min, a rate which was similar to that of BBB+AREI mRNA. The subsequent decay of poly(A)-tail-shortened RNA proceeded with a much slower rate ($t_{1/2}$ = ~86 min) than that of the mRNA carrying wt ARE ($t_{1/2}$ = ~21 min). Therefore, inversion of the sequence encoding domain II not only caused a loss of its deadenylation-enhancing ability but also led to a gain of a decay-impeding effect.

DISCUSSION

In this study, we report a detailed description of the critical structural features necessary for the destabilizing function of the c-*fos* ARE and several novel findings that have important implications for furthering our understanding of decay mechanisms mediated by different AREs. Combining extensive mutagenesis of the c-*fos* ARE with an in vivo analysis, we have identified and characterized several types of mutations that exhibit kinetic phenotypes characteristic of the biphasic decay pattern for a two-step mechanism; namely, prior to the onset of degradation of mRNA body, there is a lag during which the poly(A) tail is shortened to a length of 30 to 60 nt. Mutations altering the rate of poly(A) shortening lead to a corresponding change in this temporal lag. Our results show that mutations affecting either the poly(A) shortening rate or the decay rate or mRNA body or both result in a change of the overall half-life of the corresponding mRNA, indicating the feasibility of using the overall half-life to describe the the ultimate stability of an ARE-carrying mRNA.

Furthermore, we show that the c-*fos* ARE is composed of two functionally and structurally separable regions, which we termed domain I (or AREI) and domain II (or AREII). Domain I contains features that form the basic unit for the ARE to function as an RNA destabilizing element. These include the AUUUA motifs and the high general AU content. Conversely, domain II by itself is unable to confer instability to a stable message. However, it serves two important roles in determining the destabilizing function of the c-*fos* ARE. Its presence can enhance the destabilizing effect of domain I by accelerating the deadenylation process. Deletion of domain II from BBB+ARE does not affect the decay rate of the second step (Fig. 1), indicating that in the wt ARE domain II is involved only in the first step of mRNA decay pathway. However, when comparing the stabilizing effect of domain I mutations in the presence or absence of domain II, it is striking to discover that domain II can stimulate both the first and the second steps of the mutant domain I-mediated mRNA decay. Therefore, domain II has one additional role of buffering decay-impeding effects caused by mutations introduced within domain I. Together, these observations suggest a simple model for building a decay apparatus at the c-*fos* ARE. Domain I may carry essential sequence features, such as AUUUA motifs and AU richness, necessary for assembly of a general decay apparatus, which contains auxiliary decay factors and basic machineries for deadenylation and decay of the RNA body. Domain II may facilitate basic machineries and decay factors to make initial contacts that start the assembly of the decay apparatus at domain I. A recent study by Savant-Bhonsale and Cleveland (30) lends a support for the presence of the hypothesized decay apparatus. They identify a >20S complex that specifically forms with messages carrying the GM-CSF ARE and propose that formation of the >20S

