AUUUA Is Not Sufficient To Promote Poly(A) Shortening and Degradation of an mRNA: the Functional Sequence within AU-Rich Elements May Be UUAUUUA(U/A)(U/A)

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AU-rich elements (AREs) in the 3' untranslated regions of several cytokine and oncogene mRNAs have been shown to function as signals for rapid mRNA degradation, and it is assumed that the many other cytokine and oncogene mRNAs that contain AU-rich sequences in the 3' untranslated region are similarly targeted for rapid turnover. We have used a chimeric gene composed mostly of growth hormone sequences with expression driven by the c-*fos* promoter to investigate the minimal sequence required to act as a functional destabilizing element and to monitor the effect of these sequences on early steps in the degradation pathway. We find that neither AUUUA, UAUUUA, nor AUUUAU can function as a destabilizing element. However, the sequence UAUUUAU, when present in three copies, is sufficient to destabilize a chimeric mRNA. We propose that this sequence functions by virtue of being a sufficient portion of the larger sequence, UUAUUUA(U/A)(U/A), that we propose forms the optimal binding site for a destabilizing factor. The destabilizing effect depends on the number of copies of this proposed binding site and their degree of mismatch in the first two and last two positions, with mismatches in the AUUUA sequence not being tolerated. We found a strict correlation between the effect of an ARE on degradation rate and the effect on the rate of poly(A) shortening, consistent with deadenylation being the first and rate-limiting step in degradation, and the step stimulated by destabilizing AREs. Deadenylation was observed to occur in at least two phases, with an oligo(A) intermediate transiently accumulating, consistent with the suggestion that the degradation processes may be similar in yeast and mammalian cells. AREs that are especially U rich and contain no UUAUUUA(U/A)(U/A) motifs failed to influence the degradation rate or the deadenylation rate, either when downstream of suboptimal destabilizing AREs or when alone.

The half-lives of mRNAs in eukaryotic cells vary widely, ranging from minutes to days, and it is now recognized that the control of mRNA degradation rate is an important level at which gene expression can be regulated (for recent reviews, see references 6 and 43). Many transiently expressed cellular growth regulators (for example, proto-oncogene products and cytokines) are encoded by messages with half-lives as short as 1 h or less. In addition, many cytokine mRNAs can be stabilized considerably, for example, in response to proinflammatory agents (4, 31). Such short but regulatable half-lives, in conjunction with changes in transcription rate, allow these growth factor mRNAs to be produced in a transient burst or reach a new steady-state level very rapidly.

One class of instability element, found in the 3' untranslated regions (3'UTRs) of many unstable cytokine and oncogene mRNAs, is the AU-rich element (ARE). This is a loosely defined sequence, with the general feature of being U rich with interspersed A's. A regulatory role for AREs was first postulated by Caput et al. (15), who identified a consensus sequence, UUAUUUAU, in a number of cytokine and oncogene mRNA sequences available at that time, although the function of this sequence was not tested. The observation of oncogenic forms of *fos* and *myc* with the region containing the ARE deleted from the gene (1, 26, 37) further suggested a role for these

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sequences in the control of mRNA stability. This hypothesis was tested by Shaw and Kamen (45), who showed that the 51-nucleotide (nt) ARE from human granulocyte-macrophage colony-stimulating factor mRNA could destabilize the normally stable rabbit β-globin mRNA when inserted into its 3'UTR. This effect was ablated by disrupting the AUUUA motifs within the ARE, directly implicating this sequence in destabilization of mRNA. The AREs from c-*fos*, c-*myc*, beta interferon, and tissue factor have also been shown to be destabilizing (3, 29, 48, 52). However, as AREs vary considerably in size and sequence, the salient features of a functional ARE have not been obvious, and it cannot necessarily be assumed that the presence of an AU-rich region is sufficient to destabilize an mRNA. The motif AUUUA is present in the 3'UTRs of many unstable mRNAs, and the notion that it is a destabilizing sequence has permeated the literature. However, there is to date no direct evidence that this motif alone can function. Indeed, it is possible that the destabilizing function of an ARE is mediated not by the primary sequence but by a secondary structure, since the strings of U present in many AREs could conceivably interact with the poly(A) tail (45, 53). (Precedents for an involvement of 3' stem-loop structures in the regulation of mRNA stability are found in the transferrin receptor [16] and histone [40] mRNAs.) In addition, the minimal number of motifs required to destabilize an mRNA, whether additional copies have an additive effect, and the effect of the variable spacing observed between individual AU-rich sequences are all largely unknown. Since an understanding of these sequence requirements may contribute to determining the mechanism by which AREs enhance mRNA

degradation, we have used a model system to assess the destabilizing potential of various small, defined AU-rich sequences by inserting them into the 3'UTR of a stable mRNA.

A general model for the pathway of mRNA degradation which incorporates the results of recent studies in the yeast *Saccharomyces cerevisiae* (21, 36, 38) has been proposed (21, 38, 43). This model is based on the suggestions that mRNAs are protected from digestion by the binding of poly(A)-binding protein (PABP) to the poly(A) tail (7, 11, 12) and that the first and perhaps rate-limiting phase of degradation is a progressive shortening of the poly(A) tail (12, 47, 53). The yeast poly(A) nuclease requires PABP for its activity, and after a slow initial phase, deadenylation proceeds more rapidly and in a distributive manner until the tail reaches an oligo(A) form, which may be sufficient to still bind one molecule of PABP (36). The body of the mRNA can then either be degraded immediately or first be completely deadenylated, a process called terminal deadenylation (21). In both cases, the degradation of the body of the mRNA occurs very rapidly, as neither poly(A)⁻ mRNAs nor degradation intermediates are usually detectable (38). Both poly(A) shortening and decay of the oligo(A) species are more rapid in unstable yeast mRNAs (21, 38), with the first step being more processive (36). Degradation of the mRNA body was originally assumed to proceed exonucleolytically from the 3' end (28), although in *S. cerevisiae*, degradation intermediates lacking the 5' end (which must result from 5'-3' exonucleolytic degradation following 5' cap removal or endonucleolytic cleavage) have been recently identified by impeding the body degradation process (21, 51).

Degradation intermediates of several mammalian mRNAs have been observed, some of which may be products of either 3' exonucleolytic degradation or endonucleolytic cleavage (12, 32, 49) and others of which are clearly the result of endonucleolytic cleavage in the 3'UTR (9, 13). Various nucleases proposed to be involved in degradation, including a 5'-3' exonuclease (20), a sequence-specific endonuclease (13), and a nuclear poly(A) nuclease (5), have been purified. Mammalian mRNAs differ from yeast mRNAs in having considerably longer poly(A) tails, but at least for some mRNAs the process of degradation may be similar, and several studies suggest that the instability elements in yeasts and higher eukaryotes may target the same steps in a common degradation pathway (21, 38, 43). Studies of c-*fos* mRNA have suggested that deadenylation is the first step in degradation of this mRNA (53) and that the c-*fos* ARE can stimulate deadenylation of a heterologous mRNA (47). Tumor necrosis factor, c-*myc*, *gro*-α, and beta interferon mRNAs have also been shown to undergo deadenylation as the first stage in their degradation (12, 34, 35, 41, 49). The deadenylation rate decreases when these mRNAs are stabilized either by inhibiting translation (which has been shown to be necessary for degradation of ARE-containing mRNAs [2]) (34, 41, 49, 52, 53), deleting the ARE (48, 53), or inhibiting protein kinase C (35), and thus it has been suggested that AREs increase the degradation rate of an mRNA by increasing the rate of poly(A) shortening. On the other hand, experiments with the c-*fos* ARE have shown that degradation can be uncoupled from deadenylation and raised the possibility that different sequence motifs within the complex c-*fos* ARE may be responsible for the two phases of degradation (47).

