AU-rich elements target small nuclear RNAs as well as mRNAs for rapid degradation

Xinhao Cynthia Fan, Vic E. Myer,1 and Joan A. Steitz2

Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, Boyer Center for Molecular Medicine, New Haven, Connecticut 06520 USA

AU-rich elements (AREs, usually containing repeated copies of AUUUA), when present in the 3'-untranslated regions (UTRs) of many mammalian mRNAs, confer instability on their host RNA molecules. The viral small nuclear RNA (snRNA) Herpesvirus saimiri U RNA 1 (HSUR 1) also contains an AUUUA-rich sequence. Here, we report that this ARE induces rapid degradation of HSUR 1 itself and of other snRNAs including HSUR 2 and cellular U1. Mutational analyses of the viral ARE establish that sequence requirements for mRNA and snRNA decay are the same, suggesting a similar mechanism. Moreover, the in vivo degradation activity of mutant AREs correlates with their in vitro binding to the HuR protein, implicated previously as a component of the mRNA degradation machinery. Our results suggest that ARE-mediated instability can be uncoupled from both ongoing translation and deadenylation of the target RNA.

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Targeted mRNA instability represents an important mode of post-transcriptional gene regulation in eukaryotic cells. Much attention has focused on AU-rich elements (AREs) that are present in the 3'-untranslated regions (3' UTRs) of the mRNAs from many early response genes (ERGs), including cytokines, lymphokines, and proto-oncogenes (Caput et al. 1986; Shaw and Kamen 1988; for review, see Belasco and Brawerman 1993; Chen and Shyu 1995). The expression of ERGs is both sensitive to extracellular stimuli and transient, requiring rapid mRNA removal through destabilization following the cessation of transcription. The ARE is a major determinant for the rapid degradation of these mRNAs. Insertion of a 51-nucleotide AU-rich sequence derived from the GM-CSF (granulocyte–macrophage colony-stimulating factor) into the 3' UTR of β-globin mRNA greatly shortens its normally long half-life (Shaw and Kamen 1988). Conversely, removal of the ARE stabilizes the labile proto-oncogene c-fos mRNA and confers a transforming phenotype (Miller et al. 1984; Mejiljian et al. 1985; W. Lee et al. 1988). Depending on cell type, there appear to be different pathways regulating ARE-mediated mRNA decay. For instance, in a monocyte tumor cell line, c-fos mRNA is degraded constitutively, whereas the GM-CSF mRNA is stable (Schuler and Cole 1988). GM-CSF and interleukin-3 (IL-3) mRNAs have also been reported to be selectively stabilized in a human T cell line upon anti-CD28 antibody treatment (Lindsten et al. 1989).

The AREs in ERG mRNAs contain multiple copies of the sequence AUUUA (Caput et al. 1986; Shaw and Kamen 1988; for review, see Belasco and Brawerman 1993; Chen and Shyu 1995). Using a serum-inducible c-fos promoter and a reporter gene system in transiently transfected NIH-3T3 cells, two groups have performed extensive mutagenesis to identify the minimum sequence that directs mRNA degradation. Both found that an isolated single AUUUA is not active, whereas an AUUUA flanked by two Us (UUAUUUAUU) has weak destabilization activity and two separate or overlapping copies of UUAUUUAUU are highly destabilizing (Lagnado et al. 1994; Zubiaga et al. 1995). Using the same serum-inducible promoter system, Shyu and coworkers provided evidence that ARE-mediated mRNA decay proceeds in a biphasic manner, with shortening of the poly(A) tail followed by a first order degradation of the remaining RNA (Shyu et al. 1991; Chen et al. 1995). Whether the latter step involves exo- or endonucleases is not yet established. Also unknown is whether ARE-mediated mRNA decay is obligatorily coupled to ongoing translation, because evidence both in favor of and against this linkage has been reported (Koeller et al. 1991; Savant-Bhonsale and Cleveland 1992; Aharon and Schneider 1993; Chen et al. 1995; Curatola et al. 1995; Winstead et al. 1995; see below).
Although many proteins have been characterized as factors recognizing AREs (Malter 1989; Bohjanen et al. 1991, 1992; Brewer 1991; Malter and Hong 1991; Vakalopoulou et al. 1991; Hamilton et al. 1993; Zhang et al. 1993; Katz et al. 1994; Nakagawa et al. 1995), we have concentrated on an apparent 32-kDa protein, first identified by cross-linking to the c-fos ARE in HeLa cell extracts (Vakalopoulou et al. 1991). This protein also binds to several of the seven small nuclear RNAs (snRNAs) that are highly expressed in marmoset T cells transformed by Herpesvirus saimiri (Myer et al. 1992). These H. saimiri U RNAs (HSURs 1–7) are similar to cellular U RNAs in that they are transcribed by RNA polymerase II, possess 5′-trimethylated guanosine caps at their 5′ ends, and assemble into small nuclear ribonucleoprotein particles (snRNPs) of the Sm class (Murthy et al. 1986; S. Lee et al. 1988; Wassarman et al. 1989; Lee and Steitz 1990; Albrecht and Fleckenstein 1992). At their 5′ ends, HSUR 1, HSUR 2, and HSUR 5 contain four, two, and one copies of AUUUA, respectively. Characterization of the purified 32-kDa protein demonstrated that it is identical to HuR (Myer et al. 1997), a ubiquitously expressed member of the ELAV family (Embryonic Lethal, Abnormal Vision; Ma et al. 1996). Like other ELAV proteins, HuR contains three RNA recognition motifs (RRMs) and binds in vitro to ARE sequences (Levine et al. 1993; Gao et al. 1994; Ma et al. 1996; Myer et al. 1997). Detailed analyses revealed a direct correlation between the in vitro affinity of an ARE sequence for HuR and its ability to direct in vivo degradation of a reporter mRNA, suggesting the involvement of HuR in the destabilization (or stabilization) of ARE-containing mRNAs (Myer et al. 1997).

