Evidence for instability of mRNAs containing AUUUA motifs mediated through translation-dependent assembly of a >20S degradation complex

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Many short-lived mRNAs, including those encoding lymphokines, cytokines, and proto-oncogenes, contain an AU-rich sequence in their 3'-untranslated regions. These AU domains and, more specifically, AUUUA motifs within them, are widely thought to mediate the extreme instability of the corresponding mRNAs. This is most clearly true for granulocyte monocyte colony stimulating factor (GM-CSF) mRNA whose AUUUA motifs are conserved phylogenetically and whose presence in an otherwise stable β -globin mRNA results in a 50-fold decrease in accumulated mRNA level. We show that RNA instability conferred by the GM-CSF AU motif requires the mRNA to be actively translated and the AU motif to be within the 3'-untranslated region. By analysis of the sedimentation characteristics, we identify a large (>20S), divalent cation-independent complex found only on unstable RNAs. Like instability, complex formation (1) is dependent on translation of the RNA, (2) requires intact AUUUA motifs, and (3) is blocked by ribosome translocation across the AU-rich motif. We propose that RNA instability mediated by the AU motif is achieved through translation-dependent assembly of this large mRNA-destabilizing complex.

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Many highly unstable mammalian mRNAs, including those encoding proto-oncogenes, cytokines, and lymphokines, contain within their 3'-untranslated regions a common AU-rich motif of 30–80 bases. In most of these regions, besides the high AU content, the most obvious sequence motif is a variable number (3–8) of AUUUA pentamers (Shaw and Kamen 1986). In the case of granulocyte monocyte colony stimulating factor (GM-CSF), this AU-rich domain is the most highly conserved region between mouse and human GM-CSF mRNAs (Gough et al. 1985; Wong et al. 1985). The importance of the AU domain in mediating mRNA instability has been shown previously in two ways. First, insertion of 14 G and C residues into the AU domain (specifically to disrupt all AUUUA motifs) results in >10-fold higher abundance of the mRNA, probably owing to changes in RNA stability (Shaw and Kamen 1986; Vakalopoulou et al. 1991). Second, the domain appears to contain a dominant, *cis*-acting element that can destabilize the otherwise very stable rabbit β -globin mRNA (Shaw and Kamen 1986). In other labile mRNAs, similar AU-rich domains have also been implicated in mediating mRNA instability. For *fos*, a 75-base AU domain (containing three AUUUA motifs) has been clearly demonstrated to destabilize the normally stable β -globin mRNA (Shyu et al. 1989). Evidence that the AUUUA repeats are important to instability

mediated by this *fos* domain has also been provided by use of U $\rightarrow$ A substitutions to disrupt each motif: The resultant domain is inhibited dramatically in its ability to specify rapid degradation (Shyu et al. 1991).

Despite much preceding work, particularly on defining RNA domains that can destabilize other RNAs into which they are placed, the basic mechanics of the RNA destabilization machinery have yet to be defined for any higher eukaryotic mRNA. One approach to identification of the key components (the nucleases involved and mode of activation, the method and components that mediate RNA recognition, and the compartment in the cytoplasm where cleavage takes place) would be available if *in vitro* conditions could be identified where RNA instability reproduces the *in vivo* pathway. Efforts along these lines, primarily from Ross and colleagues (Brewer and Ross 1988, 1989; Bernstein et al. 1989, 1992; Brewer 1991) have characterized degradation of both histone and *c-myc* mRNAs in an *in vitro* system comprised of a ribosome-associated 3' $\rightarrow$ 5' exonuclease and polysome-bound RNAs. But the degree to which this is a faithful mimic of *in vivo* instability remains to be proven, for example, through the use of mutant RNAs known to be stable *in vivo*. A further difficulty is that addition of poly(A)-binding protein stabilizes RNAs *in vitro* (Bernstein et al. 1989), a finding apparently at odds with the

demonstration in yeast that poly(A)-binding protein is necessary for poly(A) shortening (Sachs and Davis 1989).

An alternative approach has been to search for components that bind to specific sequence motifs, such as an AU-rich motif. Gel-shift and UV-cross-linking experiments have identified a 32-kD, predominantly nuclear, protein that binds to the GM-CSF, *c-fos* and *c-myc* AU-rich sequences (Vakalopoulou et al. 1991). Two other factors have been found by a similar approach (Bohjanen et al. 1991). One (AU-B) is an inducible cytoplasmic factor that interacts with the 3'-untranslated region of GM-CSF mRNA but not with *c-myc* mRNA. The other (AU-A) is a nuclear protein found in many cell types and binds equally to the GM-CSF and *c-myc* AU-rich 3'-untranslated regions. Malter (1989) reported AUBF, a set of three small lymphocyte proteins (similar to AU-A) that bind very tightly to an in vitro-synthesized target sequence consisting of four repeats of the AUUUA motif. The role of this factor in in vivo mRNA degradation is not proven, but AUBF does bind to the AU-rich motifs of GM-CSF, *c-fos*, and *v-myc* (Gillis and Malter 1991). Finally, a pair of 37- and 40-kD cytosolic proteins purified by their ability to enhance in vitro degradation of *c-myc* have been shown to bind to an AU-rich domain of *c-myc* RNA (Brewer 1991).

To compliment these in vitro efforts, we have now used an in vivo approach to focus on the mechanism of RNA degradation mediated by the 51-base GM-CSF AU-rich domain. We show that instability mediated by the AU motif requires translation of the RNA containing it and that the AU motif cannot destabilize an RNA if it is placed in a translated region. By analyzing the sedimentation of RNAs from cells transfected with a set of β -globin gene constructs that carry this AU domain, we document the translation-dependent assembly of a large complex onto all rapidly degraded RNAs that carry this GM-CSF destabilizing domain. Disruption of the AUUUA motifs by G or C insertion or ribosome translation across the AU domain blocks complex formation and the RNAs are stable. We propose that RNA instability mediated by the AU motif is achieved through translation-dependent assembly of this $\geq 20S$ mRNA-destabilizing complex.