We describe here the use of a chimeric gene to assess the effects of a number of simple AU-rich sequences on degradation of an otherwise stable mRNA. We find that some AU-rich sequences, including one which has four copies of the motif AUUUA and others which contain strings of U and could potentially form secondary structures with the poly(A) tail, have no destabilizing effect. We find that UAUUUAU can

function when present in three copies and propose that a larger sequence, UUAUUUA(U/A)(U/A), forms the optimal binding site for a degradation factor. Sequences which stimulate degradation have a corresponding stimulatory effect on the rate of poly(A) shortening, both increasing the processivity of the first phase of the deadenylation reaction and increasing the rate of degradation of the oligoadenylated RNA which transiently accumulates prior to body degradation.

MATERIALS AND METHODS

Cell culture and transfection. Mouse NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum (FCS; Gibco). Cells (2×10^6 in 0.8 ml) were transfected with 20 µg of plasmid (linearized with *Sal*I) by electroporation (Bio-Rad Gene Pulser, 275 V, 960 µF) and grown for 3 days, and then colonies were selected in 400 µg of G418 sulfate (Geneticin; Gibco) per ml. After 10 to 12 days, colonies were pooled, and the pools were maintained in the presence of 200 µg of G418 sulfate per ml. For analysis of mRNA decay, 0.75×10^6 to 1.0×10^6 cells were plated onto 9-cm-diameter dishes, grown for 48 h in DMEM–10% FCS, then washed three times in phosphate-buffered saline, and serum starved in DMEM–0.5% FCS for 48 h prior to stimulation with DMEM–15% FCS.

Plasmid constructions. Where appropriate, 5' or 3' DNA protruding ends were made blunt by treatment with the Klenow fragment of DNA polymerase I or T4 DNA polymerase, respectively. Plasmid pfGH was prepared in multiple steps as follows. Plasmid pRcCMV (Invitrogen) was digested with *Eco*RI and *Kpn*I, blunt ended, and ligated shut to generate plasmid pRcCMVΔ2. Plasmid pG4BGH was prepared by inserting a 241-bp *Pvu*II-*Bcl*I fragment of pRcCMV, containing the bovine growth hormone 3'UTR and polyadenylation signals, into the *Eco*RI site of pGEM4Z (Promega). Plasmid pG4HGHBGH was prepared by inserting a 660-bp *Nco*I-*Sma*I fragment from plasmid pHGH (kindly provided by A. Robins, Bresatec Pty. Ltd., Adelaide, South Australia, Australia), containing the coding region of human growth hormone, into the *Sma*I site of pG4BGH. Plasmid pCMVGH was prepared by insertion of a 1,081-bp *Pvu*I-*Bam*HI fragment from pG4HGHBGH (containing the human growth hormone coding region, bovine growth hormone polyadenylation sequence, and SP6 promoter) into the *Eco*RI site of RcCMVΔ2. Plasmids pCMVGH7 to pCMVGH15 were prepared by insertion of oligonucleotides encoding the various ARE sequences shown in Fig. 1 between the *Kpn*I and *Sac*I sites of plasmid pCMVGH. Plasmids pCMVGH33, pCMVGH44, and pCMVGH55 were prepared by double insertion of oligonucleotides into the *Sac*I site of plasmid pCMVGH to encode the appropriate ARE sequences as shown in Fig. 1. These plasmids were digested with *Nru*I and *Hin*dIII to delete the cytomegalovirus promoter, and a 700-bp *Eco*RI-*Hin*dIII fragment of plasmid pfos3CAT (kindly provided by H. Iba and D. Cohen), containing the chicken c-*fos* promoter, was inserted to yield plasmids pfGH7 to pfGH15, pfGH33, pfGH44, and pfGH55. Plasmid pfGH733 was prepared by ligating a 1.4-kb *Sac*I fragment of pfGH7 to a 4.4-kb *Kpn*I fragment of pfGH33. Plasmids pfGH833 and pfGH1033 were prepared by ligating a 3.5-kb *Sac*I-*Pvu*I fragment of plasmids pfGH8 and pfGH10, respectively, to a 3.6-kb *Kpn*I-*Pvu*I fragment of plasmid fGH33. All inserted ARE sequences were confirmed by dideoxynucleotide sequencing.

Plasmid pGAPM, containing a portion of the coding region of the human *GAPDH* cDNA, was prepared by inserting a 400-bp *Sac*II-*Hin*dIII fragment of plasmid pHcGAP (ATCC
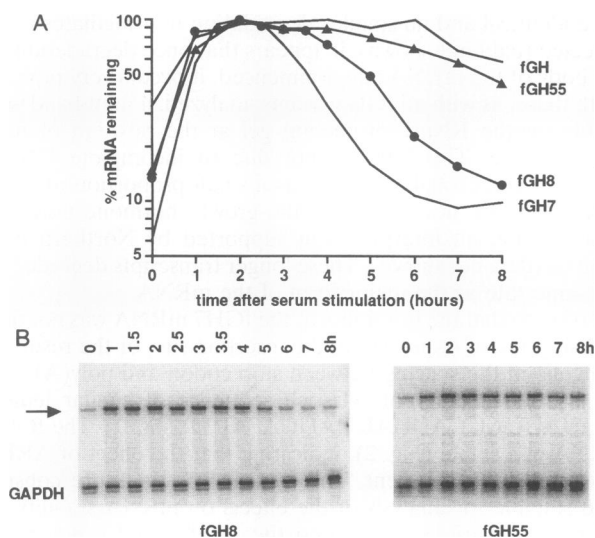
FIG. 1. The fGH gene and sequences of the various AREs inserted in the 3′UTR. (A) Schematic representation of the fGH gene. Boxes indicate segments of the gene that were derived from the chicken c-fos gene (c-fos), human growth hormone cDNA (hGH), and bovine growth hormone cDNA (bGH); plasmid-derived sequences are indicated by a line. The translated region is indicated by shading. The transcription start site, site of insertion of AREs, and the polyadenylation site are indicated. Also shown are the relative location of the SP6 promoter, used to make 3′ probes for RNase protection assays, and the neomycin resistance gene. The regions protected by the 5′ and 3′ RNase protection probes are shown as zigzag lines. (B) Sequences of AREs inserted between the KpnI and SacI sites (ARE7 to ARE15) or into the SacI site (ARE33 to ARE55). AUUUA motifs are underlined, and sequences from restriction sites used to insert the AREs are shown in lowercase.

57090) between the HincII and HindIII sites of plasmid pGEM-1 (Promega).

Analysis of mRNA decay and deadenylation. Total RNA was isolated at various times after serum stimulation of NIH 3T3 cells by the guanidine thiocyanate procedure of Chomczynski and Sacchi (18). Transcripts derived from transfected DNA and cellular *GAPDH* were detected by RNase protection analysis using in vitro-synthesized complementary RNA probes. The various fGH (growth hormone chimeric gene; Fig. 1) mRNAs were detected by using probes synthesized from the appropriate pfGH plasmids digested with PvuII, using SP6 RNA polymerase and 200 Ci of [α-^{32}P]UTP per mmol (23). Depending on the size of the ARE inserted in the pfGH plasmid, the probes were complementary to 165 to 211 nt of the 3′ terminus of the mRNA. The *GAPDH* mRNA was detected by using a probe complementary to a 120-nt internal fragment of the mouse *GAPDH* mRNA, synthesized from DdeI-digested plasmid pGAPM, using T7 RNA polymerase and 100 Ci of [α-^{32}P]UTP per mmol. Poly(A)$^-$ mRNA was prepared by using oligo(dT) and RNase H as described previously (12).