We set out to test a model whereby the HSURs function to stabilize mRNAs normally targeted for rapid degradation by competitively binding cellular components of the mRNA degradation machinery (Myer et al. 1992). By transiently expressing HSUR 1, which has a strong affinity for HuR (Myer et al. 1992), in mouse L929 cells, we expected to observe elevated levels of ARE-containing mRNAs. Instead, we discovered that wild-type HSUR 1 is expressed at a much lower level than a mutant HSUR 1 at one-sixth to one-tenth the level of the mutant HSUR 1 at one-sixth to one-tenth the level of the mutant (Fig. 1B, lane 2) was significantly lower (one-eighth) than that of the mutant (lane 3) when normalized to the cotransfected control HSUR 3. Comparable results were obtained in other cell lines of human, monkey, or mouse origin; HeLa cells, HEK-293 cells, Jurkat cells, COS cells, and NIH-3T3 cells all produced wild-type HSUR 1 at one-sixth to one-tenth the level of the mutant (data not shown).

To determine whether the lower level of wild-type compared with mutant HSUR 1 was attributable to greater instability or to some unexpected effect of the internal HSUR 1 sequence on promoter activity, we performed run-on transcription assays. Whole cell rather than the standard nuclear assay was used because of the low [32P]UTP incorporation into snRNAs in the nuclear system (Sheldon et al. 1993). Transfected mouse L929 cells were permeabilized and incubated with [32P]UTP at 30°C for 10 min (Sheldon et al. 1993). Total RNAs were isolated and hybridized to excess double-stranded DNA that had been denatured and immobilized on a nylon membrane. The cotransfected HSUR 3 and nontransfected HSUR 4 sequences served as positive and negative controls, respectively. Figure 1C shows that wild-type HSUR 1 has a transcription rate comparable (95%) with that of the mutant. Similar results (±10%) were obtained in repeated experiments (data not shown). We conclude that the lower level of wild-type HSUR 1 in transfected L cells is attributable to more rapid decay and that the ARE, which...
Figure 1. ARE-mediated HSUR 1 degradation in vivo. (A) HSUR RNA sequences and expression plasmid configuration. The HSUR 1 ARE and the nucleotides altered in Mut are underlined. The U1 promoter in the pUC–U1 vector is the 423-bp human genomic sequence upstream of U1 transcription initiation site, whereas the 3' box is the 30-bp transcription termination signal downstream of the U1 coding sequence (Yuo and Weiner 1989). (B) T1 RNase protection analysis of wild-type and mutant HSUR 1 levels in transient transfection assays. The pUC–U1–HSUR 1 constructs were transiently cotransfected with a pUC–U1–HSUR 3 plasmid into mouse L929 cells (see Materials and Methods). Total RNA collected 48 hr after transfection was subjected to RNase T1 protection assays with wild-type and mutant HSUR 1 antisense RNA (lanes 2 and 3, respectively), together with antisense HSUR 3 RNA as an internal control. One-fiftieth of the amount of the anti-wild-type and anti-mutant HSUR 1 RNA probes used is shown in lanes 4 and 5. The data were quantitated with a Molecular Dynamics PhosphorImager and normalized to HSUR 3. Wild-type HSUR 1 levels were reproducibly one-eighth of those of mutant HSUR 1. (C) Whole-cell run-on assays of wild-type and mutant HSUR 1 transcription. The pUC–U1–HSUR 1 construct containing wild-type or mutant HSUR 1 sequences was cotransfected with pUC–U1–HSUR 3 into L cells, and whole-cell run-on assays were performed (see Materials and Methods). Total RNA was hybridized to nylon membranes that had been dot-blotted with wild-type (top) or mutant (bottom) HSUR 1 and HSUR 3 DNA fragments. Untransfected HSUR 4 DNA was also dotted as a negative control. The patterns of dots are illustrated at right; hybridizations with the run-on RNAs are at left. After quantitation and normalization against the cotransfected positive control HSUR 3 (also subtracting the untransfected negative control HSUR 4), the wild-type HSUR 1 (left dot, top) and the mutant (left dot, bottom) were found to have similar transcription rates (wild type/mutant = 0.95). (D) Immunoprecipitation of wild-type and mutant HSUR 1 from transfected mouse L929 cells. L cells were transiently cotransfected with the pUC–U1 constructs containing wild-type or mutant HSUR 1 genes, and whole-cell extracts were prepared by sonication. Equal amounts of extract were precipitated with anti-Sm monoclonal antibody Y12 or anti-U1 70K monoclonal antibody H111 as a control. RNA was harvested from the immunoprecipitation pellets (lanes 1,3,5,7) and supernatants (lanes 2,4,6,8), and wild-type (left) and mutant (right) HSUR 1 were assayed by T1 RNase protection. For both wild-type and mutant HSUR 1s, >90% of the RNA was in the anti-Sm precipitate (lanes 3,7), whereas >99% of the HSUR 1s remained in the supernatant with the anti-U1 70K antibody (lanes 2,6).
is altered in the mutant, is a cis-acting instability sequence.