Results

Reduced accumulation of mRNAs containing the GM-CSF AU domain requires translation of those mRNAs

A wide series of preceding experiments on transiently expressed, unstable mRNAs have shown that translation inhibitors almost invariably increase abundance of the mRNA. More specifically, this property has been shown to be conferred to rabbit β -globin mRNA by introduction of a 51-base AU-rich destabilization domain from GM-CSF (to produce gene $R\beta G^{AT}$) but not to a control globin mRNA ($R\beta G^{GC}$) in which the AUUUA motifs in the GM-CSF domain are disrupted by insertion of G and C residues (Fig. 1A; Shaw and Kamen 1986). To address

whether translation per se plays a role in controlling the level of RNAs containing this AU-destabilizing domain, we prepared a series of genes based on the constructs of Shaw and Kamen but in which translation of the mRNA was altered either by premature termination or by translation into the destabilizing domain. Schematic drawings of these genes are displayed in Figure 1B. Positions of predicted translation initiation and termination are marked. In naming each construct, the superscript number refers to the predicted number of translated codons, and the superscript letters denote whether the AU domain is intact (AT) or has been disrupted by G and C residues (GC).

To test accumulated levels of each RNA after stable DNA transfection, polyclonal cell lines were generated by transfection of each construct into mouse L cells or tsAF8 cells, a line of baby hamster kidney cells that contains a temperature-sensitive RNA polymerase II (Meiss and Basilico 1972; Rossini et al. 1980; Jaskulski and Baserga 1988). After selection for G418 resistance, >200 colonies were pooled for each construct. Total cytoplasmic RNAs were prepared, and the amount of β -globin RNA accumulated from each transfected construct was determined with an RNase protection assay using a ^{32}P -labeled β -globin RNA probe. As expected from the earlier work (Shaw and Kamen 1986), a large difference in abundance was seen when the $R\beta G^{AT}$ and $R\beta G^{GC}$ RNAs were compared (Fig. 2A–C). The level of the AU-containing RNA was undetectable when 10 μ g of RNA was assayed (Fig. 2A), although the comparable GC construct showed a strong signal. When 50 μ g of RNA from cells transfected with $R\beta G^{AT}$ was analyzed, a signal could be detected, but even here it was only about one-tenth of the level of the GC construct. By analyzing dilutions of purified β -globin RNA to provide quantitation standards and an S1 nuclease assay for γ -actin RNA levels to ensure that comparable amounts of RNA had been analyzed, $R\beta G^{AT}$ RNAs in both tsAF8 cells (Fig. 2B) and L cells (Fig. 2C) were seen to be 40- to 50-fold less abundant than their $R\beta G^{GC}$ counterparts. (Because of this large difference in accumulation, for all subsequent experiments we routinely analyzed five times more RNA from cells expressing the AU-containing gene constructs than we used for those expressing the homologous GC-containing constructs.)

When translation was shortened prematurely by introduction of a termination codon at position 100 of the 147-amino-acid β -globin sequence, again the AU-containing RNA accumulated to less than one-fortieth of the similarly terminated GC-containing control (Fig. 2B,C). When a translation terminator was introduced at codon 22 ($R\beta G^{AT22}$), this too yielded less than one-thirtieth of the level of the $R\beta G^{GC22}$ control. These results indicate clearly that translation of the majority of the coding region of the mRNA is not required for the low RNA accumulation phenotype conferred by the AU motif of GM-CSF.

Blockage of translation by mutation of the ATG initiator methionine codon to a TAG (to produce construct $R\beta G^{AT1}$), however, resulted in a 10-fold higher level of

Figure 1. Sequence of the AU-rich motif from GM-CSF and schematic diagrams of β -globin genes carrying this motif in different positions. (A) The 62-base sequence containing the AU-rich motif from the 3'-untranslated region of GM-CSF mRNA and a similar sequence (denoted GC) in which all AUUUA motifs have been disrupted by 14 G or C substitutions. (B) Schematic drawings for the various β -globin genes used in this study. Plasmid names are shown at left, where AT/GC indicates the presence of either the 62-base AT-rich segment or the 62-base GC sequence, respectively, from A. (Solid area) exons; (open area) introns; (wavy lines) flanking sequences; (stippled area) inserted synthetic sequences (AT-62 or GC-62). (PyEP) Polyoma early promoter; (pA) the globin polyadenylation site. The predicted translation initiation codon and translation termination codons are marked; the numbers denote the respective codon positions. Arrows indicate the transcription initiation sites.

accumulation compared with the fully translated AU-containing RNAs (although the level was still only one-third of the homologous $R\beta G^{GC1}$ control). If all genes are transcribed at comparable rates (as expected), and the $R\beta G^{AT1}$ construct does not initiate translation efficiently at an alternative site (considered in detail below), these results demonstrate that the GM-CSF AU domain confers a low RNA phenotype primarily through a translation-dependent mechanism.

Ribosome translocation across the GM-CSF AU motif blocks its ability to specify a low RNA phenotype

To test further the mechanism through which the AU motif confers a low RNA phenotype, we examined whether translation across the domain affected its ability to yield the low RNA phenotype. We did this in two ways. First, we constructed $R\beta G^{AT156}$ by insertion of 4

bases at codon 121 of $R\beta G^{AT}$. This produced a frameshift that extended translation to a TAA sequence that lies 26 bases within the AU motif. Despite the presence of an intact AU motif in essentially the same position of the mRNA, the AU domain no longer functioned to yield a low RNA phenotype; rather, the $R\beta G^{AT156}$ RNA was >20 times more abundant than $R\beta G^{AT}$ RNA and only slightly less abundant than the $R\beta G^{GC153}$ control (Fig. 2). Second, because the 51-base AU motif contains an open reading frame in reading frame 2, we tested whether it could confer a low RNA phenotype when moved to within the normal β -globin-translated domain. We ligated the AU domain at codon 99 of $R\beta G$, producing $R\beta G^{ATC}$, whose translation product ends at the normal β -globin terminator but carries 21 additional codons from insertion of the AU domain within the translated region. As seen in Figure 2, the AU element does not yield a low RNA phenotype when present in this actively translated domain.

