

# An AU-rich sequence in the 3'-UTR of plasminogen activator inhibitor type 2 (PAI-2) mRNA promotes PAI-2 mRNA decay and provides a binding site for nuclear HuR

Fabienne Maurer, Marcus Tierney and Robert L. Medcalf\*

Monash University Department of Medicine, Box Hill Hospital, Box Hill, Victoria 3128, Australia

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## ABSTRACT

The plasminogen activator inhibitor type 2 (PAI-2) gene is regulated by transcriptional and post-transcriptional processes. We have previously shown that insertion of the 3'-untranslated region (3'-UTR) of PAI-2 mRNA into the 3'-UTR of a  $\beta$ -globin reporter mRNA reduces constitutive  $\beta$ -globin mRNA expression and that this requires, at least in part, an AU-rich motif. Here we have directly assessed the role of this motif in PAI-2 mRNA stability using both chimeric and non-chimeric reporter systems. We first show that the full-length PAI-2 mRNA is indeed unstable with a half-life of 1 h. Using the c-fos promoter-driven human growth hormone (HGH) mRNA as a reporter, we demonstrate that the 580 nt 3'-UTR of PAI-2 accelerates chimeric HGH mRNA decay in a process which is dependent on the intact AU-rich sequence. Furthermore, disruption of this motif within a constitutively expressed PAI-2 cDNA produces a 2.5- and 2.7-fold increase in PAI-2 mRNA and protein levels in HT-1080 cells, respectively. RNA electrophoretic mobility shift and supershift assays indicate that this motif provides a specific binding site for cellular proteins that include nuclear HuR. Taken together, these data show that a correlation exists between the binding of HuR to the AU-rich motif *in vitro* and the destabilizing properties conferred by this sequence *in vivo*.

## INTRODUCTION

The timely and regulated proteolytic generation of plasmin by the plasminogen activating (PA) enzymes is involved in many physiological processes, including fibrinolysis, development, wound healing and cell migration (1). In the extravascular compartment, urokinase-type plasminogen activator (u-PA) is the main plasminogen activating protease, whereas tissue-type plasminogen activator (t-PA) plays a more prominent role in the circulation. The proteolytic activities of t-PA and u-PA are controlled by plasminogen activator inhibitor types 1 and 2

(PAI-1 and PAI-2, respectively). PAI-2, the main inhibitor of urokinase in the extravascular space, has been referred to as the most enigmatic serpin (2), since it exists in both intracellular and secreted forms and is implicated in keratinocyte differentiation in a process that may not involve u-PA inhibition (3).

All components of the PA system are subject to tight regulation, however, the response of the PAI-2 gene to cytokines (4,5) and tumor promoters including phorbol esters (6) and okadaic acid (7) is particularly striking. A complex array of repressor and enhancer elements reside within the first 2 kb of the PAI-2 gene promoter (8–11) and have been shown to convey transcriptional responses mediated by some of these agonists. Although nuclear run-on assays clearly established that tumor promoters and other stimuli impressively activate PAI-2 gene transcription, the relative increase in mRNA levels under the same conditions largely exceeds the magnitude of the transcriptional induction, suggesting involvement of post-transcriptional mRNA stabilization events (12).

The rate of mRNA turnover provides a major post-transcriptional step to regulate gene expression (13). Although the mechanisms of mRNA decay in the cytoplasm of mammalian cells are still poorly understood, it is widely accepted that deadenylation of the transcript occurs as an initial step, followed by degradation of the RNA body (14). This process is essentially controlled by interactions between *cis*-acting sequences and their cognate *trans*-acting factors. The best characterized *cis*-acting determinants are AU-rich elements located within the 3'-untranslated region (3'-UTR) of numerous labile mRNAs encoding cytokines and oncoproteins. Such sequences usually harbor repeats of AUUUA or UUAUUUAUU motifs (15,16) and have been classed in three groups, depending on their particular AU-rich sequence content (17,18). Stability determinants are not restricted to the 3'-UTR as elements have been identified within the coding regions of  $\beta$ -tubulin (19), c-fos (20,21), c-myc (22,23) and urokinase receptor mRNAs (24). Decay determinants have also been reported within 5'-UTRs, however, the proteins recognizing these sites are poorly described (25,26).

A number of mammalian *trans*-acting factors which interact with AU-rich sequences have been identified (27) and cloned, such as AUF-1 (28), Hel-N1 (29), AUH (30) and HuR (31). These

\*To whom correspondence should be addressed. Tel: +61 3 9895 0318; Fax: +61 3 9895 0332; Email: robert.medcalf@med.monash.edu.au

factors have been correlated with either mRNA destabilizing (32–34) or stabilizing mechanisms (35,36). Interestingly, some proteins associated with mRNA turnover possess additional biological functions (30,37–39), indicating that the control of mRNA metabolism may be coupled with other physiological events.