We further investigated whether the transfected viral snRNAs assemble into Sm-precipitable snRNP. Extracts of L cells transfected with wild-type or mutant HSUR 1 were precipitated either with anti-Sm monoclonal antibodies (Y12) or as a negative control, with anti-U1 70K monoclonal antibodies (H111). Comparison of the anti-Sm pellets (Fig. 1D, lanes 3,7) and supernatants (lanes 4,8) reveals that >90% of both the wild-type and mutant HSUR 1 assemble with Sm proteins. More than 99% remains in the supernatants in the control anti-U1 70K immunoprecipitations (lanes 2,6). We conclude that in transfected L cells, the more stable mutant as well as wild-type HSUR 1 become assembled into Sm snRNP particles.

Mutational analyses of the HSUR 1 ARE

Because detailed mutational studies have shed light on the exact sequence requirements for functional AREs in mRNA 3' UTRs, we performed similar analyses of the HSUR 1 5'-end sequence. We first introduced two or three U→G substitutions into the HSUR 1 ARE, leaving either one intact copy of UUAUUUAUU in mutant M1 or one copy of AUUUA flanked by Gs in M2 (Fig. 2A). Both of these mutations yield high levels of HSUR 1 in mouse L cells (Fig. 2B, lanes 2,3) although the amount of M2 (10-fold relative to wild type) is slightly greater than that of M1 (7-fold relative to wild type). We then made single point mutations in the HSUR 1 5' end, each disrupting only one copy of the AUUU repeat (M3–M6; see Fig. 2A). M3, with two copies of AUUUAU flanked by Us, is the only mutant expressed at low levels (Fig. 2B, lane 4); yet its amount is reproducibly higher than that of wild-type HSUR 1 (1.8-fold; Fig. 2B, lane 1). M4, M5, and M6 contain only one copy of UUAUUUAUU, as does M1; their RNA levels are similar to that of M1 (Fig. 2B, lanes 5–7; 5.8-, 6.4-, and 6.1-fold of wild type, respectively) and are lower than that of M2. Similar results were obtained in repeated experiments. These results suggest that at least two copies of UUAUUUAUU and the activity of the ARE as an snRNA destabilization element in vivo.