Figure 2. Relative steady-state levels of β -globin mRNAs containing an intact or disrupted AU motif from GM-CSF. (A) Levels of β -globin RNAs determined by RNase protection analysis of total cytoplasmic RNA isolated from pools of stably transfected derivatives of cell line tsAF8. (Lanes 1,2) 10 and 50 μ g of RNA from $R\beta G^{AT}$; (lane 3) 10 μ g of RNA from $R\beta G^{GC}$; (lanes 4,5) 10 and 50 μ g of RNA from $R\beta G^{AT100}$; (lane 6) 10 μ g of RNA from $R\beta G^{GC100}$; (lanes 7,8) 10 and 50 μ g of RNA from $R\beta G^{AT22}$; (lane 9) 10 μ g of RNA from $R\beta G^{GC22}$; (lane 10) 10 μ g of RNA from $R\beta G^{AT1}$; (lane 11) 10 μ g of RNA from $R\beta G^{GC1}$; (lane 12) 10 μ g of RNA from $R\beta G^{AT156}$; (lane 13) 10 μ g of RNA from $R\beta G^{GC153}$; (lane 14) 10 μ g of RNA from $R\beta G^{ATC}$; (lane 15) 10 μ g of RNA from $R\beta G^{GCC}$. (B) Relative RNA abundance was determined by densitometry of the analyses in A. (C) Relative RNA abundance was determined from RNase protection analyses similar to that in A, except that all cell lines were stable transformants of L cells.

Taken together, these experiments demonstrate that for the low RNA phenotype to be conferred by the AU domain, the RNA itself must be translated (e.g., the results of $R\beta G^{AT1}$), but translation cannot extend into or across the AU motif (as shown by $R\beta G^{AT156}$ and $R\beta G^{ATC}$).

The low RNA phenotype conferred by the GM-CSF AU motif is mediated by translation-dependent mRNA instability

The differences in mRNA accumulation among the various transcripts examined could be explained either by changes in cytoplasmic mRNA stability or if markedly different rates of RNA production were obtained in different stably transfected cells. Although this latter possibility seemed unlikely because the transcriptional control elements are the same in all of the genes and in all cases we analyzed pools representing >200 individual transformants, we directly tested transcription rates using isolated nuclei. Nuclei from all polyclonal pools were incubated in vitro with [α - 32 P]UTP to allow elongation of already initiated RNA transcripts. After isolation, the labeled RNAs were hybridized to immobilized, single-stranded DNAs specific for the transfected globin, endogenous β -actin, and endogenous β -tubulin RNAs.

The resultant autoradiograms were quantified by densitometry. After normalizing to set actin transcription to a constant value in all cell lines, the relative transcription rate of each transfected AT- or GC-containing construct was measured and tabulated in Table 1. Transcription rates of the various transfected globin genes are similar in magnitude in all lines, with a maximum measured difference of only about twofold. (A similar fluctuation in the level of tubulin transcription was also seen, suggesting that the actual transcription differences among the various globin genes are less than twofold.) In any event, because differences in accumulated steady-state levels of the globin mRNAs are much larger than these modest transcriptional variations, differences in transcription cannot explain the marked changes in accumulated level.

To test directly whether the differences in RNA levels could be attributed to changes in cytoplasmic RNA stability, we followed the kinetics of RNA loss after inhibiting transcription either by adding actinomycin D or shifting tsAF8 derivatives from 34°C to 40.5°C to inactivate the temperature-sensitive polymerase II. For this latter approach, we first used two independent methods to verify that polymerase II activity in tsAF8 derivatives declines rapidly upon temperature shift. Using pulse-labeling with [3 H]uridine, we determined that overall

Table 1. Transcription rates of rabbit β -globin mRNA by nuclear run-on assay

Cell lines	Transcription rate			Steady-state globin RNA
	actin	globin	tubulin	
R β G ^{AT}	1.0	0.6	0.24	1.0
R β G ^{GC}	1.0	0.75	0.39	50.0
R β G ^{AT100}	1.0	0.85	0.31	0.6
R β G ^{GC100}	1.0	0.7	0.4	47.0
R β G ^{AT22}	1.0	0.5	0.24	1.7
R β G ^{GC22}	1.0	1.2	0.45	23.5
R β G ^{AT1}	1.0	0.9	0.31	11.0
R β G ^{GC1}	1.0	0.68	0.36	55.0
R β G ^{AT156}	1.0	0.5	0.2	25.0
R β G ^{GC153}	1.0	0.65	0.34	38.4
R β G ^{ATC}	1.0	0.45	0.2	59.6
R β G ^{GCC}	1.0	0.76	0.22	39.4

Transcription rates were measured by nuclear run-on assay in isolated nuclei. Quantitation of data was obtained by scanning the autoradiograph with a densitometer. Relative rate of transcription was calculated by setting the transcription rate of actin to be 1. Steady-state RNA levels were corrected for apparent transcription rate.

RNA synthesis declines 40% within 2 hr after temperature shift. Because polymerases I and III usually account for >50% of overall RNA synthesis (e.g., Lewin 1980) and because we determined uridine incorporation in isolated nuclei to decline 40% after the addition of 5 μ g/ml of α -amanitin to selectively inhibit polymerase II (not shown), the 40% decline must indicate a very significant, rapid inhibition of polymerase II activity. Second, we used RNA blotting to examine the well-studied, temperature-dependent, transcriptional induction of *hsp70* RNA (for review, see Lindquist 1986). As shown in Figure 3, the fivefold increase in the *hsp70* RNA level that occurs within 2 hr of shifting wild-type hamster cells to 40.5°C was inhibited completely in the tsAF8 mutant line. This must mean that within 2 hr after temperature shift, polymerase II activity declines by at least 80%. Taken together, we conclude that in the tsAF8 line, polymerase II activity is rapidly inactivated at the restrictive temperature.