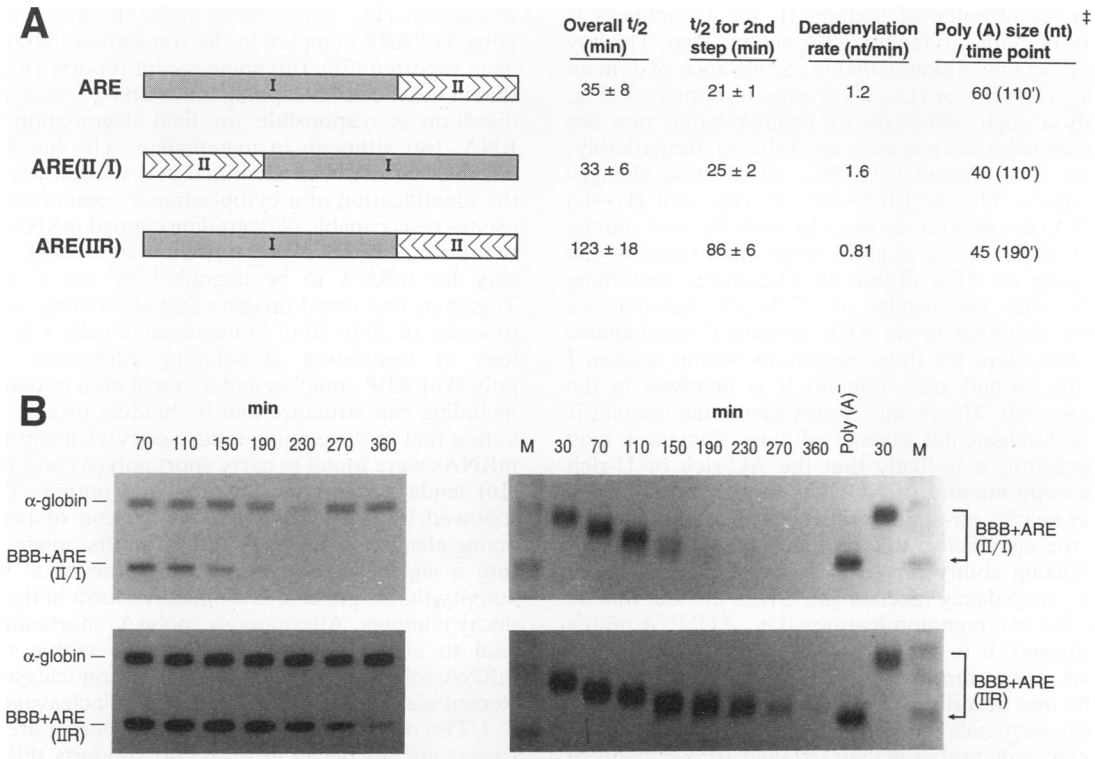


FIG. 4. Domain II can function when placed upstream of domain I but not when the sequence encoding domain II is inverted. (A) Left panel, schematic diagram showing the arrangements of domain I and domain II in the ARE(II/I) and ARE(IIR) mutants. Right panel, summary of mRNA decay rates and deadenylation for the mutant AREs. Quantitation of data was obtained by scanning the radioactive gels or blots (shown in panel B) with a Betascope blot analyzer (the Betagen). (‡) This column shows the size of the poly(A) tail that still remained in the corresponding mRNA when decay of the RNA body occurred at the time point given in parentheses. (B) RNA isolation and time course experiments were carried out as described in the legend to Fig. 1. Left panel, results of RNase protection analyses. Right panel, results of Northern blot analyses. The times given on the top correspond to minutes after serum stimulation. α-Globin mRNA serves as an internal standard for RNase protection experiments. Poly(A)$^-$ RNA was prepared in vitro by treating RNA samples from 30-min time points with oligo(dT) and RNase H. M, molecular size standards.

complex may be involved in the GM-CSF ARE-directed rapid mRNA decay. Recent findings that in yeast cells deadenylation is a common first requirement for decay of both labile and stable mRNAs (10) and that two regions within the 3′ UTR of yeast MFA2 mRNA specify its rapid decay (22) suggest that a similar mechanism is conserved from yeast cells to mammals. Moreover, our novel finding that domain II has a general buffering capacity in terms of rescuing mutations within domain I has an important implication that mammalian cells may have evolved a way of uniting the two domains such that a functional and more mutation-resistant ARE of c-*fos* mRNA can be maintained to ensure the rapid decay of c-*fos* mRNA.

The finding that domain II can retain its decay-enhancing ability when placed upstream of domain I (Fig. 4) further augments our definition of AREI and AREII regions as two functionally distinct and separable domains. Our observation that domain II cannot exert its function when the sequence encoding domain II is inverted (Fig. 4) suggests that accelerated assembly of the hypothesized decay apparatus at domain I may involve recognition of U richness of domain II. Previously, we have identified four U-rich sequence binding proteins (URBPs) that specifically interact with domain II and have proposed that the binding may be involved in the first step of the c-*fos* ARE-mediated decay pathway (38). Notably, the AREII-URBP interactions can be abolished by addition of poly(U) ribohomopolymers but not by poly(A), poly(G), or poly(C) (38). Since the inversion changes the extraordinary U richness of domain II into A richness, these observations together suggest that loss of the deadenylation enhancing ability of domain II by inversion may be due to the loss of the deadenylation enhancing ability of domain II by inversion may be due to the loss of specific domain II-URBP interactions. Moreover, when the sequence encoding domain II is inverted, it may become recognized by poly(A) binding proteins (PABPs), which in turn might interfere with the assembly of a decay apparatus at domain I in a way that the second step is significantly slowed down.