The HSUR 1 ARE can also target a β-globin reporter mRNA for degradation

Because the AU-rich 5' end of HSUR 1 is similar to the AREs found in the 3' UTRs of ERG mRNAs, we transplanted sequences from wild-type HSUR 1 and mutants M1–M6, all driven by the same U1 promoter, were each transiently cotransfected with HSUR 3 into mouse L929 cells, and the RNA levels were assayed by T1 RNase protection (lanes 1–7, respectively). The antisense HSUR 1 probe covered a 120-nucleotide region at the 3' end, which is common to wild-type HSUR 1 and all six mutants. When quantitated and normalized to HSUR 3, the mutant M1–M6 levels were found to be 7.0-, 10.1-, 1.8-, 5.8-, 6.4-, and 6.1-fold that of wild-type HSUR 1.
As is evident from Figure 3, the positive control pB-ARE mRNA, which contains the c-fos ARE and has been shown previously to be functional in NIH-3T3 cells (Zubiaga et al. 1995; Myer et al. 1997), is unstable in L cells with levels below 17.5% 5.5 hr postinduction. On the other hand, the negative control mRNA from the parental pBBB plasmid is stable (Zubiaga et al. 1995; Myer et al. 1997), exhibiting an RNA level >80% after 5.5 hr. The sequence from the 5'8 end of wild-type HSUR 1 is even more destabilizing than the c-fos ARE, reducing mRNA levels below 8% by the 5.5-hr time point. In contrast, both the M1 and M2 mutant sequences are ineffective as destabilization elements, with 74.5% and 88% RNA remaining 5.5 hr postinduction. Only the sequence from mutant M3 confers partial instability, with 37% of the RNA remaining after 5.5 hr. These results parallel the data shown in Figure 3: Wild-type HSUR 1 exhibits the lowest RNA level and M3 is the second lowest, whereas M1 and M2 levels are high and not detectably different.

Hence, the viral ARE and its mutants appear to function equivalently in the 5' end of HSUR 1 and in the 3' UTR of a reporter mRNA, conferring rapid degradation on both RNA molecules.

Binding of the HuR protein correlates with the in vivo cis-acting degradation activity of the viral ARE

Because the AU-rich sequences from HSUR 1 and its mutants tested here are different from those analyzed previously (Myer et al. 1997), we performed UV cross-linking experiments to assess their binding to HuR. Relative affinities for the HuR protein were measured by the ability of each sequence to compete at 10-, 20-, and 50-fold molar excess with a radioactively labeled wild-type HSUR 1 transcript in HeLa cell nuclear extract. In the absence of competitor RNA (Fig. 4, lanes 1,14), the lowest band (arrow) in the UV cross-linking profile has been identified as HuR (Myer et al. 1992); its cross-link-
When transiently expressed in mouse L cells from copies of AUUU interrupted only by a guanosine (G) (Fig. 5A), we also made two other mutants as controls, M2 and M3 (Fig. 5A), which have substituted the G with CC and CA, respectively, at the same position. When transiently transfected into mouse L cells, HSUR 2 M1 (Fig. 5B, lane 2) exhibits a RNA level one-fifth that of the wild-type HSUR 2 (lane 1), whereas the control M2 and M3 levels are comparable with the wild-type HSUR 2 (lanes 3, 4, and 1, respectively). We conclude that tandem copies of AUUU can also target HSUR 2 for rapid degradation in vivo.

Because HSUR 1 and HSUR 2 are both viral snRNAs, we further investigated whether the (AUUU)$_4$A sequence would confer instability on a cellular snRNA. It is known that the 5' splice site recognition sequence of U1 (nucleotides 3-10) is dispensable and that substitution of this sequence does not affect U1 snRNA stability (Yuo and Weiner 1989). We therefore replaced this region with (AUUU)$_4$A (mutant AU3–U1; Fig. 5C) and with the nonfunctional mutant ARE sequence of HSUR 1 M2 (mutant AGU–U1; Fig. 5C), which contains four copies of AUUUUA interrupted by three Gs. These U1 constructs were transiently cotransfected with HSUR 3 into mouse L cells. In this case, U1 levels were analyzed by primer extension using a labeled oligonucleotide complementary to the 3' end of U1 (nucleotides 155-164); the extension product from endogenous U1 is 164 nucleotides in length, whereas those for the two mutants are 176 nucleotides. HSUR 3, assayed by T1 RNase protection, served as an internal control. As shown in Figure 5D, AU3–U1 is expressed at much lower levels than AGU–U1. When normalized against HSUR 3 and averaged between the duplicate experiments, the AU3–U1 level was found to be one-fourth that of the AGU–U1. Hence, the HSUR 1 ARE is also transferable to a cellular snRNA, conferring rapid degradation.

**Figure 4.** Binding of viral ARE sequences to HuR. The binding of the HuR protein to $[^{32}P]$UTP-labeled wild-type HSUR 1 transcript in the presence of unlabeled wild-type or mutant HSUR 1 competitor RNA was assayed by UV cross-linking in HeLa cell nuclear extract. After RNase A treatment, label transfer was visualized by electrophoresis in a 12.5% SDS-polyacrylamide gel. The competitor RNAs, whose sequences are given in Figs. 1A and 2A, are indicated at top of the gel. (Lanes 1-14) Proteins cross-linked in the absence of specific competitor RNA. For each set of competitors, the first lane (lanes 2, 5, 8, 11, 15, 18, 21, 24) contains a 10-fold molar excess, the second lane (lanes 3, 6, 12, 16, 19, 22, 25) a 20-fold molar excess, and the third lane (lanes 4, 7, 10, 13, 17, 20, 23, 26) a 50-fold molar excess over the labeled HSUR 1 RNA. The cross-linked HuR protein is indicated with an arrow.