Using either actinomycin D or the temperature shift to inhibit polymerase II, the abundance of RNAs from each R β G construct was assayed by RNase protection and quantified by densitometry. Each point represents the average of three measurements from two independent experiments. Both methods revealed that insertion of the AU domain into β -globin significantly lowered the RNA half-life to $\sim$ 3 hr (Fig. 4A) compared with that of the R β G^{GC} control RNA, which did not diminish over a 6-hr time course. This half-life is similar to that documented previously (Shaw and Kamen 1986) for R β G^{AT} RNA (although in this earlier study, RNA loss deviated significantly from a first-order decay). Like the initial R β G^{AT} construct, RNAs bearing premature translation termination codons (R β G^{AT100} and R β G^{AT22}) also showed apparent half-lives of $\sim$ 3 hr (Fig. 4B,C), whereas

the homologous GC-containing RNAs were completely stable over the 6-hr observation period. Although the precise magnitude of the difference in stability between the AU- and GC-bearing RNAs could not be assessed because the GC-containing RNAs did not decay measurably, these findings are consistent with the AU domain mediating cytoplasmic RNA degradation, in this instance lowering $t_{1/2}$ to $\sim$ 3 hr. The ability of this AU-rich motif to destabilize β -globin RNA is, however, disrupted if ribosomes translocate across it. Movement of the AU motif to the translated domain (in R β G^{ATC}) yielded an RNA that remained fully stabilized (Fig. 4F), with no detectable loss after 6 hr.

Although RNA accumulation levels for the preceding RNAs can be explained easily by differences in half-lives, examination of the R β G^{AT1} and R β G^{AT156} RNAs yielded unexpected results. Because these RNAs are transcribed at rates comparable to the others (Table 1), but accumulate to levels between 10- and 30-fold higher than R β G^{AT}, R β G^{AT22}, and R β G^{AT100} RNAs (Fig. 2), correspondingly 10- to 30-fold longer half-lives would be needed to explain the differences in abundance. Although modestly extended apparent half-lives were measured (4.0–4.2 hr compared with 3 hr for those that accumulated to much lower levels; Fig. 4D,E), these differences are far too small to account for the marked differences in accumulation. Because in all cases blockage of transcription using either actinomycin D or temperature shift yielded indistinguishable decay kinetics, we do not believe that the quantitative disagreement results from an artifact of the temperature shift or of leaky polymerase II transcription. Rather, the simplest explanation is that both in the temperature-sensitive lines and in the L cells the AU motif-mediated RNA degradation pathway may require ongoing transcription, presumably because a short-lived RNA is involved (directly or indirectly) in the degradation pathway. In any event, we conclude that the AU motif can specify rapid RNA degradation, but only if (1) the mRNA containing it is translated, and (2) ribosomes do not translocate across the AU motif.

Figure 3. Rapid loss of RNA polymerase II activity blocks transcriptional induction of *hsp70* mRNA in tsAF8 cells shifted to the restrictive temperature. Mutant (tsAF8) and parental (BHK-21) cells were grown at 34°C and shifted to 40.5°C for 2 hr. Total cytoplasmic RNA was extracted, and accumulated *hsp70* RNA levels before and after temperature shift were examined by RNA blot analysis using a ³²P-labeled human *hsp70* probe (Wu et al. 1985).

Figure 4. Stability of β -globin RNAs carrying the GM-CSF AU motif measured following inhibition of transcription using temperature shift or actinomycin D. Levels of β -globin RNA remaining following inhibition of transcription were measured using RNase protection to analyze total cytoplasmic RNA. Levels of globin mRNA were measured by densitometry, and the log of the amount remaining was plotted as a function of time (hr). Each point represents the average of three measurements from two independent experiments. Transcription was inhibited in the tsAF8 polyclonal lines (described in the legend to Fig. 2) by shifting the cultures to the nonpermissive temperature (40.5°C). In L-cell derivatives, transcription was blocked by addition of actinomycin D. (A) R β G^{AT} and R β G^{GC} RNAs; (B) R β G^{AT100} and R β G^{GC100} RNAs; (C) R β G^{AT22} and R β G^{GC22} RNAs; (D) R β G^{AT1} and R β G^{GC1} RNAs; (E) R β G^{AT156} and R β G^{GC153} RNAs; (F) R β G^{ATC} and R β G^{GCC}. (Boxes) GC RNA levels; (circles) AU RNA levels. (Solid symbols) Measurements following temperature shift of tsAF8 polyclones; (open symbols) measurements following addition of actinomycin D to L cell polyclones.

Selective translation-dependent assembly of a large, presumptive degradation complex onto unstable, AU motif-containing RNAs

To examine further the mechanism of translation-dependent RNA instability (and to verify the translated properties of each of our constructs), the distribution of each

R β G mRNA in polyribosome profiles was examined. To do this, postnuclear cell lysates were centrifuged on sucrose gradients. Fractions were collected and RNA recovered from each was assayed by RNase protection. The results, shown in Figures 5 and 6, generally confirmed the predictions as to which RNAs would be translated efficiently. RNAs from R β G^{AT}, R β G^{GC}, R β G^{AT156},

A complex mediates AUUUA-dependent RNA stability

Figure 5. Detection of a rapidly sedimenting complex bound to unstable rabbit β -globin mRNAs carrying the GM-CSF AU motif. Polysome profiles were generated from lysates of tsAF8 polyclonal stable cell lines described in Fig. 2. The tops of the gradients are to the *left*. Positions of the 40S, 60S, and 80S ribosomal subunits are marked. Each fraction was treated with proteinase K (200 μ g/ml), RNA was extracted and β -globin RNAs were identified by RNase protection. (A) $R\beta G^{AT}$; (B) $R\beta G^{GC}$; (C) $R\beta G^{AT100}$; (D) $R\beta G^{GC100}$; (E) $R\beta G^{AT22}$; (F) $R\beta G^{GC22}$.

$R\beta G^{GC153}$, $R\beta G^{ATC}$, and $R\beta G^{GCC}$ were all found predominantly attached to multiple ribosomes. For example, $R\beta G^{GC}$ mRNAs (which contain the entire 147-

amino-acid β -globin-coding domain) were primarily associated with 6–8 ribosomes (Fig. 5B), as are those encoded by $R\beta G^{GC153}$ (not shown). RNAs from $R\beta G^{GCC}$,

Figure 6. Absence of rapidly sedimenting complexes on stable RNAs that carry the GM-CSF AU motif. Polysome profiles and RNase protection assays were performed as in Fig. 5. (A) $R\beta G^{AT1}$; (B) $R\beta G^{GC1}$; (C) $R\beta G^{ATC}$; (D) $R\beta G^{GCC}$.

which contains an additional 21 codons as a consequence of insertion of the AU motif into the translated domain, were attached to 7–8 ribosomes (Fig. 6D). This is as expected for efficiently translated mRNAs with dense ribosome packing every 30 codons (Yonath et al. 1987). For mRNAs with shorter translated domains [e.g., $R\beta G^{GC100}$ (Fig. 5D) and $R\beta G^{GC22}$ (Fig. 5F)], appropriately fewer ribosomes were attached. A mutation of the initiating ATG codon in $R\beta G^{GC1}$ yielded an mRNA sedimenting primarily with the 80S peak (Fig. 6B), indicating at most a transient attachment to a single ribosome followed by rapid translation termination.