We have previously demonstrated that insertion of the complete 3'-UTR of PAI-2 into the 3'-end of a rabbit  $\beta$ -globin reporter gene markedly reduced steady-state expression levels of the chimeric mRNA in transfected HT-1080 cells. However, mutagenesis of a nonameric AU-rich sequence (UUAUUUAUU) located 304 nt upstream of the poly(A) tail substantially abrogated this effect (12). Limitations of the mRNA reporter system used in that study precluded the direct demonstration that this motif played a role in controlling PAI-2 mRNA stability. Here, we have addressed the relationship between this motif and PAI-2 mRNA stability in human cell lines. We demonstrate that the full-length PAI-2 mRNA possesses a half-life of ~1 h and that the AU-rich motif plays an important role but is not solely responsible for this effect. We further demonstrate that disruption of this motif within the context of a constitutively expressed PAI-2 cDNA increases both PAI-2 mRNA and protein expression. RNA supershifting experiments indicate that this sequence provides a specific binding site for cellular factors that are preferentially nuclear in location and include HuR.

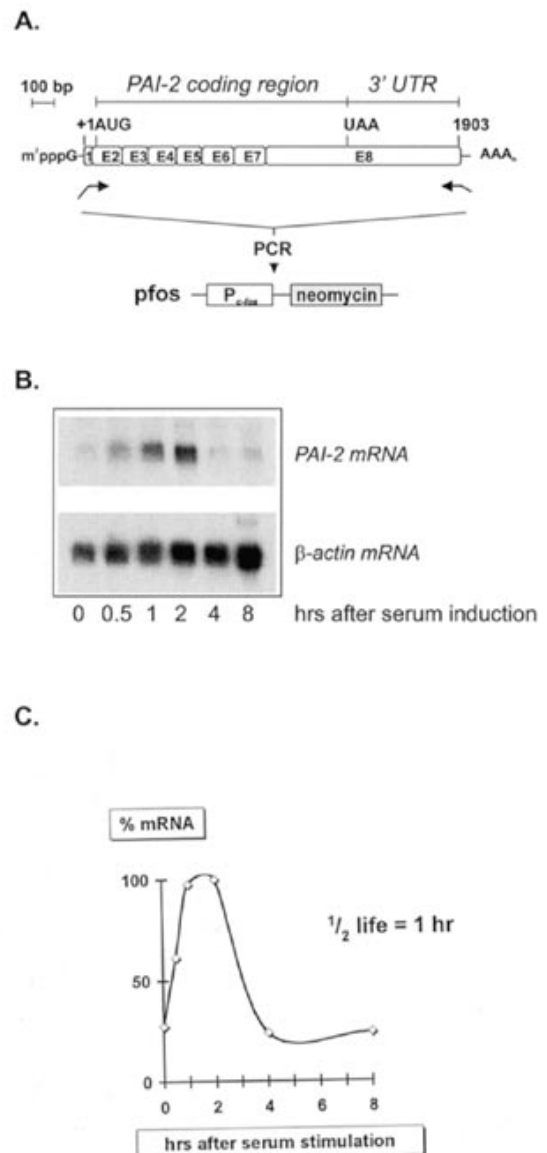
## MATERIALS AND METHODS

### Cell culture

Mouse NIH 3T3 and human HT-1080 fibrosarcoma cells (American Type Culture Collection, Rockville, MD) were grown according to standard techniques as described (12). For mRNA decay experiments,  $3 \times 10^5$  NIH 3T3 cells were plated onto 20 cm<sup>2</sup> plates, incubated for 16 h at 37°C in 10% HI-FCS-DMEM, then washed three times in phosphate-buffered saline (PBS) solution and serum starved in 0.5% HI-FCS-DMEM for 48 h prior to stimulation with 15% HI-FCS. Cells were then harvested at various times up to 8 h (15).

### Plasmids and polymerase chain reaction (PCR)

*c-fos promoter driven PAI-2 constructs.* Plasmid pfoSHGH (15) was kindly provided by Dr Gregory Goodall (Hanson Centre, Adelaide, Australia). This vector harbors the human growth hormone gene (HGH) placed under the control of the serum-responsive chicken *c-fos* promoter as well as the neomycin resistance gene. Plasmid pfoS was generated by removing the HGH insert from pfoSHGH using the restriction enzymes *Hind*III and *Sac*I (see Fig. 1A). A full-length PAI-2 cDNA was prepared by PCR using plasmid pJ7 (6) as a template. This was obtained using a 5' megaprimer which introduced the missing 50 nt in the 5'-UTR of pJ7 into the PCR product and a 3' primer complementary to the last 17 nt of the 3'-UTR immediately upstream from the poly(A) tract. The 5' and 3' primers had *Hind*III and *Sac*I extensions to allow subcloning. The sequences of these primers are: 5' megaprimer, 5'-CTATGACTCGAGAAGCTTTAACAAC-TCTCAGAGGAGCATTGCCCGTCAGACAGCAACTCAGA-GAATAACCAGAGAACAACCAGA-3'; 3' primer, 5'-CAGCTT-GAGAGCTCTTGTGTTGGAAGCAGGTT-3'. This PCR product was introduced into the plasmid pfoS, creating plasmid pfoPAI-2 and confirmed by sequencing.



**Figure 1.** The full-length PAI-2 mRNA is unstable. The decay rate of PAI-2 mRNA was assessed in serum-treated NIH 3T3 cells stably transfected with pfoPAI-2. (A) Generation of plasmid pfoPAI-2. Arrows indicate the position of the primers used for PCR amplification of the PAI-2 cDNA. (B) NIH 3T3 cells transfected with pfoPAI-2 were subjected to 0.5% serum starvation for 48 h, then treated with 15% serum for various periods of time up to 8 h, as indicated. PAI-2 mRNA levels were assessed by northern blot analysis using a <sup>32</sup>P-labeled PAI-2 probe. The same filter was stripped and rehybridized for  $\beta$ -actin mRNA. (C) The decay curve for PAI-2 mRNA was established by quantitating the mRNA signals corrected for changes in  $\beta$ -actin mRNA by phosphorimaging. The half-life was estimated to be 1 h. This was calculated by determining the time taken for the intensity of the signal to reduce to 50% of the maximal level of induction.