Through the investigations of 12 different AUUUA mutations, we were able to identify a mechanistic role of the AUUUA motifs. Our data show that these motifs are involved in both steps of the c-*fos* ARE-mediated decay pathway (Table 1). However, it is worth noting that all of these mutations display a more profound stabilizing effect on the second step than on the first step. Each of the three AUUUA motifs appears to play an equivalent role in determining the ARE destabilizing ability since regardless which AUUUA motifs are mutated, a similar effect is always observed for any single point mutations or for any double point mutations. In addition, there is an additive effect of these AUUUA motifs and the three AUUUA motifs together give the most significant destabilizing effect.

The general U richness of domain I displays an interesting

property. In the absence of domain II, the U richness is necessary for domain I to facilitate the second step. The key result supporting this notion is that in the absence of domain II, a (U→A), (U→G), or (U→C) change of domain I (Fig. 3B) has only a slight effect on its deadenylation rate but significantly impedes the second step (Table 2). Remarkably, when domain II is present (Fig. 3C), all of these changes have little effect. The ARE(U→A), (U→G), and (U→C) mutant mRNAs decay with the rates for both the first and the second steps similar to or slightly faster than those of the mRNA carrying wt ARE (Table 2). Therefore, combining these results with the results of AUUUA mutants, we conclude that although in wt ARE domain II participates only in the first step, for those mutations within domain I that affect the second step, domain II is involved in the second step as well. The results clearly show that domain II can cause a fundamental change of how domain I may behave. Therefore, it is likely that the AU-rich or U-rich content, the copy number of AUUUA motif, and the presence of other nearby *cis*-acting sequences all are involved in elaborating the specificity, differential regulation, and ultimate destabilizing ability for ARE to mediate RNA decay through a two-step decay mechanism. Given the fact that all AREs share the two common features (i.e., AUUUA motifs and AU richness), it is likely that different AREs may all target mRNA decay through a two-step decay mechanism similar to the one described for the c-*fos* ARE even though no significant sequence homologies can be found among them. However, differences in their detailed arrangements of some common sequence features, such as the AUUUA motifs, general AU or U richness, and the presence or absence of oligo(U) stretches, may be required by mammalian cells to achieve differential regulation among different AREs in response to cell differentiation, cell growth, and cell type-specific gene expression (see below).

Nearly all the investigated mRNAs still carry a poly(A) tail with a size ranging from approximately 30 to 60 nt when decay of the transcribed portion starts. Some labile messages such as BBB+ARE, BBB+ARE(U→A), and BBB+ARE(U→G) mRNAs still undergo poly(A) shortening even when the second step commences (Fig. 1 and 3). Moreover, no decay intermediates in a form of poly(A)$^-$ RNA are detected even in the case of mutations that significantly slow down the second step. These observations favor the notion that it may not be necessary to undergo a complete removal of the poly(A) tail before the body of the RNA can be degraded. This is consistent with a recent report by Decker and Parker (10) showing that in yeast cells poly(A) shortening leads to either internal cleavage or decapping followed by 5′ to 3′ exonuclease digestion of certain stable and labile mRNAs. Identification of decay intermediates that are derived from the second step and still retain oligo(A) tails leads these authors to conclude that complete removal of the poly(A) tail is not necessary for the mRNA body to be triggered for rapid degradation.