Compared with the corresponding GC-containing RNAs, however, all AU-containing RNAs that display the low RNA phenotype (i.e., $R\beta G^{AT}$, $R\beta G^{AT100}$, $R\beta G^{AT22}$) showed a reproducible difference in their sedimentation characteristics: In each case, the AU-containing RNA sedimented faster than the GC-containing RNA. For example, the distribution of $R\beta G^{AT}$ mRNAs was offset by one faster sedimenting fraction compared

with the control $R\beta G^{GC}$ RNA (Fig. 5, cf. A and B). By examination of the accompanying absorbance profiles, this increase in the sedimentation coefficient corresponds roughly to that expected for attachment (on average) of $\frac{1}{2}$ –1 additional ribosome per $R\beta G^{AT}$ mRNA. A similar shift was observed for $R\beta G^{AT100}$ versus $R\beta G^{GC100}$ RNAs (Fig. 5C,D). The majority of the $R\beta G^{GC100}$ RNA sedimented in fractions 6–7 (containing 3- to 5-mer polysomes), but about half of $R\beta G^{AT100}$ RNAs sedimented in fraction 8 (containing 6- to 7-mer polysomes). A shift in sedimentation was even more prominent in the case of $R\beta G^{AT22}$ (Fig. 5E) and $R\beta G^{GC22}$ RNAs (Fig. 5F). Although the majority of $R\beta G^{GC22}$ RNAs sediment in fractions 4 and 5 (containing mono-ribosomes through 3-mer polysomes), almost all $R\beta G^{AT22}$ RNAs sediment with 3- to 4-mer polysomes (fraction 6). Because in each case ribosome packing on the GC-containing RNAs is as expected for efficiently translated RNAs, the increased sedimentation of the AU-containing RNAs demonstrates that there is an AU

motif-dependent assembly of a rapidly sedimenting complex whose presence correlates perfectly with the low RNA phenotype.

The differential sedimentation properties between GC and AU motif-bearing RNAs are dependent on RNA translation. The $R\beta G^{AT1}$ mRNA carrying the authentic AU motif but whose translation is blocked by mutation of the AUG initiation codon to a UAG sediments indistinguishably from its translation-inhibited GC-containing control mRNA (Fig. 6A,B). Nonetheless, despite the apparent requirement for translation, the complex responsible for the more rapid sedimentation of AU-bearing RNAs does not form efficiently if the AU domain is translocated from the 3'-untranslated region into the coding domain. When placed in the correct frame at codon 99, translation of the resultant mRNA ($R\beta G^{ATC}$) proceeds across the AU motif, stopping at the normal β -globin termination site. Not only do $R\beta G^{ATC}$ RNAs and the control $R\beta G^{GCC}$ RNAs accumulate to similar levels (see Table 1), but they also sediment indistinguishably (Fig. 6, cf. C and D). Similarly, no differences in sedimentation could be detected between $R\beta G^{AT156}$ and $R\beta G^{GC153}$ RNAs in which translation extends into the AU motif (not shown). Clearly, in both of these latter cases in which the intact AU motif does not destabilize the mRNA, the AU-dependent complex fails to form, does so inefficiently, or is disrupted by ribosome transit across the AU domain.

Increased sedimentation of unstable, AU motif-containing RNAs is not due to changes in ribosome loading but is the result of a >20S complex whose binding is divalent cation independent

To examine the characteristics of the components responsible for the more rapid sedimentation of unstable, AU motif-bearing RNAs, we examined whether increased sedimentation was maintained following puromycin-

mycin- or EDTA-induced ribosome release in vitro. Post-nuclear lysates of cells expressing $R\beta G^{AT22}$ or $R\beta G^{GC22}$ were treated with puromycin or with EDTA and sedimented on sucrose gradients, and the positions of the corresponding RNAs were determined. Following release with puromycin, polysomes were dissociated completely as expected (Fig. 7A). Even in the absence of bound ribosomes, however, the $R\beta G^{AT22}$ RNA sedimented on average one fraction faster than the corresponding $R\beta G^{GC22}$ RNA (Fig. 7A). This difference in sedimentation was even more prominent when EDTA was used to remove the ribosomes and any other divalent cation-dependent binding proteins before sedimentation (Fig. 7B). A high proportion of AU motif-containing RNAs again sedimented $\sim 20S$ more rapidly than the analogous GC-containing RNA (Fig. 7B). Indistinguishable results were found with $R\beta G^{AT}$ and $R\beta G^{GC}$ mRNAs. These findings eliminate the possibility that additional ribosomal loading can account for the shift in sedimentation of $R\beta G$ RNAs that carry the AU motif-destabilizing domain; rather, the increase in sedimentation must represent binding of a large complex within the AU motif, and at least a portion of the complex must bind in an EDTA-insensitive manner. Furthermore, the complex remaining in EDTA is sufficiently large to alter RNA sedimentation by $\sim 20S$.