*c-fos promoter-HGH-PAI-2 reporter constructs.* To create plasmid pfoHGH-PAI-2, the 3'-UTR of the human PAI-2 gene was amplified by PCR using the plasmid pJ7 as a template and inserted into the *Kpn*I and *Sac*I sites of pfoHGH. The PCR primers, synthesized with *Kpn*I and *Sac*I extensions to allow the

sub-cloning, have the sequences: 5'-end primer, 5'-GCACTGACGGTACCAACTAAGCGTGCTGCTTC-3'; 3'-end primer, 5'-CAGCTTGAGAGCTCTTGTTTGGAAGCAGGTT-3'.

**PAI-2 expression constructs.** To prepare a vector constitutively expressing wild-type PAI-2, the 1.8 kb *EcoRI* cDNA fragment of pJ7 was sub-cloned into the *EcoRI* site of the pCI-neo mammalian expression plasmid (Promega) producing pCI-J7. This vector was also used as a template to generate a mutant PAI-2 (pCI-J7mut) which possessed a 4 nt substitution within the AU-rich motif (see below).

**Generation of constructs for *in vitro* transcription.** DNA templates for the *in vitro* transcription of labeled RNAs for the RNA electrophoretic mobility shift assays (REMSAs) were prepared. Two 5'-phosphorylated oligonucleotides encompassing the sense and antisense sequences of either the wild-type or mutant AU-rich element present in the 3'-UTR of PAI-2 were annealed and inserted into the *SacI* site of pBluescript II KS<sup>+</sup> (Stratagene). The orientation and copy number of these inserts were checked by DNA sequencing. The following oligonucleotides were used (sequences underlined indicate substituted nucleotides): sense, wild-type, 5'-CTTTACTTTGTTATTTATTTATTTATATAGAGCT-3'; sense, mutant, 5'-CTTTACTTTGTTAAAGCTTATTTATATAGAGCT-3'; antisense, wild-type, 5'-CTATATAAAATAATAAATAACAAAGTAAAGAGCT-3'; antisense, mutant, 5'-CTATATAAAATAAGCTTTAAACAAAGTAAAGAGCT-3'.

### Mutagenesis

Site-directed mutagenesis of the PAI-2 AU-rich nonameric sequence within pFHGH-PAI-2 and pCI-J7 was performed using the Transformer DNA kit (Clontech), following the manufacturer's instructions. The mutagenic primer was designed to insert a *HindIII* restriction site into the core element of the AU-rich motif, creating a 4 nt substitution (underlined below): mutagenic primer (PAI-2 3'-UTR), 5'-CTTTGTTAAAGCTTATTTATATATAATGG-3'. The selection primer used to prepare pFHGH-PAI-2mut was designed to replace the *Bam* HI site in pFHGH-PAI-2 with an *EcoRI* site (underlined below): selection primer (pFHGH), 5'-CATGTCTGAATTCCGTCGACCTCG-3'. The selection primer used to prepare pCI-J7mut was designed to replace the *XhoI* site in pCI-J7 with a *PstI* site (underlined below): selection primer (pCI-J7), 5'-CTATAGGCTAGCCTGCAGAATTCCTCCAGAG-3'.

### Transfection studies

Stable transfection of plasmids into HT-1080 or NIH 3T3 cells was performed by the calcium phosphate precipitation procedure (40) using 5 µg of DNA. Transfected clones were selected in medium supplemented with 400 or 600 µg/ml of G418 (Life Technologies), respectively, and resistant colonies (≥200) were pooled by trypanization.

### Northern blot analyses

Total RNA was purified from transfected cells as described by Chomczynski and Sacchi (41). Aliquots of 5 µg of RNA were electrophoresed through 1% agarose gels containing 20% formaldehyde and subsequently transferred to Hybond-N<sup>+</sup> membranes (Amersham). The filters were hybridized with the <sup>32</sup>P-labeled DNA probes as described (42). Membranes were processed by standard techniques and exposed to Kodak BioMax

film (Eastman Kodak) at -80°C with two intensifying screens. Signals were quantitated using a Fujix BAS 1000 phosphorimager.

The labeled inserts used for hybridization were obtained as follows: the 1.8 kb *EcoRI* cDNA fragment of plasmid pJ7 containing the full-length PAI-2 cDNA (6); the 679 bp *Bam*HI-*Hind*III HGH cDNA fragment of pfosHGH containing the human growth hormone cDNA (15); the 1.2 kb *PstI* cDNA fragment of mouse β-actin (43).

### Nuclear run-on transcription assays

This method was performed as described by Greenberg and Ziff (44,45) using nuclei extracted from 10<sup>7</sup> cells. Nitrocellulose filter strips containing 2 µg of slotted plasmid DNA were prepared as described (45) using a HYBRI-SLOT manifold slot-blot apparatus (Bethesda Research Laboratories). Filter strips were hybridized with labeled RNA for 36 h at 65°C, then washed, RNase treated, air dried and exposed to X-ray film.