RNase protection assays were performed essentially as described previously (23), using 10 μg of RNA and digestion with RNase A (10 μg/ml) at 0°C, and analyzed on 6% polyacrylamide–8 M urea gels. For analysis of poly(A) tail length, the digestion products were dissolved in 10 μl of loading dye (0.04% bromophenol blue, 0.04% xylene cyanol, 10% glycerol, 1× Tris-borate-EDTA) and electrophoresed on nondenaturing 6% polyacrylamide gels in 1× Tris-borate-EDTA. It was not possible to measure actual poly(A) tail lengths on these gels, as appropriate fGH constructs encoding

mRNAs with poly(A) tails of defined lengths were not available. Gels were run for appropriate times to ensure that the poly(A)$^-$ markers migrated to identical positions.

The amount of specific mRNA was quantitated by Phosphor-Imager analysis using Imagequant version 3.21 (Molecular Dynamics, Sunnyvale, Calif.) and normalized with respect to the *GAPDH* internal standard. Since the amount of *GAPDH* mRNA increased linearly for 8 h following serum stimulation, a regression line was fitted to the pooled *GAPDH* mRNA data and used to calculate the expected *GAPDH* mRNA level in each sample. Levels of fGH mRNA were normalized by multiplying by the ratio expected *GAPDH*/observed *GAPDH*. This correction avoids overestimating the instability of individual mRNAs and does not change the relative destabilizing effect of the various AREs.

RESULTS

A model system for studying the effects of AU-rich sequences on mRNA stability. To compare the effects on mRNA stability of various 3′UTR sequences, we constructed a chimeric gene (fGH; Fig. 1) containing the promoter and 5′UTR from c-fos, a translated region encoding human growth hormone, and a 3′UTR and downstream polyadenylation signals from bovine growth hormone. The *fos* promoter responds transiently to a shift from low to high serum in the culture medium, resulting in a pulse of transcription lasting less than 1 h, and has been used by others in studies of mRNA degradation (3, 17, 47, 48). This inducible promoter was used in preference to a constitutive promoter to allow mRNA degradation rates to be mea-

FIG. 2. Degradation of fGH mRNA is enhanced by insertion of ARE7. RNA was isolated at the times indicated following serum stimulation of NIH 3T3 cells stably expressing the fGH transcript with ARE7 (fGH7), ARE9 (fGH9), or no ARE (fGH) in the 3'UTR. (A) Time course of degradation. Specific mRNAs were quantitated by PhosphorImager analysis. (B) Phosphorimage of RNase protection gels. The fGH mRNA protection product is indicated by an arrow. The asterisk indicates the position of a larger transcript believed to result from usage of a downstream polyadenylation site.

sured without using transcription inhibitors, which have been shown in a number of situations to prolong the life of unstable mRNAs (3, 33, 46, 48, 52). A short synthetic sequence containing unique restriction sites was included within the 3'UTR to allow the insertion of various chemically synthesized oligonucleotides. For simplicity, we call each inserted sequence AREn, with insertion of AREn into fGH, generating fGHn. All sequences are presented in the RNA form. Polyclonal NIH 3T3 lines expressing the various chimeric genes were established, and the stability of the mRNAs was determined by using RNase protection analysis to quantitate the amount of specific transcript remaining at various times after induction of transcription.

The fGH transcript, which contains no AU-rich insert, decayed little during the 8 h of the time course (Fig. 2). This was expected since growth hormone mRNA, which makes up most of the transcript, has a half-life greater than 9 h (25, 39). To confirm that insertion of an ARE could destabilize the fGH mRNA, we inserted ARE7 (Fig. 1), containing the sequence AUUUAUUUAUUUAUUUAUUUA. This sequence is part of the granulocyte-macrophage colony-stimulating factor ARE that has been shown to destabilize β-globin mRNA when inserted into its 3'UTR (45). We used Northern (RNA) blot analysis to confirm that these and all other fGH variants used in this study express a single major mRNA species of the expected size (data not shown). As expected, the fGH7 mRNA was unstable; transcript levels were maximal between 1.5 and 2.5 h after induction with serum and declined rapidly thereafter (Fig. 2). Since the pulse of transcription lasts only 40 min (data not shown), there is a significant lag before the onset of degradation. We confirmed that the degradation rate of the 3'UTR measured in these experiments using the 3' RNase protection probe accurately reflected the degradation rate of the transcript by repeating the assays with a coding-region probe (Fig. 1). Since the rates obtained with the two probes

were identical and no smaller degradation intermediates were detected (data not shown), it appears that once degradation of the body of the mRNA has commenced, it occurs very rapidly. With these, as with all fGH variants analyzed, a faint band was visible on the RNase protection gel at the position of full-length probe. This band is not due to incomplete RNase digestion and probably represents a small proportion of transcripts that are not cleaved at the growth hormone polyadenylation site, an interpretation supported by Northern blot analysis (data not shown). These longer transcripts degraded at the same rate as the major form of the mRNA.

To verify that the instability of the fGH7 mRNA was not due to disruption of sequences at the insertion site, or the result of a change in the spacing between stop codon and poly(A) tail, we inserted a different AU-rich sequence of similar length (AUUAUUAUUAUUAUUAUUA, ARE9; Fig. 1). The fGH9 mRNA was stable (Fig. 2), indicating that the effect of ARE7 was sequence dependent. This system was therefore considered suitable for analysis of the effects on mRNA stability of insertion of various AREs into the 3'UTR and was further used to define the sequence requirements of such destabilizing elements.

Multiple isolated copies of AUUUA do not destabilize. The motif AUUUA is present in the 3'UTRs of many unstable cytokine mRNAs and is often assumed to be the sequence within the ARE that is responsible for targeting the mRNA for rapid degradation. To test whether the sequence AUUUA in any context can target an mRNA for degradation, we prepared ARE55, which contains four copies of AUUUA separated by three to nine bases (G<u>AUUUA</u>AAA<u>AUUUA</u>GAGCUGCA G<u>AUUUA</u>AAA<u>AUUUA</u>G), and ARE8, in which five copies of AUUUA lie adjacent to each other (CAUUUAAUUUAA UUUAAUUUAAUUUAC). The choice of sequence to separate the AUUUA motifs in ARE55 was arbitrary, in that it was not based on any naturally occurring sequences; the central region is largely derived from restriction sites used in the construction of the ARE, and the AAA sequences were chosen to maintain AU richness in the region without creating a sequence similar to ARE7.

Little degradation of fGH55 mRNA was observed during 8 h (Fig. 3), showing that an appropriate context is required for the sequence AUUUA to affect the stability of an mRNA. The fGH8 mRNA was unstable but did not degrade as rapidly as fGH7 mRNA (Fig. 3) even though both AREs have five copies of AUUUA, further indicating that the effect of an ARE on mRNA stability is not determined solely by the number of AUUUA motifs that are present but depends on the context in which the AUUUA motifs reside. Since the sequence AUUUA alone is not sufficient to destabilize an mRNA, the flanking sequences in fGH7 and fGH8 must somehow contribute to their destabilizing effect. These flanking sequences may either contribute to the destabilizing sequence or provide a more optimal spacing between two such sequences, perhaps allowing a cooperative effect between the binding of two proteins, or both.

ARE7 and ARE8 increase the rate of poly(A) tail removal. By analyzing the poly(A) distribution profiles of mRNAs at various times after serum induction, it is possible to correlate the effects of different AREs on both deadenylation and degradation of the fGH mRNA and thus draw conclusions about whether the same sequences direct both shortening and degradation and about the relationship between these two processes. We therefore compared the rates of deadenylation of the unstable mRNAs, fGH7 and fGH8, with those of the stable mRNAs, fGH, fGH55, and fGH9.