Discussion

Our studies provide evidence for formation of a large complex on translated mRNAs containing the GM-CSF AU-rich destabilizing element in their 3'-untranslated regions. Inhibition of translation by mutating the first methionine codon, moving the AU element into the translated region, or disrupting the AUUUA motifs results in inefficient formation or absence of the complex and a 30- to 40-fold increase in accumulated RNA levels. For most of these, direct measurement of RNA stability

Figure 7. The rapidly sedimenting complex on unstable mRNAs carrying the GM-CSF AU motif remains bound following puromycin- or EDTA-induced ribosome release. (A) Cell lysates from $R\beta G^{AT22}$ and $R\beta G^{GC22}$ cell lines were treated with 1 mg/ml of puromycin for 10 min at 37°C. (B) Cell lysates from $R\beta G^{AT22}$, $R\beta G^{GC22}$, $R\beta G^{AT}$, and $R\beta G^{GC}$ were treated with 10 mM EDTA. After either treatment, lysates were centrifuged at 38,000 rpm, 4°C, on 10–45% sucrose density gradients in 500 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4). RNA from each fraction was analyzed for the presence of rabbit β -globin RNA by RNase protection assay (fractions 12–16 were pooled into a single fraction).

demonstrates that much, and quite possibly all, of these changes in RNA abundance result from changes in stability.

Linkage of continued translation with RNA instability has been found repeatedly for short-lived mRNAs. Typically seen as an ~10-fold increase in mRNA abundance after the addition of protein synthesis inhibitors, this "superinduction" effect has been reported for *c-myc* (Linial et al. 1985), *c-myc* (Thompson et al. 1986), *c-fos* (Greenberg et al. 1986), and *c-jun* (Ryseck et al. 1988) and can be conferred to heterologous mRNAs by substituting the AU-rich domain from the 3'-untranslated region of GM-CSF (Shaw and Kamen 1986) or *c-fos* (Wilson and Treisman 1988; Koeller et al. 1991) but not *c-myc* (Wisdom and Lee 1991).

This linkage of continued translation with RNA instability could derive from either of two causes (which are not mutually exclusive). First, the RNA may be recognized as a substrate RNA only when that RNA is undergoing translation itself. We have shown this possibility to be true for instability mediated by the GM-CSF AU motif. Alternatively, RNA degradation may involve one or more highly unstable protein components whose abundance drops rapidly after the cessation of protein synthesis. There is now precedent for both of these possibilities in degradation of other eukaryotic mRNAs. β -Tubulin mRNAs (Pachter et al. 1987; Yen et al. 1988) and cell cycle-dependent histone mRNAs (Graves et al. 1987) have been reported to be degraded cotranslationally, possibly by a ribosome-bound nuclease (Cleveland 1988; Marzluff and Pandey 1988). For other transiently expressed, short-lived RNAs, a general conclusion cannot yet be drawn. For *myc* RNA, like GM-CSF, direct translation seems required for instability, because disruption of translation by mutation of the translation initiation codon yields a stable RNA. However, the only domain demonstrated in *c-myc* RNA to confer this cotranslational instability is a 105-codon segment from the carboxy-terminal translated region, rather than the AU-rich 3'-untranslated domain (Wisdom and Lee 1991). Further complicating the issue is the situation for *c-fos*. Here, the 3'-untranslated region of *c-fos* RNA confers instability to transferrin receptor mRNA whether or not translation initiation of this mRNA is specifically blocked (Koeller et al. 1991). *fos* mRNA increase following blockage of protein synthesis is thus most consistent with loss of a labile component of the degradative machinery.

Although in most cases we have demonstrated AU motif-dependent changes in mRNA stability, we cannot be certain whether instability can quantitatively account for all of the differences in accumulated RNA levels mediated by the GM-CSF AU motif. This is most clearly true for $R\beta G^{AT1}$ and $R\beta G^{AT156}$, each of which accumulates to a level 10-fold higher than predicted from the measured half-lives. We believe that the most likely possibility to explain the quantitative discrepancy is that half-life measurements are themselves suspect after inhibition of polymerase II transcription. An alternative possibility, proposed previously for dihydrofolate

reductase (Urlaub et al. 1989) and triose phosphate isomerase (Cheng et al. 1990) mRNAs, which were found to accumulate only to low levels when their translation terminated prematurely, is that translation near to the end of the mRNA is necessary for proper nuclear splicing and/or RNA transport. Although such a scenario could explain the low accumulation of $R\beta G^{AT1}$, this seems unlikely because premature translation termination in $R\beta G^{GC1}$ would also be predicted to yield low accumulation. This latter RNA accumulates to a level similar to that of the fully translated β -globin RNA. Furthermore, this processing/transport model certainly cannot account for the low RNA accumulation of $R\beta G^{AT156}$ in which translation is prolonged.

In any event, the perfect correlation between the presence of the complex responsible for the more rapid sedimentation and the lower steady-state level only of translated RNAs bearing the GM-CSF AU motif in the 3'-untranslated region suggests that formation of the complex is an early step in the control of RNA instability mediated by this AU domain. That complex formation is disrupted by ribosome translocation through the AU domain is easily understood. Less obvious is why translation is required for complex formation. One possibility is that translation-dependent displacement of cytoplasmic ribonucleoproteins may make the AU domain accessible to components of the complex. Alternatively, one or more factors may themselves be peripheral ribosomal components that transfer efficiently to the mRNA only during translation.

Both the identity of the factors that assemble into this translation-dependent complex and the mechanism through which instability is conferred remain to be identified. For both *c-fos* and *c-myc*, deadenylation has been argued to precede degradation of the body of the mRNA (Laird-Offringa et al. 1990; Shyu et al. 1991). We have tried to determine whether there is a corresponding deadenylation of unstable mRNAs bearing the GM-CSF AU domain, but their low abundance has precluded a careful examination thus far (S. Savant-Bhonsale and D.W. Cleveland, unpubl.). In any event, whether poly(A) loss is linked to rapid RNA turnover *in vivo* is not firmly established: Deadenylated *c-myc* RNAs turn over no more rapidly than do those with normal poly(A) tails (Laird-Offringa et al. 1990), and mutations in the *fos* AU motif that have no effect on the rate of poly(A) shortening stabilize the RNA (Shyu et al. 1991), a finding fully consistent with the absence of linkage of tail loss and instability.