The following plasmids were used in the run on assays: pCI-J7 containing the 1.8 kb cDNA insert of PAI-2, as described above; a vector harboring the mouse β-actin cDNA.

### Assessment of PAI-2 and HuR antigen in cell extracts

Cytoplasmic and nuclear protein extracts were prepared from cells by lysis in 0.5% NP-40 as previously described (45,46). Western blot analysis was performed by SDS-PAGE (10%) using 30 µg of protein extracts followed by electroblotting onto PVDF membranes. PAI-2 antigen was detected on the membrane using a monoclonal anti-PAI-2 antibody (American Diagnostics) at a 1:1000 dilution. Recombinant PAI-2, run in parallel as a control, was a kind gift of Delta Technologies. HuR antigen was detected using an anti-HuR monoclonal antibody (clone 165A) kindly provided by Dr Henry Furneaux (Sloan Kettering Institute, New York, NY). Monoclonal antibodies specific for AUF-1 were kindly provided by Dr Gary Brewer (University of North Carolina, Winston-Salem, NC). Primary antibodies were detected using a goat anti-mouse IgG coupled to horseradish peroxidase (1:10 000 dilution). Immunocomplexes were revealed by Enhanced Chemiluminescence (ECL; Amersham) following the manufacturer's instructions. PAI-2 antigen was quantitated using a PAI-2 specific ELISA assay (Biopool). Total protein concentration of cell extracts was determined using the Bio-Rad protein dye reagent (Bio-Rad).

### *In vitro* transcription and RNA electrophoretic mobility shift assays (REMSAs)

The DNA templates used to transcribe the RNA probes *in vitro* were first linearized with *XbaI*. For *in vitro* transcription, 1 µg of template was incubated for 2 h at 37°C in the presence of 50 µCi [α-<sup>32</sup>P]UTP (DuPont), 10 µM UTP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 20 U RNase inhibitor (Promega) and 50 U T7 RNA polymerase. RNA probes were purified on a 6% polyacrylamide-urea denaturing gel, eluted in a 500 mM NH<sub>4</sub>CH<sub>3</sub>COO, 1 mM EDTA solution for 6 h at room temperature, ethanol precipitated at -80°C and resuspended in water (500-1000 c.p.s./µl).

Unlabeled RNA competitors were prepared by *in vitro* transcription. As an additional control, competition titration experiments were performed using *in vitro* transcribed unlabeled RNA identical to the 54 nt iron-responsive element (IRE) which is recognized by the iron regulatory protein (IRP-1) (47). The

plasmid containing the IRE insert was provided by Dr Peter Leedman (Perth, Australia). The relative concentrations of the cold RNAs were estimated by ethidium bromide staining on agarose gels. When used in the binding assays, similar fold excesses of the competitors were pre-incubated with the protein extracts for 15 min at room temperature prior to adding the labeled probe. It was estimated that the cold competitor was used at a minimum of either 50- or 250-fold molar excess over the labeled probe in the competition experiments (see figure legends). However, it is difficult to calculate precisely the fold excess of the cold RNA over the labeled counterpart because of the different methodologies used during the *in vitro* transcription reactions. The complete sequences of the probes used in REMSAs are given in the legend to Figure 4.

To prepare protein extracts for the REMSAs, confluent cells were collected by trypsinization, washed three times with PBS, then lysed for 5 min on ice in 100  $\mu$ l/10<sup>6</sup> cells of cytoplasmic extraction buffer (CEB; 10 mM HEPES, pH 7.1, 3 mM MgCl<sub>2</sub>, 14 mM KCl, 0.2% NP-40, 1 mM DTT, 2  $\mu$ g/ml aprotinin, 0.5 mM PMSF and 10  $\mu$ g/ml leupeptin). The nuclei were pelleted for 1 min at 1000 *g* at 4°C and the supernatant containing the cytosolic fraction was aliquoted, snap frozen in liquid nitrogen and stored at -80°C. Nuclear protein extracts were prepared from nuclei as previously described (46). Protein concentration of cell extracts was determined by the Bio-Rad protein dye reagent.

For the binding assays, 2–5  $\mu$ g of protein extract were preincubated with 150  $\mu$ g of heparin (Sigma) for 10 min at room temperature in CEB before addition of the RNA probe (500–1000 c.p.s.). After a 30 min incubation at room temperature, samples were electrophoresed through a 6% native polyacrylamide gel and protein–RNA complexes visualized by autoradiography.

### Ultraviolet cross-linking studies

Binding reactions were performed identically as for the REMSAs then transferred to microtiter plate wells and put on ice. The samples were placed 7 cm from a UV source (model UVB-20; Ultra-Lum) and cross-linked for 15 min, then digested with 3 U of RNase T1 for 20 min at room temperature and finally resolved on 10% SDS–PAGE gels under reducing conditions, then dried. Labeled RNA–protein complexes were detected by autoradiography.

### REMSA supershift assays

Binding reactions were prepared as described for the REMSAs. Following the incubation of protein extracts with the RNA probe, antibodies were added to the tubes and the reactions incubated for 1 h on ice, prior to analysis on a native 6% polyacrylamide gel. The concentration of antibody used is provided in the figure legends. These experiments were performed in the presence of RNase inhibitor (Promega).