An interesting question then arises as to why the poly(A) tail needs to be shortened to a size around 30 to 60 nt before the onset of RNA decay. The size is apparently long enough to bind one or two PABPs. For discussion purposes, we propose one possibility that requires 5′ exonuclease attack as a necessary step and also takes into account the close link of the poly(A) tail with translation (for reviews, see references 18, 23, 26, and 27). The key observations supporting the two considerations are the following: (i) it has been shown that in mammalian cells the 3′ end poly(A) tail and the 5′ cap structure work in a synergistic way to enhance translation (13); (ii) in yeast cells, the participation of the poly(A)-PABP complex in the translation initiation has also been reported (28); (iii) some recent reports (10, 17) suggest that in yeast cells decapping followed by 5′ to 3′ exonuclease digestion is responsible for final degradation of oligo(A) RNA; (iv) although in mammalian cells the 5′ decapping enzyme has not been identified yet, a recent report showing the identification of a cytoplasmic 5′ exonuclease in mouse ascites cells capable of degrading capped mRNA (9) suggests that prior removal of cap structure from RNA is not necessary for mRNA to be degraded by the 5′ exonuclease. Together, one could imagine that shortening of poly(A) tail to a size of 30 to 60 nt in mammalian cells might result in a loss of translation stimulating interaction between 3′ poly(A)-PABP complex and 5′ translation initiation complex including cap structure and its binding protein. The observation that in developing mouse oocytes many untranslated mRNAs were found to carry short poly(A) tails of 30 to 50 nt (16) lends support to the proposed notion. This change, followed by the subsequent recognition of necessary *cis*-acting elements such as AUUUA motifs, might then constitute a signal for activation or release of 5′ exonuclease activity that is present as an inactive form in the translation/decay complex. Alternatively, poly(A) shortening could also lead to an endonucleolytic cleavage within the body of mRNA followed by both 5′ and 3′ exonuclease digestions. Recent identification of endonucleolytic cleavage sites in the 3′ UTRs of several eukaryotic mRNAs that are believed to trigger mRNA decay (2, 3, 6, 34) supports this possibility, though deadenylation is not a prerequisite in these cases. However, it is important that Koeller et al. (20) showed that when the c-*fos* ARE was inserted into the 3′ UTR of transferrin receptor mRNA to replace its original RNA destabilizer, the destabilizing function of c-*fos* ARE was not affected by inhibition of ongoing translation, suggesting translation coupling may not be necessary for the ARE to direct rapid mRNA decay. Nevertheless, given the intriguing way that the mutation-resistant destabilizing function of the c-*fos* ARE is determined by its distinct structure features, it seems possible that the c-*fos* ARE has the ability to mediate rapid mRNA decay through more than one pathway which have different requirements for translation coupling. If this is the case, it will serve as a fail-safe mechanism to ensure complete removal of the "dangerous" c-*fos* mRNA from cytoplasm when the c-*fos* mRNA is no longer needed.

Finally, we would like to propose a model to summarize how the critical structural features described and characterized in this report are involved in the c-*fos* ARE-directed mRNA decay pathway. As shown on the left of Fig. 5, rapid shortening of RNA poly(A) tail to a size of ~30 to 60 nt (step I) precedes decay of mRNA body (step II). Both domain II and the three AUUUA motifs within domain I are necessary for the ARE to achieve a high rate for the first step. Triggering and subsequent completion of the second step requires the three AUUUA motifs. Although domain I alone can also function as a potent RNA destabilizing element, this AREI-directed pathway (Fig. 5, right) exhibits characteristics somewhat different from those of the ARE-directed pathway. The three AUUUA motifs and the AU richness are required for domain I to direct accelerated deadenylation. The deadenylation rate when compared with that of the ARE-directed pathway is slightly reduced because of the absence of domain II. Therefore, domain II can enhance the deadenylation function of AUUUA motifs. For the second step of AREI-directed mRNA decay pathway, both the three AUUUA motifs and the high general U richness of domain I