Discussion

It has been known for some time that an ARE within the 3' UTR confers instability on its host mRNA (for review, see Belasco and Brawerman 1993; Chen and Shyu 1995). Our study provides the first evidence that AREs are also able to destabilize snRNAs including viral HSUR 1, HSUR 2, and cellular U1 (Figs. 1 and 5). SnRNAs differ from mRNAs in that they are not polyadenylated and do not associate with ribosomes; the AREs we have analyzed are located in the snRNAs near the 5' end, a region known to be accessible within the snRNP particles for interaction with trans-acting molecules (S. Lee et al. 1988). We show that the viral ARE and its mutants exhibit comparable destabilization activities when transferred from the snRNA to the 3' UTR of a reporter mRNA (Figs. 2 and 3). Because an ARE sequence's in vitro binding affinity for the HuR protein correlates with its ability to target in vivo degradation of both snRNAs (Figs. 2–4) and mRNAs (Myer et al. 1997), the HuR pro-
tein may participate in ARE-mediated decay of both snRNAs and mRNAs.

ARE-mediated snRNA decay and Sm snRNP biogenesis

Both mRNAs and the U snRNAs studied here are transcribed by RNA polymerase II. mRNAs then have their introns removed and become polyadenylated in the nucleus before transport to the cytoplasm, where they are translated on polysomes (for review, see Dreyfuss et al. 1996). The maturation and trafficking of U snRNAs, however, are quite different. After transcription, precursor snRNAs are exported to the cytoplasm, where they assemble with the core Sm proteins; the 5' m7G cap becomes hypermethylated, and the 3' end is often trimmed. Sm snRNPs are then transported back into the nucleus, where they participate in splicing or other nuclear functions (for review, see Dahlberg and Lund 1988; Parry et al. 1989).

Here, we have shown that the ARE at the 5' end of wild-type HSUR 1 confers instability (Fig. 1B). Yet, in transiently transfected L cells, 90% of both the unstable wild-type and stabilized mutant HSUR 1 are assembled with Sm proteins (Fig. 1D), which appear to contribute to the stabilization of snRNAs (Jones and Guthrie 1990; Terns et al. 1993; Fischer et al. 1994). Thus, the instability conferred by the HSUR 1 ARE appears to oc-

Figure 5. AUUUA repeats target other snRNAs for rapid decay. (A) The 5' ARE of wild-type HSUR 2 and its mutants. The single guanosine that interrupts the AUUUA repeat sequence in HSUR 2 was mutated to UA, CC, and CA (underlined) in HSUR 2 M1, HSUR 2 M2, and HSUR 2 M3, respectively. (B) RNA levels of wild-type and mutant HSUR 2s. Wild-type HSUR 2 or mutant M1, M2, or M3, all controlled by the same U1 promoter, were each transiently cotransfected with HSUR 3 into mouse L929 cells and analyzed by T1 RNase protection. The level of mutant M1 (lane 2), which has four tandem copies of AUUUU, is one-fifth that of wild-type HSUR 2 (lane 1), whereas controls M2 and M3 (lanes 3 and 4, respectively), which have been mutated at the same two positions as M1, have levels 1.3- and 1.1-fold of the wild-type HSUR 2, respectively. (C) The 5'-end sequences of wild-type U1 and two U1 mutants. The 5' splice site recognition sequence of U1 and the sequences replacing it in the mutants are underlined. The AU3-U1 mutant has four tandem copies of AUUU, whereas in the AGU-U1 mutant, there are four AUUU repeats interrupted by 3 Gs (the same sequence as in HSUR 1 M2). (D) Levels of U1 RNA levels were analyzed by primer extension using an oligonucleotide complementary to the most 5' 20 nucleotides of U1 (nucleotides 155-164), whereas HSUR 3 was assayed by T1 RNase protection assay as above. When normalized to HSUR 3 and averaged between the duplicate transfection experiments, the AU3-U1 level was found to be one-fourth that of AGU-U1.
cur rapidly, possibly even before assembly into Sm snRNPs. We speculate that there may be a competition for binding of HSUR 1 transcripts by RNA destabilization factors versus Sm proteins. Whether decay occurs in the nucleus or in the cytoplasm is a problem for future investigation.