## RESULTS

### Direct evidence that PAI-2 mRNA is unstable

We previously observed that inhibitors of transcription could not be used to investigate PAI-2 mRNA stability, which precluded definitive demonstration that the 3′-UTR in general or the AU-rich motif in particular could promote PAI-2 mRNA decay (12). We therefore used a mRNA decay system based on the transient responsiveness of the chicken *c-fos* promoter to serum

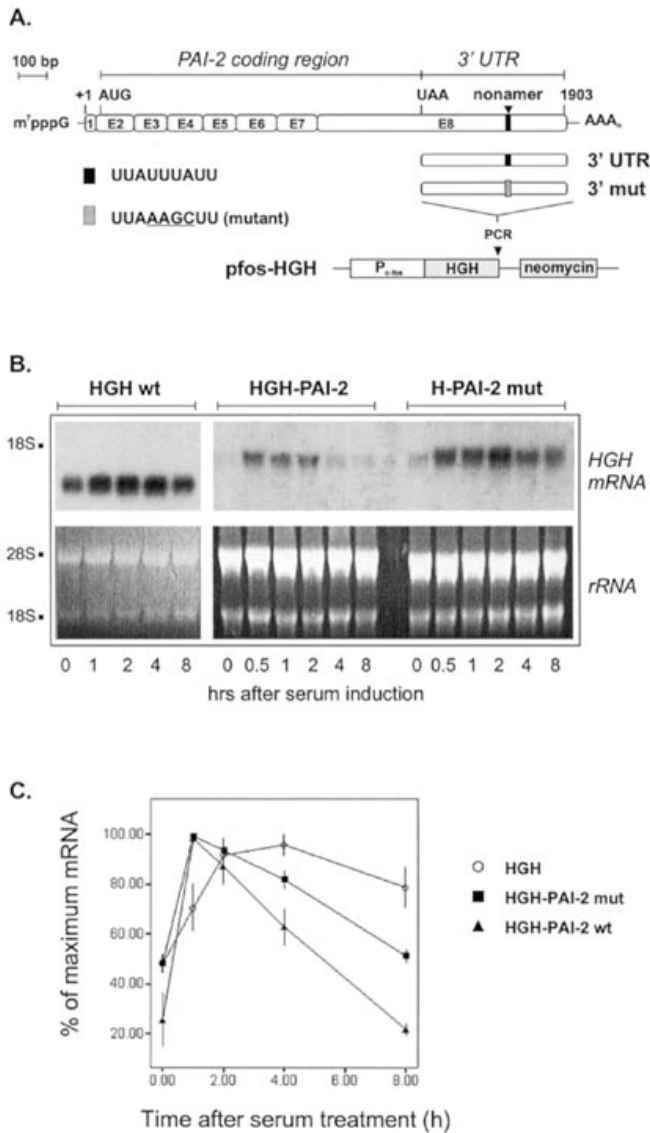
to analyze the destabilizing properties of the full-length PAI-2 mRNA. In this approach, the full-length PAI-2 cDNA was placed under control of the *c-fos* promoter, creating pPAI-2 (Fig. 1A), and stably transfected into NIH 3T3 cells. Cells were subjected to serum deprivation (0.5%) for 48 h to repress the *c-fos* promoter (15). The *c-fos* promoter was transiently activated by supplementing culture medium with 15% serum and the cells harvested after 0.5, 1, 2, 4 or 8 h. As shown in Figure 1B, the full-length PAI-2 mRNA was markedly induced by serum within a 2 h period. This response was transient with PAI-2 mRNA returning to basal levels within 4 h. The half-life of the transcript was estimated to be 1 h (Fig. 1C).

### The AU-rich motif within the 3′-UTR of PAI-2 promotes mRNA decay

To assess the effects of the 3′-UTR of PAI-2 mRNA and the role of the AU-rich region in the decay process, the full-length 3′-UTR was fused to the 3′-end of the HGH reporter gene placed under the control of the *c-fos* promoter (pFHGHPAI-2; Fig. 2A). The expression profiles of chimeric HGH-PAI-2 mRNAs in stably transfected NIH 3T3 cells were analyzed by northern blotting after serum induction and compared with that of the wild-type HGH reporter transcript. As shown in Figure 2B, wild-type HGH mRNA levels remained stable over the entire time course, consistent with the characteristic stability of this transcript. Insertion of the 580 nt PAI-2 3′-UTR into the 3′-UTR of HGH resulted in a lower level of constitutive mRNA expression. However, in response to serum, this construct produced a strong but transient increase in chimeric mRNA expression. To assess the contribution of the nonameric motif in the decay process, a similar construct was prepared which contained a 4 nt substitution introduced into this element (pFHGH-PAI-2mut) and the same serum time course experiment performed. As shown in Figure 2B, disruption of the nonamer resulted in a prolongation in the levels of the chimeric transcript following serum treatment. This experiment was repeated three times and mRNA decay curves were calculated. As shown in Figure 2C, the half-life of the HGH transcript alone was calculated to be >8 h, while insertion of the full-length 3′-UTR of PAI-2 into HGH reduced the chimeric mRNA half-life to 3 h. Introduction of the 4 nt substitution into the 3′-UTR of PAI-2 extended the chimeric HGH mRNA half-life to 4.5 h.