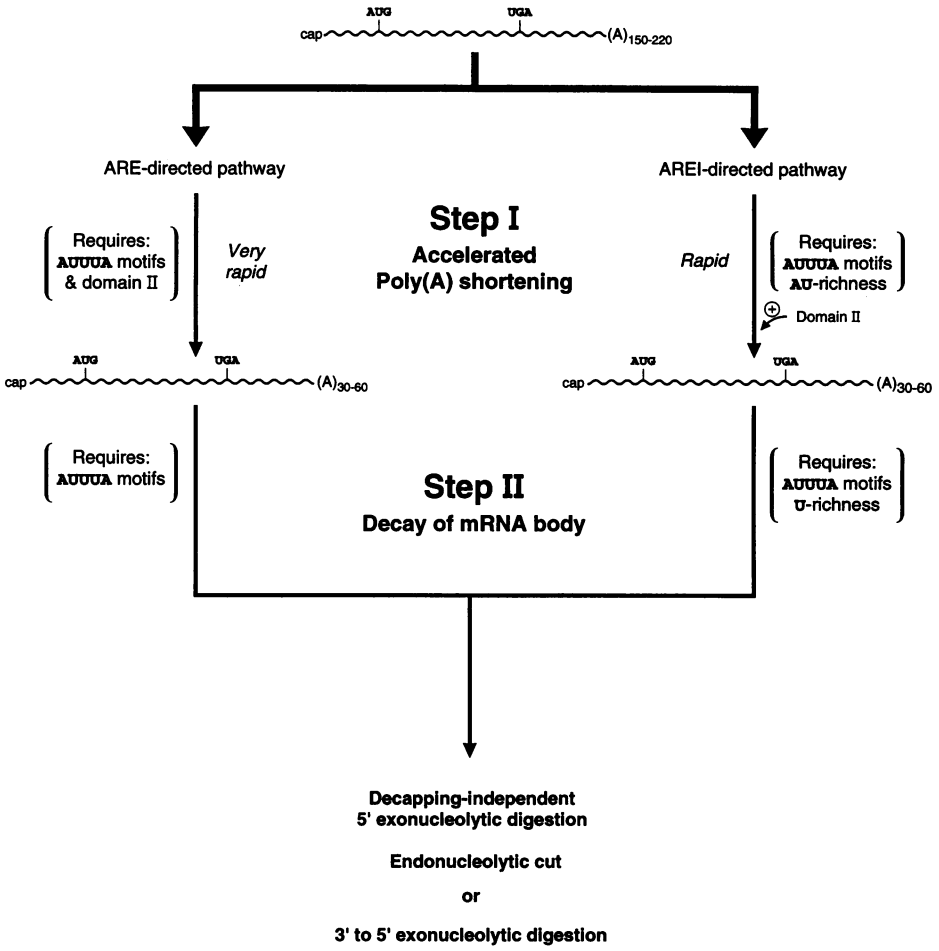


FIG. 5. Two-step model for mRNA decay directed by the c-*fos* ARE or AREI. In this model, accelerated shortening of mRNA poly(A) tail to a size of ~30 to 60 nt (step I) is followed by decay of mRNA body (step II). See the text for details.

are required. As discussed above, we favor that recognition of these structural features will then lead to degradation of the RNA body either by an decapping-independent 5′ exonuclease or by an endonucleolytic cleavage followed by exonuclease digestion. However, it is still a formal possibility that accelerated deadenylation might be able to trigger 3′ to 5′ exonucleolytic digestion of the RNA body as well.

One important implication of our model is that each different ARE has its own distinct identity and is presented as a unique composition of these structural features, which may gradually develop through evolution to suit the special function of the corresponding mRNA. For example, unlike the c-*fos* ARE, the GM-CSF ARE has no long stretch of U sequence and contains highly reiterated motifs of AUUUA (31). Recently, three seemingly discrepant reports show that disturbance of the ongoing translation has an opposite effect on the destabilizing function between the c-*fos* ARE and GM-CSF ARE (1, 20, 30). We propose that the observed different effects might be derived from the structurally critical differences between the two AREs. Thus, the two steps proposed in our model may constitute a common yet primary pathway for ARE-directed mRNA degradation in general, whose activity can be modulated by *cis*-acting elements through their interaction with the cognate regulatory factors that are distinct from the essential RNA-degrading enzymes. Depending on the availability of *trans*-acting factors, the cell physiological conditions, and cell growth and differentiation states, individual ARE may fulfill its diversified roles in the regulation of gene expression through mRNA decay and translation.

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