Comparison of ARE-mediated snRNA and mRNA degradation

It has been demonstrated that ARE degradation signals within the 3' UTR function independently of the host mRNA coding sequence and are transferable to other mRNAs (for review, see Belasco and Brawerman 1993; Chen and Shyu 1995). We have found that the 5' ARE of HSUR 1 can also be transplanted to other snRNAs (Fig. 5), demonstrating the independence of the cis-degradation signal from the host snRNA sequence. Mutational analyses of ARE sequences located at the 5' end of HSUR 1 (Fig. 2) or within the 3' UTR of β-globin mRNA (Fig. 3) revealed comparable destabilization activities (Figs. 2 and 3), suggesting that common sequences orchestrate both decay processes.

Mutational analyses of the HSUR 1 ARE (Fig. 2A,B) further indicated that the shortest degradation sequence among those we tested in mouse L929 cells is two overlapping copies of UUAUUUAUU, namely UUAUUU-AUUAAUU (as in mutant M3). This sequence targets β-globin mRNA weakly for destabilization in the same cell line (pB–H1 M3; Fig. 3A,B). In NIH-3T3 cells, the minimum mRNA degradation signal has been reported to be UUAUUUAUU (Zubiaga et al. 1995; Lagnado et al. 1994), which was confirmed in our previous studies (Myer et al. 1997). Our data therefore suggest some cell-type specificity, because UUAUUUAUU is unable to confer instability on either mRNA (data not shown) or snRNA (HSUR 1 M1, Fig. 2A,B) in L929 cells. At least two overlapping (pB–H1 M3; Fig. 3) or two separate copies of UUAUUUAUU sequences (data not shown) are required to destabilize the reporter β-globin mRNA in L929 cells.

We have shown previously that the in vitro affinity of an ARE sequence for the HuR protein correlates with its in vivo ability to target an mRNA for rapid degradation (Myer et al. 1997). Here, we report that the same correlation exists for snRNA destabilization and HuR binding (Figs. 3 and 6), arguing that HuR is likely to play an important role in ARE-mediated decay of snRNAs as well. Because the HuR protein has no detectable nuclease activity (Myer et al. 1997), it is possible that it recognizes and initiates the assembly of degradation complexes on ARE sequences. The observation that HuR protein is present in both nuclear (Vakalopoulou et al. 1991; Myer et al. 1992, 1997) and cytoplasmic extracts (Vakalopoulou et al. 1991) leaves open the question of whether decay occurs in the nucleus, the cytoplasm, or both. However, it is also conceivable that HuR may be a stabilizing factor that escorts mRNAs and snRNAs from the nucleus to the cytoplasm, providing protection from nucleases until its binding is actively reversed. Future studies involving altering HuR levels in vivo and producing monospecific anti-HuR antibodies will provide further insights into the role of HuR in RNA destabilization. Because ARE-mediated snRNA and mRNA degradation share the same cis-destabilization signals as well as a putative trans-acting factor, HuR, other components involved in the two degradation pathways may also be shared.

One controversial issue in ARE-mediated mRNA degradation is whether or not it is coupled to ongoing translation; there have been reports supporting both alternatives. Abolishing translation initiation by mutating the AUG translation start codon or introducing a strong stem-loop structure into the 5' UTR has been shown to stabilize mRNAs containing an ARE (Savant-Bhonsale and Cleveland 1992; Aharon and Schneider 1993). Degradation can be re-established when translation is restored by introducing an internal ribosomal landing sequence downstream of the stem-loop (Aharon and Schneider 1993), providing evidence for translational coupling. However, it was demonstrated later that inserting a strong stem-loop structure anywhere upstream of an ARE sequence stabilizes the mRNA (Curatola et al. 1995). Similarly, placing the iron response element (IRE) in the 5' UTR to regulate mRNA translation has produced conflicting results: Destabilization of an α-globin reporter mRNA by the c-fos or GM-CSF ARE appeared dependent on polysome loading and translation in growing NIH-3T3 cells (Winstall et al. 1995), whereas the destabilization of transferrin receptor reporter mRNA by the c-fos ARE was observed to be independent of its translation (Koeller et al. 1991). Finally, using a serum-inducible c-fos promoter and inserting a hairpin structure into the 5' UTR to inhibit translation, Chen and coworkers (1995) observed that destabilization of a β-globin reporter mRNA by the c-fos or GM-CSF ARE can be uncoupled from their translation.

If ARE-mediated mRNA and snRNA decay indeed utilize the same or overlapping pathways, our data favor the idea that the mRNA decay is not obligatorily coupled to translation. Because snRNAs do not contain open reading frames, ARE-promoted snRNA degradation cannot require translation. Moreover, even though the first step of mRNA degradation is deadenylation (Shyu et al. 1991; Chen et al. 1995), our observation that nonpolyadenylated snRNAs are destabilized by the same sequences suggests that interactions with the poly(A) tail are not always essential to initiate decay. Additional evidence that AUUUA-containing untranslated RNAs and mRNAs may be degraded by the same mechanism has been reported based on studies in murine macrophages (S. Kaszuba, D. Angerer, T. Said, A.V. Gabain, and M. Baccarini, in prep.).