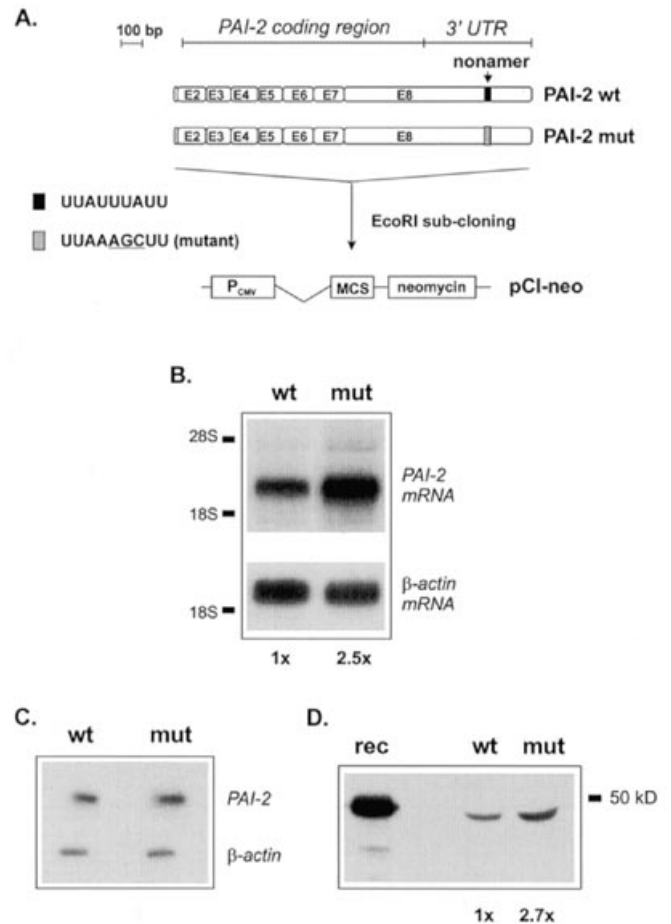
### Disruption of the nonameric AU-rich motif in the 3′-UTR of PAI-2 mRNA increases PAI-2 mRNA and protein expression levels

We established a model to directly study the influence of the AU-rich motif on the post-transcriptional regulation of PAI-2. For this purpose, we cloned the 1.8 kb PAI-2 cDNA fragment of pJ7 (6) into the pCI-neo expression plasmid, producing pCI-J7 (Fig. 3A). A 4 nt substitution was introduced into the AU-rich motif producing pCI-J7mut (Fig. 3A). Plasmids pCI-J7 and pCI-J7mut were stably transfected into HT-1080 fibrosarcoma cells. Northern blot, run-on transcription and western blot assays were performed on pooled pCI-J7 and pCI-J7mut clones to measure PAI-2 mRNA, transcription and antigen levels, respectively. PAI-2 ELISA assays were also performed to quantitate levels of PAI-2 antigen in the cytoplasmic fraction. As shown in Figure 3B, cells transfected with pCI-J7mut produced 2.5-fold higher levels of steady-state PAI-2 mRNA as compared with cells expressing