With regard to identifying degradation components, a telling clue may have emerged from our finding that inhibition of transcription, either by use of actinomycin D or inactivation of a temperature-sensitive polymerase II mutant, yields measured half-lives that cannot account for differences in RNA accumulation. The simplest resolution is that ongoing transcription may be required for the rapid mRNA degradation pathway, presumably because a short-lived RNA is involved directly or indirectly in the AU-mediated degradation pathway. If so, then the apparent half-lives of RNAs $R\beta G^{AT}$, $R\beta G^{AT100}$, and

RβG^{AT22} will obligatorily be overestimated by any method that inhibits transcription. Consistent with this view is the original evidence of Shaw and Kamen (1986), who used actinomycin D to block transcription: RβG^{AT} RNA loss in their cells did not follow a first-order decay and plateaued at 20–30% of the initial level. The requirement for a nucleic acid component has also been supported experimentally during *in vitro* degradation of *c-myc* mRNA: A soluble factor responsible for accelerated loss of *c-myc* RNA was shown to be sensitive to micrococcal nuclease digestion (Brewer and Ross 1989).

The combination of the potential of an RNA component in AU-dependent mRNA degradation and an apparently large degradation complex are reminiscent of one preceding example of an RNA-dependent cleavage complex. Mammalian RNase P, which cleaves sequences from the 5' ends of precursors of tRNAs to produce the mature 5' termini of the tRNAs (Altman et al. 1986), is a labile, ~15S nuclear complex that can be dissociated into and reconstituted from RNA and protein components (Gold and Altman 1986; Pace and Smith 1990). With this example in mind, key questions now remaining are identification of the components of the AU-binding complex and determination of whether it contains any nucleic acid component.

Materials and methods

Cell culture and transformations

The baby hamster kidney cell line tsAF8 (Rossini et al. 1980; obtained from J. Corden, Johns Hopkins University School of Medicine, Baltimore, MD) was maintained at 34°C in Dulbecco's modified Eagle medium (DMEM) with 1 gram/liter of glucose and supplemented with 10% fetal calf serum and 10% CO₂. To obtain stable polyclonal lines, 18–24 hr before transfection, ~10⁶ cells were seeded per 100-mm culture dish. Ten micrograms of DNA plus 15 μg of *Escherichia coli* genomic carrier DNA were transfected by the calcium phosphate coprecipitation method (Graham and van der Eb 1973). Colonies were selected in DMEM containing 10% fetal calf serum and 0.5 μg/ml of G418 (GIBCO), and pooled after 14 days.

Plasmid construction and DNA probes

The plasmids pNeoRβG^{AT} and pNeoRβG^{GC} (renamed pRβG^{AT} and pRβG^{GC}) were gifts from R. Kamen (Shaw and Kamen 1986). Site-directed mutagenesis of pRβG^{AT} and pRβG^{GC} was used to prepare plasmids pRβG^{AT1}, pRβG^{GC1} and pRβG^{AT22}, pRβG^{GC22}. To do this, appropriate domains were subcloned into M13, and the uracil incorporation method with the *dut*-deficient bacterial strain was performed as described (Kunkel et al. 1987). To create pRβG^{AT1} and pRβG^{GC1}, the initiator methionine ATG codon of these parental plasmids was changed to TAG. To make pRβG^{AT22} and pRβG^{GC22}, the twenty-second codon GAA (glutamine) was changed to TAA (stop). Plasmids pRβG^{AT100}, pRβG^{GC100} and pRβG^{AT156}, pRβG^{GC153} were generated by nucleotide insertions into available restriction sites to produce translational frameshift mutations. For pRβG^{AT100} and pRβG^{GC100}, pRβG^{AT} and pRβG^{GC} were cleaved at the unique *Bam*HI site in β-globin exon 2, and the ends were blunted using Klenow polymerase and all four deoxynucleotides. pRβG^{AT156} and pRβG^{GC153} were made in an analogous manner after cleav-

age at the *Eco*RI site at codon 122 of the β-globin gene. Plasmids pRβG^{ATC} and pRβG^{GCC} were made by first replacing the 0.6-kb *Eco*RI fragment containing sequences from codons 99 to 147 plus the 3'-untranslated region (including the GM-CSF AU domain) and part of the vector sequences from pRβG^{AT} with the equivalent 0.76-kb *Eco*RI fragment from the authentic rabbit β-globin gene (isolated from pUK4.7; Hardison et al. 1979). This produced the intermediate plasmid pRβG. The AU and GC domains in pRβG^{AT} and pRβG^{GC} were obtained by polymerase chain reaction (PCR) using oligonucleotide primers that add *Bam*HI restriction sites at both ends. Following digestion with *Bam*HI, pRβG^{ATC} and pRβG^{GCC} were made by ligation of the AU or GC PCR products into the *Bam*HI site at codon 99 of pRβG. For all constructs, sequences at and surrounding the site of each mutation were verified by DNA sequencing.

RNA preparation and analysis

Total cytoplasmic RNA was prepared by a modification of the method of Favaloro et al. (1980). Cells were washed (twice) with ice-cold, phosphate-buffered saline and scraped in 200 μl/100-mm plate of lysis buffer (0.14 M NaCl, 1.5 mM MgCl₂, 10 mM Tris-Cl at pH 8.6, 0.5% NP-40, 10 mM vanadyl-ribonucleoside complex). The resultant cell suspension was vortexed briefly and cell debris and nuclei were pelleted by centrifugation at 2000g for 2 min at 4°C. The supernatant was recovered and diluted with an equal volume of a proteinase K solution [0.2 M Tris-Cl at pH 7.5, 25 mM EDTA, 0.3 M NaCl, 2% (wt/vol) SDS] and 200 μg/ml of proteinase K. After incubating at 37–45°C for 30 min, proteins were extracted first with phenol-chloroform and, finally, with chloroform alone. The aqueous phase containing the total cytoplasmic RNA was precipitated using 0.3 M sodium acetate (pH 5.2) and 2.5 volumes of cold ethanol. RNA was resuspended in dH₂O, and the concentration was determined by A₂₆₀ and stored at –80°C.

S1 nuclease mapping was done essentially as described previously (Gay et al. 1987). DNA probes were 5'-end-labeled with ³²P and hybridized in 80% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA, and 0.4 M NaCl at 50°C overnight. The hybridization mixture was diluted 10-fold by the addition of ice-cold S1 nuclease buffer (0.2 M NaCl, 30 mM sodium acetate at pH 4.5, 5 mM ZnCl₂, 50 μg/ml of denatured salmon sperm DNA) and incubated at room temperature in the presence of 100 units of S1 nuclease (BRL) for 60 min. The protected fragments were resolved on 9 M urea/6% polyacrylamide gels and visualized by autoradiography. To measure γ-actin levels, plasmid mAc (Gay et al. 1989) was digested with *Asp*718, dephosphorylated by treatment with alkaline phosphatase, and end-labeled with [γ-³²P]ATP for use in protection analyses.