Biological significance of HSUR 1 degradation

HSUR genes are conserved between oncogenic subgroups of H. saimiri (Biesinger et al. 1990) and are expressed only during latent infection (Murthy et al. 1986; S. Lee et al. 1988). Genetic analysis of subgroup A has
identified a viral transforming protein, called STP-A11, that is required for viral-induced T-cell immortalization in culture (Desrosiers et al. 1985; Murthy et al. 1989; Junge et al. 1991). The ATP-A11 gene does not continue to be expressed in transformed T cells, suggesting that it may be important for the induction but not the maintenance of viral transformation (Kamine et al. 1984; Murthy et al. 1989). Viral deletion mutants lacking HSUR 1, HSUR 2, and HSUR 5 are still able to transform T cells in culture. However, the transformed cells grow much more slowly than those transformed by wild-type H. saimiri (doubling time of 1 week vs. 1.5 days), arguing that these HSURs may be important only for the maintenance of viral transformation (Murthy et al. 1989).

Why then does H. saimiri produce an snRNA (HSUR 1) that is quickly degraded, especially because viruses are known for evolving economical modes of gene expression? Our study suggests that rapid HSUR 1 degradation may be significant for maintaining viral transformation: HSUR 1 shares and may compete with mRNAs for the cellular degradation machinery, sacrificing itself, whereas host mRNAs with AREs are in turn stabilized. Elevated levels of these mRNAs, encoding proto-oncoproteins, lymphokines, and cytokines, would then enhance the viral transformed state. An alternative possibility is that HSUR 1, HSUR 2, and HSUR 5 bind HuR and retain it in the nucleus, as proposed previously (Myer et al. 1992). It will therefore be interesting to investigate whether expression of HSURs in either transformed T cells or transfected mammalian cell lines alters the localization of HuR and other putative components of the RNA degradation machinery, such as an RNase E-like activity that cleaves AUUUA sequences (Wenborg et al. 1995).

It is quite possible that in addition to HSUR 1, other viral products (either proteins or other HSURs) are required to elevate the levels of cellular ARE-containing mRNAs. This may explain why transiently expressing HSUR 1 alone in mouse L929 cells did not produce higher levels of β-globin mRNA with the GM-CSF ARE at its 3' UTR (X. C. Fan, V. E. Myer, and J. A. Steitz, unpubl.). It is also possible that HSUR 1 may function to stabilize proto-oncogene and growth factor mRNAs only in certain host cells, such as marmoset T cells. Lack of a negative-control cell line (a non-viral-infected marmoset T cell line with similar growth rates to the transformed 1670 cells) makes it difficult to test this hypothesis directly. Future exploration of the model will involve analysis of point mutations within the HSUR 1 gene in the H. saimiri genome and testing the transformation efficiency and maintenance of these mutant viruses in marmoset T cells.

Materials and methods

Cell culture, nuclear extracts, and transient transfections

Murine L929 fibroblasts were passed in minimal essential medium (MEM, GIBCO) supplemented with 10% horse serum (HS; GIBCO). HeLa cells were maintained in RPMI 1640 medium (GIBCO) with 10% fetal bovine serum (FBS; GIBCO). NIH-3T3, COS, HEK-293, and Jurkat cells were grown in 10% FBS-Dulbecco’s modified Eagle medium (DMEM; GIBCO). HeLa cell nuclear extracts were prepared as described (Dignam et al. 1983), modified as in Myer and Steitz (1995).

L929 cells were grown in 100-mm dishes to between 50% and 70% confluence and then switched to MEM medium supplemented with 10% NuSerum (GIBCO) for DEAE-dextran transfection. Plasmids (4–6 µg) were resuspended in 40 µl of TBS, mixed with 80 µl of 10 mg/ml DEAE-dextran (Sigma)/TBS, and then overlaid on cells. After incubation for 4 hr, cells were shocked with 10% DMSO/Phosphate-buffered saline (PBS) for 1 min and allowed to recover in 10% HS-MEM. For investigation of β-globin mRNA stability, 3 µg of β-globin plasmid and 2 µg of control pEF-BOS-CAT plasmid were used. Transfected L929 cells were recovered in 10% HS-MEM overnight and serum-starved in 0.5% HS-MEM for 24 hr. Cells were then serum-stimulated by addition of 20% HS-MEM, and RNA was isolated at 0, 1, 2, 5, 4, and 5.5 hr.