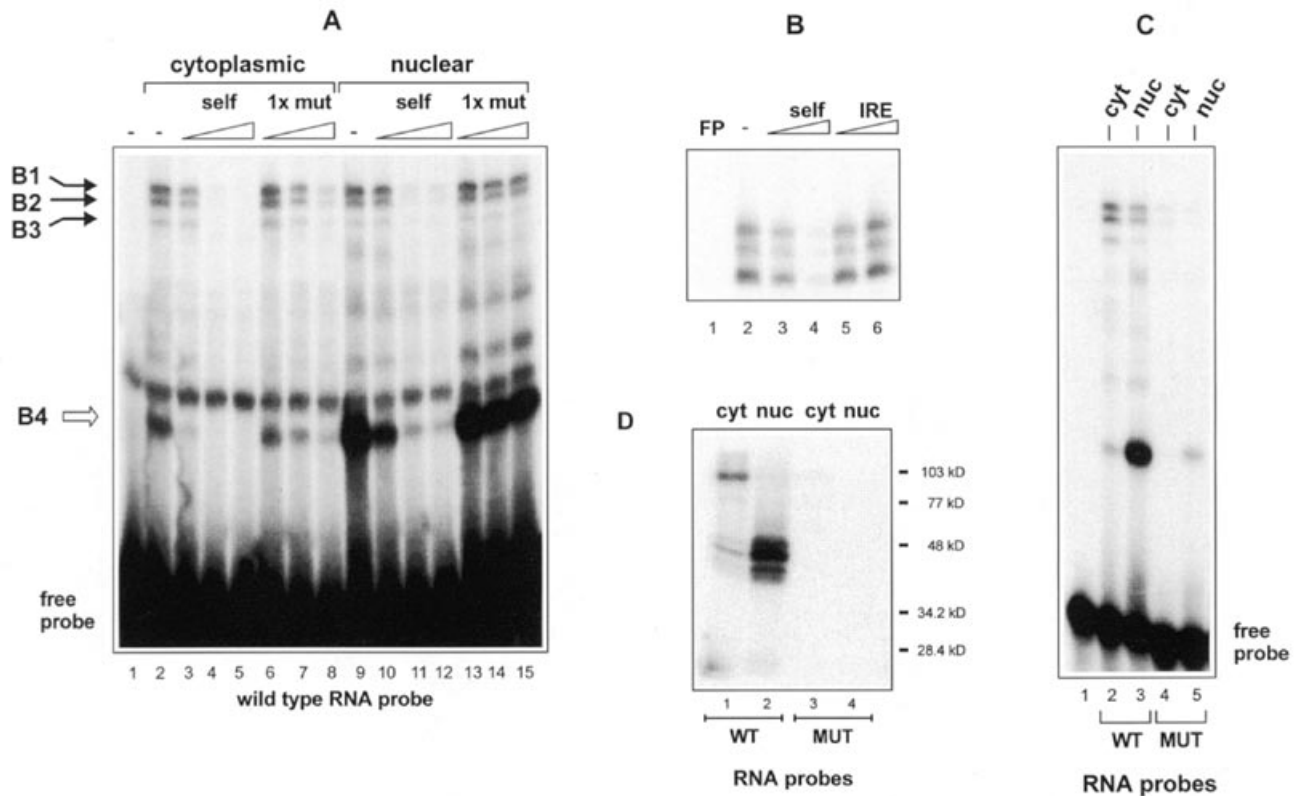
**Figure 2.** Introduction of the PAI-2 3'-UTR into the 3'-UTR of a HGH reporter gene accelerates chimeric mRNA decay. (A) Constructs prepared for stable transfection into NIH 3T3 cells. The full-length 3'-UTR of PAI-2 was inserted into the 3'-UTR of HGH, producing plasmid pHGH-PAI-2. A 4 bp substitution was then introduced into the nonameric element producing pHGH-PAI-2 mut. (B) NIH 3T3 cells stably transfected with wild-type pfosHGH (HGH wt), pHGH-PAI-2 (HGH-PAI-2) or the pHGH-PAI-2 mutant construct (H-PAI-2 mut) were stimulated with serum for up to 8 h, as described in the legend to Figure 2. Wild-type HGH and chimeric HGH-PAI-2 mRNA levels were assessed by northern blot analysis using a <sup>32</sup>P-labeled HGH probe. Ethidium bromide staining of the gels indicated below the figure was performed to demonstrate similar RNA loading to each lane. (C) The mRNA decay curves were calculated for each group by measuring the intensity of the mRNA signals by phosphorimaging. Results are expressed as means ± SEM of three separate serum time course experiments. The calculated mRNA half-lives are as follows: wild-type HGH mRNA, >8 h (open circles); HGH-PAI-2 mut, 4.5 h (closed squares); HGH-PAI-2 wild-type (wt), 3 h (closed triangles).

pCI-J7, while both populations displayed a similar magnitude of P<sub>CMV</sub>-driven PAI-2 gene transcription (Fig. 3C). Finally, western blot (Fig. 3D) and ELISA assays indicate that the pCI-J7mut cells



**Figure 3.** Disruption of the UUAUUUAUU motif in the PAI-2 3'-UTR results in an increase in constitutively expressed PAI-2 mRNA and protein in stably transfected HT-1080 cells. (A) Description of wild-type and mutant PAI-2 expression vectors (PAI-2 wt and PAI-2 mut, respectively). (B) An aliquot of 5 µg of total RNA prepared from HT-1080 cells stably transfected with either the wild-type or mutant PAI-2 expression vectors (PAI-2 wt or PAI-2 mut) was subjected to northern blot analysis. PAI-2 mRNA was assessed by hybridization with a <sup>32</sup>P-labeled PAI-2 cDNA insert. After autoradiography, the same membrane was stripped and hybridized with a <sup>32</sup>P-labeled β-actin probe. The relative increases in PAI-2 mRNA (after adjustment for β-actin) are presented below the figure. (C) Run-on transcription assays show similar levels of P<sub>CMV</sub>-driven PAI-2 gene transcription in HT-1080 cells expressing either wild-type or mutant PAI-2. (D) Cell-associated protein (30 µg) extracted from HT-1080 cells expressing wild-type (wt) or mutant (mut) PAI-2 were subjected to SDS-PAGE and western blot analysis. Rec, recombinant PAI-2. The marker to the right of the figure indicates the position of the 50 kDa protein molecular weight standard. Quantification of PAI-2 antigen by ELISA indicated a 2.7-fold increase in PAI-2 antigen in cells transfected with the mutated PAI-2 compared with cells transfected with the wild-type PAI-2 [indicated below (D)].

produced 2.7-fold more PAI-2 antigen (1465 ng/mg cell-associated protein) as compared with the pCI-J7 population (540 ng/mg cell-associated protein). A western blot experiment performed using cytoplasmic extracts prepared from the same cells on a different occasion showed a similar fold increase in PAI-2 antigen in cells overexpressing pCI-J7mut compared with cells overexpressing pCI-J7.