To compare poly(A) shortening rates, the products of the

FIG. 3. Degradation of fGH mRNA is enhanced by ARE8 but not ARE55. RNA was isolated at the times indicated following serum stimulation of stably transfected NIH 3T3 cells expressing the fGH transcript with ARE55 (fGH55) or ARE8 (fGH8) in the 3'UTR. (A) Time course of degradation of the fGH55 and fGH8 mRNAs. Specific mRNAs were quantitated by PhosphorImager analysis. For comparison, the time courses of degradation of fGH and fGH7, from Fig. 2, are also shown. (B) Phosphorimage of RNase protection gels. The fGH8 mRNA protection product is indicated by an arrow.

RNase protection assays were run on nondenaturing gels. Because the probes used protected the 3' end of the mRNA up to the polyadenylation site, and RNase A does not cleave the poly(A) tail, the migration of the protected digestion products

on a nondenaturing gel depends on the length of the tail. The stable mRNAs fGH, fGH55, and fGH9 were deadenylated very slowly (Fig. 4). RNA synthesized in the first hour following serum stimulation of the cells ran as a high-molecular-weight band, indicating the presence of a long poly(A) tail. During the subsequent 4 to 5 h, the tail length of these transcripts progressively decreased. During this time, the band appeared to broaden, which could indicate some increase in the heterogeneity of tail length during shortening or could be a consequence of the enhanced separation of smaller compared with larger molecules during gel electrophoresis. Between 6 and 8 h after serum stimulation, an accumulation of transcripts with short tails was evident; however, little or no poly(A)⁻ mRNA was seen. This result suggests that this oligo(A) form is either degraded directly or further deadenylated to poly(A)⁻ mRNA which does not accumulate because it is rapidly degraded.

There are several differences in the profile of deadenylation of the unstable fGH7 mRNA compared with the stable mRNAs. The fGH7 mRNA was rapidly deadenylated compared with the slow rate of poly(A) tail shortening seen with fGH, fGH55, and fGH9 (Fig. 4). To visualize steps in the rapid deadenylation of fGH7, samples were prepared at half-hourly intervals, instead of hourly as for the stable mRNAs. The heterogeneity of fGH7 poly(A) tail lengths increased markedly as shortening proceeded. The broadening of the band with time was greater than that seen with the stable fGH, fGH9, and fGH55 transcripts and so cannot be ascribed solely to the greater separation of smaller molecules by gel electrophoresis. This increase in heterogeneity could result from a more processive mechanism of exonucleolytic shortening of the fGH7 poly(A) tails. Some accumulation of oligo(A) mRNA could be seen between 2.5 to 4 h after stimulation, but little remained at 5 h poststimulation, and the shortest transcripts
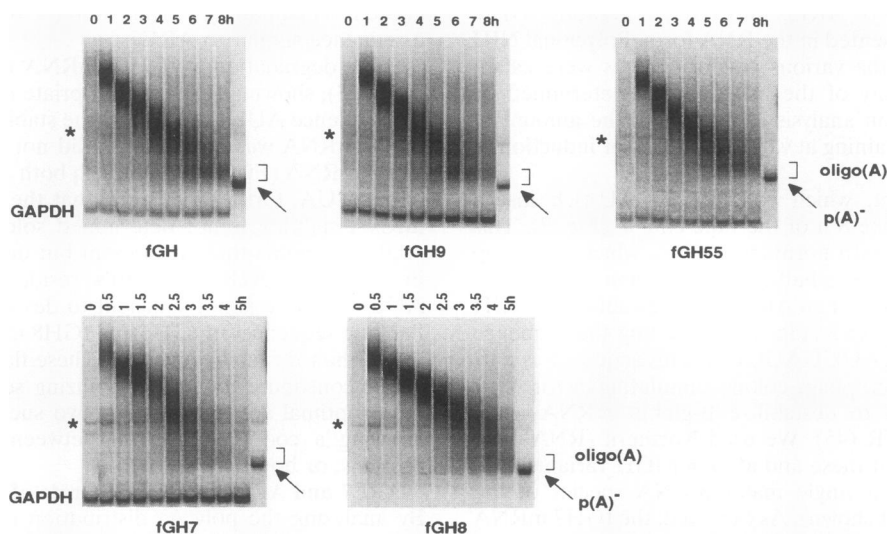
FIG. 4. Deadenylation of fGH7 and fGH8 mRNAs is rapid compared with that of the stable mRNAs fGH, fGH9, and fGH55. RNA was isolated at the times indicated following serum stimulation of NIH 3T3 cells stably transfected with the indicated constructs and was analyzed by RNase protection assay under conditions that do not cleave the poly(A) tail. The products were electrophoresed on nondenaturing polyacrylamide gels and visualized by PhosphorImager analysis. A GAPDH coding region probe was included as an internal quantitation standard except for the fGH8 experiment shown here, where its omission demonstrates that it does not obscure degradation products. The last track of each gel shows the migration of poly(A)⁻ mRNA [p(A)⁻], generated by treating RNA from serum-stimulated cells with RNase H in the presence of oligo(dT). The bracket indicates the position of the oligo(A) intermediate that can be seen late in the time courses. The asterisk indicates the position of a band that is not an intermediate in the deadenylation process but results from protection of full-length probe by a transcript believed to result from usage of a downstream polyadenylation site.
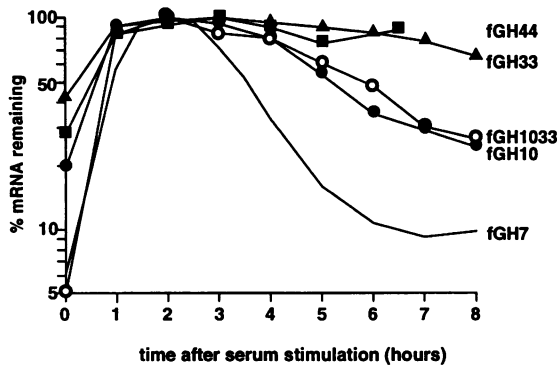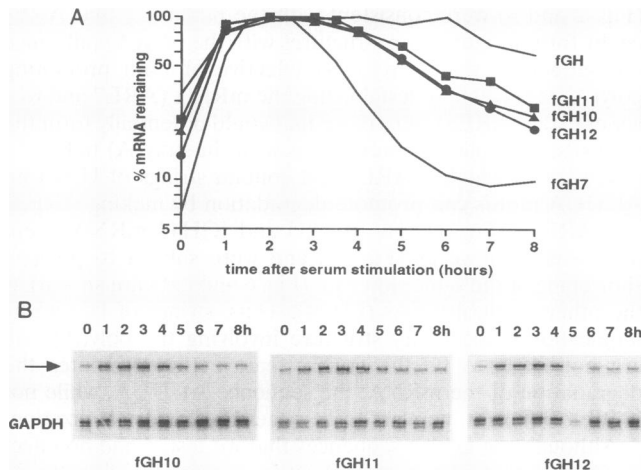
FIG. 5. Degradation of fGH mRNA is enhanced similarly by ARE10 and ARE1033 but not by ARE33 or ARE44. RNA was isolated at the times indicated following serum stimulation of NIH 3T3 cells stably transfected with the constructs indicated. For comparison, the time course of degradation of fGH7 mRNA, from Fig. 2, is also shown.

comigrated with the poly(A)⁻ marker rather than as oligo(A) (Fig. 4). Thus, both the initial rate of poly(A) shortening and the degradation of the oligo(A) form are faster than the rates of these two steps seen with the stable transcripts, fGH, fGH55, and fGH9.

The other unstable mRNA, fGH8, was also deadenylated rapidly, although at a slightly slower rate than fGH7. In both cases, transcripts with no or very short poly(A) tails first appeared at approximately 2.5 h, correlating well with the end of the lag preceding the onset of rapid degradation (Fig. 2 and 3). This observation is consistent with the hypothesis that deadenylation is required before degradation of the body of the mRNA can occur (12, 21, 38, 47, 53) and that AREs increase the overall degradation rate by increasing the rate of poly(A) shortening.