RNase protection analyses were done according to Melton et al. (1984). An RNA probe specific for globin sequences was obtained by subcloning the 490-bp *Xho*I–*Bam*HI fragment of the rabbit β-globin gene (containing codons 1–99) into pGEM-2 to create pGEM-β-globin. After cleavage with *Bam*HI, a uniformly labeled RNA transcript was synthesized with SP6 polymerase. Each sample RNA to be analyzed was mixed with probe, evaporated to dryness, and redissolved in 20 μl of 80% formamide, 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl. This mixture was heated to 85°C for 5 min (to denature the RNA), transferred rapidly to 54°C, and incubated overnight. Ribonuclease digestion was carried out for 1 hr at 30°C in 10 mM Tris-Cl (pH 7.5), 300 mM NaCl, 5 mM EDTA containing 40 μg/ml of ribonuclease A and 2 μg/ml of ribonuclease T1. Following RNase treatment, proteinase K was added for 15 min at 37°C, and protein was extracted with phenol-chloroform. RNA was precipi-

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tated with ethanol and analyzed on a denaturing 9 M urea/6% polyacrylamide gel.

For RNA blot analysis, total cytoplasmic RNA was electrophoresed on a 1% formaldehyde-agarose gel. RNA was blotted onto GeneScreen Plus (DuPont) and hybridized to the ³²P-labeled human *hsp70* DNA probe in the hybridization buffer (33% formamide, 500 mM phosphate buffer at pH 7.2, 1 mM EDTA, 1% albumin, 7% SDS) at 42°C for 16 hr. The blot was washed once with 2× SSC (1× SSC = 15 mM sodium citrate, 150 mM NaCl), 0.1% SDS for 30 min at room temperature, washed twice with 0.2× SSC, 0.1% SDS, at 55°C (30 min each wash), and then exposed to X-ray film.

Nuclear run-on assay to measure transcription rates

Nuclear run-on assays and isolation of nuclei were performed according to Banerji et al. (1984). Briefly, to isolate the nuclei, ~10⁸ cells were harvested and lysed in several volumes of lysis buffer (10 mM NaCl, 3 mM MgCl₂, 10 mM Tris-Cl at pH 7.4, 0.5% NP-40) by vortexing briefly. Nuclei were pelleted by centrifugation, and the supernatant was discarded. The nuclei were resuspended in equal volumes of nuclei storage buffer (40% glycerol, 50 mM Tris-Cl at pH 8, 5 mM MgCl₂, 0.1 mM EDTA). The nuclei were then incubated in 25 mM HEPES (pH 7.4), 2.5 mM MgCl₂, 2.5 mM dithiothreitol, 75 mM KCl, and 5% glycerol in the presence of 0.35 mM each of ATP, GTP, CTP and 0.4 μM UTP plus 200 μCi of [α-³²P]UTP. The reaction was stopped by adding DNase I and incubating at 37°C for 10 min. Proteins in the reaction mixture were digested with proteinase K and extracted with phenol-chloroform. Nucleic acids were ethanol precipitated and resuspended in 10 mM Tris, 1 mM EDTA, and 0.5% SDS.

Single-stranded DNA probes for nuclear run-on transcription assays were (1) for β-globin, the 1240-bp *Xho*I–*Hind*III fragment (beginning at position –10 in the β-globin gene and extending to the 3′-untranslated region) was isolated from pRβG^{GC} and cloned into M13mp19; (2) for actin, the 1800-bp *Sall*–*Bam*HI fragment containing the coding region of the human βactin gene was isolated from pHβA (Gunning et al. 1983) and cloned into M13mp18; and (3) for β-tubulin, the 1200-bp *Eco*RI–*Bam*HI fragment from the coding domain of mouse mβ5 β-tubulin was isolated from pmβ5 (Sullivan and Cleveland 1986) and cloned into M13mp19.

Analysis of polysomes by sucrose density gradient

Three 100-mm dishes of tsAF8 cells at just subconfluent density were used for each polysome distribution analysis. Cells were washed twice with ice-cold phosphate buffered saline containing 100 μg/ml of cycloheximide and lysed by the addition of 200 μl of polysome lysis buffer [100 mM KCl, 5 mM MgCl₂, 10 mM HEPES (pH 7.4), 100 μg/ml of cycloheximide, 0.5% NP-40]. The lysate was transferred to a 1.5-ml microcentrifuge tube and passed two to three times through a 27-gauge needle to ensure cell lysis. Nuclei were pelleted by centrifugation at 4°C, 12,000g for 3–5 min. The resulting supernatant was layered on a linear 15–45% (wt/vol) sucrose gradient in polysome gradient buffer (100 mM KCl, 5 mM MgCl₂, 10 mM HEPES at pH 7.4), and gradients were centrifuged at 38,000 rpm for 1.5 hr at 4°C in a Beckman SW41 rotor. Gradient fractions were collected with an ISCO model 185 density gradient fractionator (ISCO, Inc. Lincoln, NE) connected to a type 6 optical unit and UA5 absorbance monitor. For puromycin or EDTA-induced in vitro release of ribosomes, initial lysates were made in high salt gradient buffer [500 mM KCl, 10 mM HEPES (pH 7.4), 0.5% NP-40], and puromycin (1 mg/ml final concentration) or EDTA (10 mM final

concentration) was added to the postnuclear supernatant. Puromycin-treated lysates were incubated at 37°C for 10 min (Blobel and Sabatini 1971). Lysates were then layered onto a 10–45% sucrose gradient in high salt polysome gradient buffer. Each gradient was centrifuged at 38,000 rpm for 3 hr in a Beckman SW41 rotor, and fractions were collected as described above.

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Evidence for instability of mRNAs containing AUUUA motifs mediated through translation-dependent assembly of a > 20S degradation complex.

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