Oligonucleotides and plasmids

The plasmid pUC-U1 was a generous gift of Dr. Alan Weiner (Yuo and Weiner 1989). Wild-type HSUR 1, HSUR 2, and HSUR 3 sequences followed by the U1 3'-end box were generated by PCR using 5' overhang oligonucleotides and cloned between the BglII and SalI sites in the pUC-U1 plasmid (Fig. 1A). Oligonucleotide-directed PCR mutagenesis was used to introduce mutations into the 5' end of HSUR 1, HSUR 2, and U1, to generate mutants HSUR 1 Mut, M1-M6, HSUR 2 M1-M3, AU3-U1, and AGU-U1 (Figs. 1A, 2A, and 5A,C). All HSURs were also PCR-cloned between the EcoRI and HindIII sites of the pSP64 vector (Promega). After linearization with EcoRI restriction enzyme, antisense HSUR RNAs for RNase protection assays were generated by transcription with SP6 RNA polymerase in the presence of [α-32P]UTP. The DNA oligonucleotide used in the U1 primer extension assay was complementary to 20 nucleotides at the 3' end of U1: CAGGGGAAAACGC-8

Plasmids for transient transfection contained a genomic copy of the rabbit β-globin gene driven by the serum-inducible human c-fos promoter. A 51-bp sequence from the c-fos 3' UTR (bp 3099 to bp 3149 from the translation start site) followed by a test degradation sequence was inserted directly after the β-globin stop codon. The plasmids pB-ARE and pBBB, as well as the internal control plasmid pEF-BOS-CAT, have been described previously (Shyu et al. 1989; Zubiaga et al. 1995; Myer et al. 1997). Plasmids pB-Wt H1, pB-H1 M1, pB-H1 M2, and pB-H1 M3 were generated by replacing the c-fos AU-rich sequence in pB-ARE with the four different 32-bp sequences (+1 to +32) from the 5' end of wild-type HSUR 1 and mutants M1, M2, and M3, respectively. The pGEM-3Z plasmids (Promega) transcribed to produce the antisense β-globin and CAT RNAs used in RNase protection assays have been described previously (Myer et al. 1997).

RNase protection and primer extension assays

Total RNA was collected from transfected cells using Trizol reagent (GIBCO) and treated with RNase-free DNase (Promega). T1 RNase protection assays were performed as described (S. Lee et al. 1988), with the following modifications: DNase-treated RNA (5–10 µg for snRNA, 20–30 µg for mRNA) was combined with 2 × 10^4 to 4 × 10^5 cpm of the appropriate [α-32P]UTP-labeled antisense probe, heated at 85°C for 5 min, incubated at 45°C overnight to allow annealing, and then digested with T1 RNase (1 U/10 µg of RNA; Calbiochem) at 30°C for 1 hr. Samples were
electrophoresed on a 6% polyacrylamide/TBE gel. Results were quantitated with a Molecular Dynamics PhosphorImager.

Primer extension assays were performed as described by Tarn and Steitz (1994) with the following modifications: Five micrograms of total RNA was mixed with 2 × 10^5 to 5 × 10^5 cpm of [α-32P]UTP kinase-labeled DNA primer, heated at 85°C for 5 min, and slowly cooled to 42°C to allow annealing. Reverse transcription was performed at 42°C for 30 min, and the samples were electrophoresed on a 6% polyacrylamide/TBE gel.

Whole cell run-on assays

Transiently transfected L929 cells in 100-mm dishes were permeabilized and collected according to Sheldon et al. (1993). After resuspension in 50 µl of buffer B, 15 µl of [α-32P]UTP (150 µCi) and 100 µl of reaction buffer were added and the mixture was incubated at 30°C for 10 min. One milliliter of Trizol solution (GIBCO) was added to terminate the reaction and to harvest total RNA, which was DNase treated, denatured in 0.16 M NaOH buffer on ice for 10 min, and neutralized in 0.3 M HEPES (pH 7.9) and 0.3 M NaOAc.

All DNA probes were purified and denatured and corresponded to the full-length snRNAs. They were titrated to ensure that they were in excess of the amount of RNA being assessed by hybridization; 100 ng of each was dot-blotted and UV cross-linked onto a nylon filter (Bio-Rad), which was incubated in hybridization solution (50% formamide, 0.5% SDS, 200 µg/ml of salmon sperm DNA, 6× SSC, 1× Denhardt’s solution) at 55°C twice for 15 min, at room temperature.

The radioactive run-on RNA harvested from a 100-mm dish was fractionated on a 12.5% polyacrylamide/1% SDS gel as described (Myer et al. 1992).

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Xinhao Cynthia Fan, Vic E. Myer and Joan A. Steitz

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