U tracts· do not destabilize. ARE55 contains AUUUA motifs but does not destabilize. Are the AUUUA motifs in ARE7 and ARE8 essential for their destabilizing effects, or may it be some other feature of these AREs, such as the preponderance of U residues, that was responsible for destabilization? Indeed, a common feature of cytokine and oncogene AREs is that they are primarily U rich with interspersed A's (for compilations of ARE sequences, see references 19 and 45). Our results with fGH7, fGH8, fGH9, fGH55, and fGH

(Fig. 2 and 3) were consistent with the possibility that AREs act by forming stem-loop structures with the poly(A) tail, since the sequences that were most effective at both promoting poly(A) removal and destabilizing the mRNA (ARE7 and to a lesser extent ARE8) were those that could potentially form the strongest base-pairing interactions with the poly(A) tail.

We tested whether AREs that contain strings of U but no AUUUA motifs can promote degradation by making ARE33 and ARE44 (Fig. 1). The fGH33 and fGH44 mRNAs were quite stable, however (Fig. 5), and were subject to poly(A) shortening at the same slow rate (Fig. 6 and data not shown) as the other stable mRNAs (Fig. 4). Thus, strings of U, and by implication a secondary structure involving the poly(A) tail, are not likely to be the crucial feature that promotes the degradation of the mRNA; the sequence AUUUA, while not in itself sufficient, appears to be necessary for destabilization.

Although the U-rich sequences that we tested had no effect on degradation of the mRNA, it is conceivable that the U strings present in many cytokine and oncogene AREs enhance the effects of proximal AUUUA-containing motifs. We considered this possibility following a report by Shyu et al. (47), who showed that mutation of the c-fos AUUUA motifs had only a small effect on the rate of deadenylation of a chimeric mRNA. This raised the possibility that one or both of the U-rich regions in the c-fos ARE act as an enhancer of deadenylation. Since our ARE33 contains a duplication of a sequence very similar to the c-fos U-rich element that lies downstream of the AUUUA motifs, we tested whether ARE33 could enhance deadenylation of several fGH variants. ARE33 was inserted downstream of ARE7, ARE8, and a truncated, less destabilizing form of ARE7 called ARE10 (see below), generating fGH733, fGH833, and fGH1033, respectively. Insertion of the extra U-rich sequences did not enhance either the degradation rate (Fig. 5 and data not shown) or the rate of poly(A) shortening (Fig. 6 and data not shown).

Delineation of a minimal functional sequence. The functional element that marks the mRNA for rapid turnover does not seem to be a stem-loop structure formed by base pairing between the poly(A) tail and U-rich sequences in the ARE. Furthermore, secondary structure predictions from cytokine and oncogene 3'UTR sequences did not reveal any common secondary structures in the vicinity of the AU-rich regions (data not shown). Thus, the AREs probably provide binding sites for one or more degradation factors that recognize a single-stranded RNA sequence. Since this sequence, or a close match to the sequence, must be present in ARE7, but not in
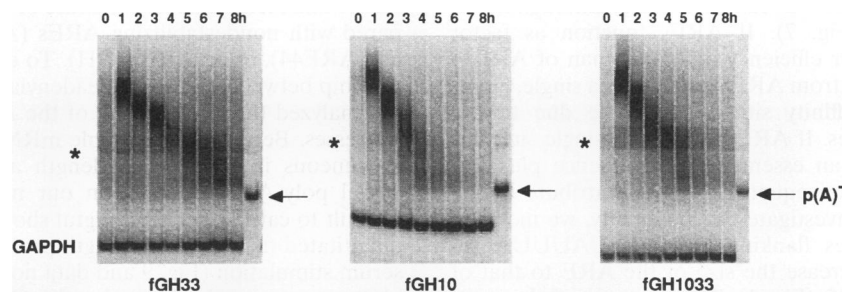
FIG. 6. A U-rich ARE does not influence deadenylation rate, either alone or in conjunction with a moderately destabilizing AUUUA-containing ARE. RNA was isolated at the times indicated following serum stimulation of NIH 3T3 cells stably transfected with the indicated constructs and analyzed by RNase protection assay under conditions that do not cleave the poly(A) tail. The products were electrophoresed on nondenaturing polyacrylamide gels and visualized by PhosphorImager analysis. The last track of each gel shows the migration of poly(A)⁻ mRNA [p(A)⁻], generated by treating the sample with RNase H in the presence of oligo(dT). The asterisk indicates the position of a band that is not an intermediate in the deadenylation process but results from protection of full-length probe by a transcript believed to result from usage of a downstream polyadenylation site.

FIG. 7. An ARE as small as 9 nt can destabilize fGH mRNA. RNA was isolated at the times indicated following serum stimulation of NIH 3T3 cells stably transfected with the indicated constructs. (A) Time course of degradation of the mRNAs. Specific mRNAs were quantitated by PhosphorImager analysis. For comparison, the time courses of degradation of fGH and fGH7, from Fig. 2, are also shown. (B) Phosphorimage of RNase protection gels. The mRNA protection product is indicated by an arrow.

FIG. 8. UAUUUAU is the smallest sequence from ARE7 that can destabilize. RNA was isolated at the times indicated following serum stimulation of NIH 3T3 cells stably transfected with the indicated constructs. (A) Time course of degradation of the mRNAs. Specific mRNAs were quantitated by PhosphorImager analysis. For comparison, the time course of degradation of fGH7 mRNA, from Fig. 2, is also shown. (B) Phosphorimage of RNase protection gels. The mRNA protection product is indicated by an arrow.

the AREs that do not destabilize (ARE33, ARE44, ARE55, and ARE9), some short sequences can be eliminated from consideration at this stage. ARE7 contains a repeating motif that can be equally expressed as $(AUUU)_n$, $(UAUU)_n$, $(UUAU)_n$, or $(UUUA)_n$, but multiple copies of all these tetranucleotide sequences are present in ARE33 and ARE44, which do not destabilize. Similarly, the pentanucleotide sequences UAUUU, UUAUU, and UUUAU that are present in multiple copies in ARE7 are present in multiple copies in ARE33 and ARE44, and the AUUUA sequence is present in ARE55. Thus, the minimal functional sequence within ARE7 must be more than 5 nt in length and must contain AUUUA. However, strict adherence to a unique sequence longer than 6 nt is not likely to be necessary for destabilizing activity, since the longest sequence that is highly conserved in cytokine and oncogene AREs is UAUUUA (33).

To more closely delimit the functional sequence, we made shorter forms of ARE7, creating ARE10 (AUUUAUUUAU UUA) and ARE11 (AUUUAUUUA) (Fig. 1). Both of these AREs increased the degradation rate of the mRNA, showing that a sequence as short as 9 nt can destabilize, although not as effectively as ARE7 (Fig. 7). If AREs function as factor binding sites, the greater efficiency of ARE7 than of ARE10 and ARE11 could result from ARE7 containing a single, larger and therefore higher-affinity site or could be due to the presence of multiple sites. If ARE7 contains a single site, this could be composed of an essential core sequence plus less specific AU-rich flanking sequences which contribute to the affinity of the site. To investigate this possibility, we included extra AU-rich sequences flanking the AUUUAUUUA sequence of fGH11 to increase the size of the ARE to that of ARE7, generating fGH12 (Fig. 1). The presence of the extra flanking AU-rich sequences had at most a small effect on the stability (Fig. 7) and did not produce an ARE as destabilizing as ARE7. The greater effectiveness of ARE7 than of ARE10, ARE11, and ARE12 is therefore probably due to the presence of more functional motifs in ARE7 than in ARE10, ARE11, and ARE12.
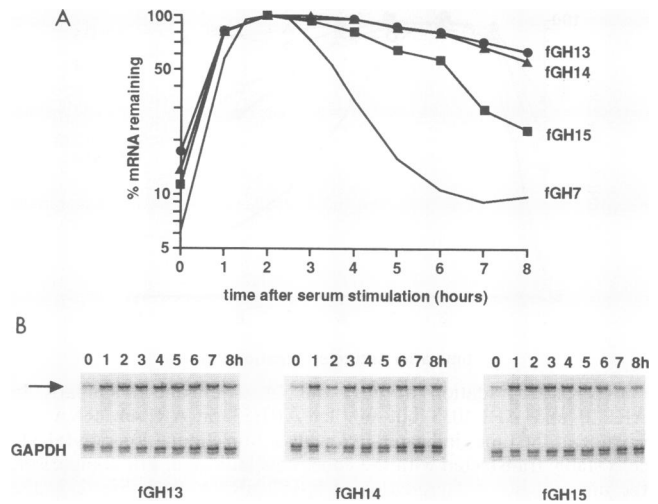
Three copies of UAUUUAU can destabilize. Since the 5-nt sequence AUUUA was necessary but not alone sufficient to destabilize, we tested whether a 6- or 7-nt sequence from ARE7 could function. To ensure that small effects would be detectable, we inserted three copies of each of these short sequences, separated by the sequence CACAC. Neither AUUUAU (ARE13) nor UAUUUA (ARE14) destabilized the fGH transcript (Fig. 8); however, three copies of UAUUUAU (ARE15) did destabilize (Fig. 8), suggesting that UAUUUAU is the smallest sequence from within ARE7 that is sufficient to destabilize an mRNA. Since ARE8 and ARE11 do not contain exact copies of UAUUUAU but are nevertheless destabilizing, it must be concluded that the sequences flanking AUUUA in these larger AREs in some way obviate a strict requirement for the UAUUUAU sequence. This conclusion is consistent with the observation that UAUUUA is the longest highly conserved sequence in cytokine and proto-oncogene AREs (33).

AREs appear to increase degradation by increasing the deadenylation rate. We showed above that the destabilizing ARE7 and ARE8 increase the rate of poly(A) shortening compared with nondestabilizing AREs (ARE55, ARE9, ARE33, and ARE44), or no ARE (fGH). To examine further the relationship between effects on deadenylation versus degradation, we analyzed the effects of all of the ARE sequences on both processes. Because the unstable mRNAs become rapidly heterogeneous in poly(A) tail length and we cannot measure actual poly(A) tail length on our nondenaturing gels, it is difficult to calculate a meaningful shortening rate. Instead, we quantitated the whole tail length profile at various times after serum stimulation (Fig. 9 and data not shown). The individual plots were normalized by using the *GAPDH* internal standard to correct for any variation in the amount of RNA run on the gel. To reduce the distortion resulting from the read-through product (Fig. 2) and the small contribution from the heterogeneous smear due to basal transcription, the steady-state profile from unstimulated cells (time zero in Fig. 4 and 6) was subtracted from each profile obtained after serum stimulation.
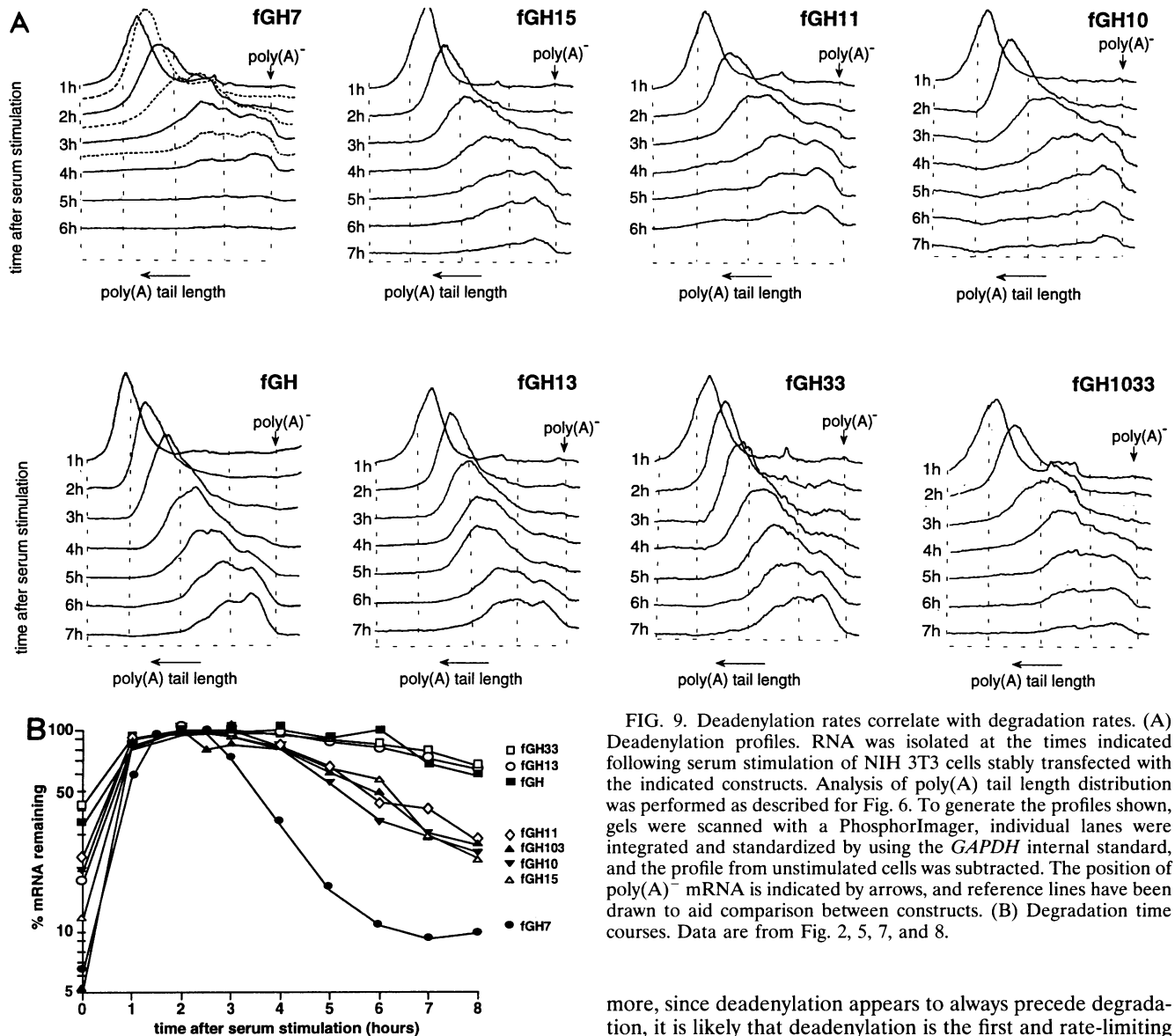
FIG. 9. Deadenylation rates correlate with degradation rates. (A) Deadenylation profiles. RNA was isolated at the times indicated following serum stimulation of NIH 3T3 cells stably transfected with the indicated constructs. Analysis of poly(A) tail length distribution was performed as described for Fig. 6. To generate the profiles shown, gels were scanned with a PhosphorImager, individual lanes were integrated and standardized by using the *GAPDH* internal standard, and the profile from unstimulated cells was subtracted. The position of poly(A)$^-$ mRNA is indicated by arrows, and reference lines have been drawn to aid comparison between constructs. (B) Degradation time courses. Data are from Fig. 2, 5, 7, and 8.

This minimizes overrepresentation of the read-through transcripts, the protected products of which run as a discrete band rather than as a smear because the antisense RNA probe does not protect the 3' end of these longer transcripts, resulting in removal of the poly(A) tail. The time intervals were hourly except for fGH7, which because of its more rapid deadenylation was sampled half-hourly.

The profiles in Fig. 9 show that the correlation between deadenylation rate and degradation rate (Fig. 2, 3, 5, 7, and 8; summarized in Fig. 9B) is maintained without exception, with those mRNAs which degraded more rapidly undergoing more rapid poly(A) shortening. The length of the lag before degradation of the mRNA body begins (varying from 1 h for fGH7 to 4.5 h for fGH) also correlates well with the time at which transcripts with no or very small poly(A) tails first appear.

Since the ARE sequences that we constructed were mostly simple reiterated sequences, the most likely explanation for this correlation is that the same putative ARE-binding factor was influencing both deadenylation and degradation. Further-

more, since deadenylation appears to always precede degradation, it is likely that deadenylation is the first and rate-limiting phase in degradation of the fGH mRNA and that the destabilizing effect of the inserted AREs results largely from their ability to increase the deadenylation rate.

DISCUSSION

A model system with which to study the effects of synthetic AREs on mRNA stability. Chimeric genes have been used in a number of studies to assess the destabilizing effect of various elements within the 3'UTRs of natural mRNAs (3, 17, 21, 45, 47, 48). Such systems have several advantages: by using a coding region from a stable mRNA (which presumably does not contain a destabilizing element such as those identified in the coding regions of the c-*fos* [30, 48] and c-*myc* [54] mRNAs) and by inserting all sequences at a fixed location in the 3'UTR, other influences on mRNA stability can be minimized, allowing the observed effects to be attributed to the insertion of these sequences. We have used such a chimeric gene, with various synthetic AU-rich sequences inserted into the 3'UTR, to analyze the sequence requirements for ARE-mediated mRNA destabilization. To perform these experiments, we found it preferable to use the inducible c-*fos* promoter, rather

than a constitutive promoter, to drive expression. In initial experiments using a constitutive promoter, we found that certain AREs reduced the steady-state level of the mRNA; however, when the mRNA half-life was measured following transcription inhibition with actinomycin D, the mRNA did not appear as unstable as expected. Subsequent experiments using the *fos* promoter confirmed that actinomycin D treatment decreased the degradation rate of the mRNA (33). Actinomycin D has also been shown to prolong the life of several other ARE-containing mRNAs (3, 46, 48, 52). On the other hand, stimulation of cells with serum (used to induced expression from the *fos* promoter) did not change the apparent half-life of an unstable ARE-containing mRNA driven from a constitutive promoter (14). The pulse of transcription from the *fos* promoter also makes it possible to determine precursor-product relationships for early intermediates in the degradation pathway, facilitating analysis of changes in poly(A) tail length during mRNA degradation.

AUUUA and oligo(U) do not destabilize, but multiple copies of UAUUUAU do. From inspection of cytokine and oncogene 3'UTR sequences, we concluded that a common feature was the presence of strings of 3 to 6 U's, often flanked by single A's. Because of this, the hypothesis that guided the initial design of AU sequences to be tested was that U richness was the functional feature of AREs, perhaps because it promoted the formation of a hairpin structure by base pairing with the poly(A) tail (45, 53). Alternatively, You et al. (55) have proposed that some U-rich AREs may be recognized on the basis of their U richness, but as single-stranded rather than double-stranded RNA. Nevertheless, in our experiments with mRNAs containing AREs with strings of U of various lengths, we found no effect of the U-rich AREs on the stability of the mRNA and concluded it was unlikely that AREs function by forming a hairpin structure. In subsequent experiments, we assumed that the ARE functioned as a single-stranded binding site for a degradation factor, and the following discussion therefore uses the terms destabilizing sequence and binding site interchangeably, although the notion that the destabilizing sequence is a factor binding site remains unproven.

We found that the sequence AUUUA is not sufficient to confer instability on an mRNA, even when present in multiple copies. The smallest sequence that destabilized in multiple copies was UAUUUAU, although this sequence is probably not optimal since ARE15, containing three separated copies of this sequence, was not as effective as ARE7, which contains only two nonoverlapping copies. For simplicity, we consider here only nonoverlapping copies of the motif because steric hindrance is likely to prevent the simultaneous binding of potential destabilizing factors to two overlapping copies of a binding site. The use of overlapping sites cannot be ruled out but would not change the conclusions that follow. The greater efficiency of ARE7 than of ARE15 could result from one or both of the two putative binding sites in ARE7 providing a better match to the full binding-site sequence. It is also possible that the greater efficiency is due to ARE7 having a more optimal spacing between its two sites, allowing cooperative interactions in the binding of two factors, or a single dimeric factor, or to a combination of better spacing and better matches to the sequence.

The full motif may be UUAUUUA(U/A)(U/A). In Fig. 10, we have attempted to deduce a consensus sequence for the putative optimal binding site, based on the relative destabilizing efficiencies of the different AREs tested in this study. Since we do not know the degree of cooperativity, if any, resulting from multiple sites, the optimal spacing between sites, or the relative effects of different mismatches in the site, our conclu-

ARE		UUAUUUAUU		UUAUUUAUU		UUAUUUAUU		effect
7	U	UUAUUUAUU	UAU	UUAUUUACU	CGA			+++
8	U	UUAAUUUAAU	U--	UAAUUUAAU	UUA			++
15	C	CUAUUUAUC	ACA	CUAUUUAUC	ACA	CUAUUUAUC	U	+
10	U	UUAUUUAUU	UAC					+
11	U	UUAUUUACU	CGA					+
12	U	AUAUUUAUU	UAU	AUUACUCGA	GCU			+
13	A	CCAUUUAUC	AC-	ACAUUUAUC	AC-	ACAUUUAUC	U	-
14	C	CUAUUUACA	CA-	CUAUUUACA	CA-	CUAUUUACU	C	-
55	C	AGAUUUAAA	---	-AAUUUAGA	G*C	AGAUUUAAA	A	-
33	A	GUUUUUAUU	U--	UUAUUUUUG	A*U	UUAUUUUUA	U	-
44	U	UUAUUUUUU	U*U	UUUUUUAUU	UUU			-
9	A	UUAUUAUUA	---	UUAUUAUUA	CUC			-

FIG. 10. Matches within different AREs to the motif UUAUUU AUU and their relative destabilizing effects. ARE sequences are aligned to maximize the fit to UUAUUUAUU. Only sequences within the AREs are shown, and matches to the motif are indicated by stippling. The asterisks in ARE55, ARE33, and ARE44 represent 3, 9, and 11 nt, respectively. ARE12 can alternatively be described as UUAUUUAUA; ARE55 has a fourth AUUUA-containing motif that is not shown.

sions remain somewhat speculative. Nevertheless, the relative efficiencies of the various AREs tested are consistent with (i) the binding site being UUAUUUAUU, (ii) the AUUUA core being necessary but not sufficient, and (iii) a total of no more than two mismatches being tolerated in the flanking positions, where their presence reduces the efficiency of the site.

The proposed requirement for AUUUA as a necessary core is based on several observations. ARE33 and ARE44 both contain at least two copies of the proposed UUAUUUAUU motif with just one or two mismatches, but the mismatches occur in the AUUUA core, and these AREs do not destabilize. Similarly, three mismatches to this motif occur in ARE9, two of which are within the AUUUA core, and this ARE also does not destabilize. The frequency with which AUUUA occurs in the 3'UTRs of unstable cytokine and oncogene mRNAs also suggests it has an important role, and in several studies in which point mutations were introduced into the AUUUA motifs of destabilizing AREs, disruption of the AUUUA sequences abolished the destabilizing effect (45, 47, 50).

The argument for inclusion of the extra U's flanking the UAUUUAU motif is based in part on the relative efficiencies of ARE8, ARE11, and ARE12 compared with ARE15. ARE15 has three UAUUUAU motifs but is no more destabilizing than ARE11 and ARE12, which probably have only one binding site (Fig. 10) and, in the case of ARE11, an incomplete UAUUUAU motif. This observation suggests that, while sufficient, the UAUUUAU motif may not be optimal. Additionally, ARE8 has no complete UAUUUAU sequences yet is more destabilizing than ARE15, indicating that UAUUUAU cannot be necessary for destabilization. The common feature between ARE8, ARE11, and ARE12 that is absent in ARE15 is U at one or both positions flanking the UAUUUAU, suggesting that U at these positions contributes to the effectiveness of the sequence as a destabilizing motif.

One copy of the proposed binding site, as in ARE10, ARE11, and ARE12, is clearly sufficient to produce a destabilizing effect, although multiple copies, as in ARE7 and ARE8, are more effective. Furthermore, the effectiveness of individual motifs probably depends on the degree of mismatching in the first two and last two positions (compare ARE7 with ARE15). Thus, AREs can potentially confer on mRNAs a wide range of half-lives, and this appears to be reflected in the variety of ARE sequences found in different cytokine and oncogene mRNAs.

A survey of 42 different cytokine and oncogene 3'UTR sequences from human and mouse genomes, containing 230

TABLE 1. Relative nucleotide frequencies at positions
flanking AUUUA sequences[a]

Base	Relative frequency (%) at indicated position in the sequence NNAUUUANN			
	1	2	8	9
U	44.3	77.4	69.1	44.8
A	26.1	16.1	26.1	31.7
C	15.7	5.7	2.2	7.8
G	13.9	0.9	2.7	15.7

[a] Derived from 42 cytokine and oncogene 3'UTR sequences from human and mouse genomes, containing 230 AUUUA motifs.

AUUUA motifs, is consistent with the functional motif being UUAUUUAUU. Where the sequence NNAUUUANN occurs, U is the preferred base at positions 1, 2, 8, and 9 (Table 1). In addition, the sequence UAUUUAU occurs at least once in 39 of the 42 3'UTRs. When U is not present at positions 8 or 9, there is a strong bias for A, suggesting that A at one or both of these positions may contribute to the affinity of the binding site, or at least be less disruptive than G or C. Since the results of experiments in which a segment from the 3'UTR of granulocyte colony-stimulating factor was inserted into fGH also suggest that A is an acceptable substitution for U at position 8 or 9 (14), we propose that the functional element is more correctly defined as UUAUUUA(U/A)(U/A). The destabilizing ability of ARE8 is also consistent with A being an acceptable substitution for U at position 8.

Since the sequence AUUUA is the essential core of a destabilizing motif but is not in itself sufficient to destabilize an mRNA, it is not necessarily correct to assume, without measurement of the half-life, that an mRNA containing AUUUA in the 3'UTR will be unstable. The larger motif that we have described should be a better indicator of the likely stability of newly identified AUUUA-containing mRNAs.

Implications for studies of binding proteins. A number of AU-binding proteins have been identified by using UV cross-linking and gel shift assays (8, 10, 22, 24, 27, 42, 50, 56). Some of these proteins bind poly(U) as well as AUUUA-containing RNAs (10, 27, 42, 50, 56), suggesting that the binding to the AUUUA-containing probes may result from affinity for oligo(U) stretches. Since the oligo(U) containing ARE33 and ARE44 did not destabilize the fGH mRNA, the primary destabilizing factor is probably not a U-binding protein. Two proteins identified by Bohjanen et al. (10) in stimulated T cells (AU-B and AU-C) appear to have binding characteristics consistent with our destabilizing data and do not bind poly(U). Thus, our data support the proposal that these proteins may have a role in ARE-mediated degradation (10).

Destabilizing AREs enhance the rate of deadenylation. We have examined the effects of various ARE sequences on both deadenylation and degradation of fGH mRNA and found that poly(A) shortening preceded degradation of both stable and unstable variants of this mRNA. In all cases, there was a correlation between the rate of deadenylation and degradation of the mRNA; all destabilizing AREs enhanced the deadenylation rate, and no ARE stimulated just one of these processes. We cannot distinguish from these experiments whether the destabilizing effect of UUAUUUA(U/A)(U/A) motifs results from stimulation of deadenylation alone (which could be rate limiting in degradation) or whether steps subsequent to deadenylation are also stimulated.

The poly(A) tails of stable mRNAs were slowly and rather uniformly shortened over a period of several hours until the tails reached an oligo(A) form (which presumably binds at least one molecule of PABP). The transient accumulation of this species indicates that a subsequent step in its degradation is slow. Although no poly(A)⁻ mRNA was evident during degradation of the fGH mRNA or the other stable variants, it cannot be concluded that these mRNAs are not terminally deadenylated. The difference between the slow rate of conversion of oligo(A) mRNA to poly(A)⁻ mRNA and the rate of the subsequent degradation of poly(A)⁻ mRNA may be too great for any significant accumulation of the poly(A)⁻ form to occur.

The more unstable mRNAs, especially fGH7, became shorter and more heterogeneous in length early in the time course compared with the stable mRNAs, consistent with deadenylation being faster and more processive, as observed for rapidly deadenylated yeast mRNAs (21, 38). Less oligo(A) mRNA accumulated, indicating that this intermediate was also more rapidly degraded. Since a poly(A)⁻ form of the fGH7 mRNA was detected, a portion at least of this unstable mRNA undergoes terminal deadenylation.

Our data show that deadenylation precedes mRNA body degradation and proceeds in at least two phases, consistent with the suggestion that the mRNA degradation pathways are similar in yeast and mammalian cells (21, 38). The simplest explanation for our observations is that AREs destabilize mRNAs by enhancing the rates of both poly(A) shortening and terminal deadenylation. However, degradation without terminal deadenylation has been observed for two yeast mRNAs by the artificial stabilization of degradation intermediates, which were found to have short oligo(A) tails (21). Thus, it could well be that not all mammalian mRNAs are terminally deadenylated prior to degradation: in addition to stimulating deadenylation, AREs may also stimulate the rate of decapping or endonucleolytic cleavage of deadenylated or oligoadenylated mRNAs.

U-rich sequences do not enhance deadenylation. Single substitutions of A for U in the AUUUA motifs of the ARE from c-fos have been shown to inhibit its ability to stimulate mRNA degradation but to have only a slight effect on the deadenylation rate (47). These studies suggested that in the more complex c-fos ARE, the AUUUA motif is required for degradation of the mRNA body but not for deadenylation, whereas our studies indicate that the UUAUUUA(U/A)(U/A) motif stimulates both processes. Another implication of the observations of Shyu et al. (47) was that the U-rich sequences proximal to the AUUUA motifs in the complex c-fos ARE may play a role in stimulating deadenylation. Since we found that two synthetic U-rich AREs (ARE33 and ARE44) had no stimulatory effect on deadenylation of the fGH mRNA, we tested whether ARE33, the sequence most resembling the downstream U-rich region of the c-fos ARE, stimulated deadenylation when placed 3' of either our most destabilizing (fGH7) or less destabilizing AREs (fGH10 and fGH8). However, no enhancement of deadenylation or degradation of these chimeric mRNAs was observed, leading us to conclude that in this context a U-rich sequence has no effect.

A further analysis of the c-fos ARE was published while our manuscript was in the final stages of preparation (17) and has shown, consistent with our findings, that the AUUUA-containing region is sufficient to promote deadenylation and degradation of a heterologous mRNA, while the downstream U-tract alone is not. In contrast to our findings, however, this U tract was found to have an enhancing effect on deadenylation when placed either 5' or 3' of the AUUUA motifs. It therefore appears that the precise nature and context of such U-rich

sequences in individual complex AREs may determine their influence on deadenylation.

Using simple synthetic AREs, we have identified a motif, UUAUUUA(U/A)(U/A), which we suggest is the binding site for a destabilizing factor that promotes two phases of mRNA deadenylation. This information should aid the understanding of the function of the more complex AREs that occur in some natural mRNAs.

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