**Figure 4.** HT-1080 cytoplasmic and nuclear extracts recognize the AU-rich probe. (A) Cytoplasmic extracts (2.5  $\mu$ g) prepared from HT-1080 cells were incubated with labeled RNA probes containing a 29 nt stretch of the 3'-UTR of PAI-2 encompassing the wild-type nonameric motif and binding assessed by REMSAs. Up to three retarded protein-RNA complexes were produced (lane 2) as indicated by the solid arrows. A faster migrating complex is also evident (open arrow). All cytoplasmic protein-RNA complexes were competed by the inclusion of unlabeled wild-type RNA (250-, 1250- and 2500-fold molar excess of 'self'; lanes 3-5, respectively). Inclusion of the mutant RNA sequence (250-, 1250- and 2500-fold molar excess of '1x mut') produced some competition at the highest concentration, but was less effective when compared with the competition seen with the self RNA (lanes 6-8, respectively). REMSAs performed using nuclear proteins also produced a relatively abundant faster migrating complex [open arrow to left of the figure (lanes 9-15)]. These complexes were specific as binding was competed by inclusion of increasing concentrations of self-competitor (250-, 1250- and 2500-fold molar excess; lanes 10-12, respectively), but not by a similar molar excess of the unlabeled mutant RNA sequence (1x mut; lanes 13-15, respectively). Lane 1, RNA probe electrophoresed in the absence of protein extracts; FP, free RNA probe electrophoresed in the absence of protein. The sequences of the RNA probes and RNA competitors transcribed *in vitro* are given below. Lower case letters, sequence arising from the pBluescript KS<sup>+</sup> vector; upper case letters, PAI-2 AU-rich region; the nonamer is given in bold. The mutations introduced into the nonamer are shown in lower case and underlined. wt, 5'-ggcgaauggagcuCUUUUACUUUGUUAUUUAUUUUUAUAGAGCUccaccgagguggcggccggucaga-3'; mut, 5'-ggcgaauggagcuCUUUUACUUUGUUAaagcUUAUUUUUAUAGAGCUccaccgagguggcggccggucaga-3'. (B) Competition titration experiments were performed with cytoplasmic extracts prepared from HT-1080 cells with a probe harboring two copies of the 29 nt sequence which includes the nonameric motif. Specific competition was observed using a 50- or 250-fold molar excess of two copies of the unlabeled self RNA (lanes 3 and 4, respectively), but not when using a 250- or 500-fold molar excess of an unlabeled RNA containing the IRE (lanes 5 and 6, respectively). (C) REMSAs were performed using HT-1080 cytoplasmic and nuclear extracts (2.5  $\mu$ g) and labeled RNA probes harboring either the wild-type (WT) or mutant (MUT) sequence of the AU-rich motif [see (A)]. The same amount of labeled probe was added to each lane. As presented (lanes 4 and 5), RNA probes possessing a 4 nt substitution within the core of the nonameric motif displayed very weak binding intensity for both cytoplasmic (cyt) and nuclear (nuc) proteins compared with the binding to the wild-type sequence (lanes 2 and 3). (D) The same cytoplasmic and nuclear extracts (2.5  $\mu$ g) and RNA probes as used in (A) were incubated and subjected to REMSA analysis then UV cross-linked and treated with RNase T1. Samples were analyzed on SDS-PAGE and detected by autoradiography. Cytoplasmic extracts (cyt) produced a complex of ~100 kDa and up to three minor complexes that ranged between 38 and 48 kDa (lane 1). Nuclear extracts (nuc) failed to generate the 100 kDa moiety but produced an abundance of the complexes migrating between 38 and 48 kDa (lane 2). Conversely, the RNA probe containing the 4 nt substitution in the nonameric AU-rich motif failed to produce any detectable UV cross-linked complexes with proteins extracted from either the cytoplasmic or nuclear compartment (lanes 3-4). Markers to the right of the figure indicate the positions of the molecular weight standards.

### The nonameric AU-rich motif provides a binding site for cytoplasmic and nuclear protein(s)

REMSAs were performed to determine whether cellular protein(s) could recognize the AU-rich motif in the 3'-UTR of PAI-2. HT-1080 cytoplasmic and nuclear extracts were incubated with a <sup>32</sup>P-labeled RNA probe containing a single copy of the wild-type 29 nt AU-rich region of PAI-2 3'-UTR. As shown in Figure 4A, up to three retarded bands (B1-B3) are produced with cytoplasmic protein extracts (solid arrows; lane 2). These

interactions were specific as determined by competition titration experiments using increasing concentrations of unlabeled single copy self RNA (lanes 3-5). Addition of unlabeled single copy mutant sequence produced some competition at the highest concentration (lanes 6-8), but this was not as effective as the wild-type competitor. As shown in lanes 9-15, nuclear proteins generated a similar pattern of slower migrating protein-RNA complexes as seen when using cytoplasmic extracts, but also produced a greater intensity of binding factors which associated with the fastest migrating complex (B4; open arrow). Competition

titration experiments verified that the binding activity of nuclear factors to the RNA probe were specific. To further confirm the binding specificity, competition experiments were performed using increasing concentrations of *in vitro* transcribed RNA harboring the 54 nt IRE (47). This particular experiment was performed using two copies of the nonameric sequence as a probe. As shown in Figure 4B, competition was observed only with the unlabeled self competitor (two copy), whereas no competition was observed using the IRE as a competitor. There were no marked differences in the binding pattern produced using a single copy or two copies of the labeled probe. However, since only a single copy of the nonamer is present in the 3'-UTR of PAI-2, subsequent experiments were performed using the single copy probe.

To substantiate the specificity of the interaction, REMSAs were performed using a mutant RNA as a probe. This probe was identical to the 29 nt wild-type sequence, but contained a 4 nt substitution within the core of the nonameric motif. As shown in Figure 4C, binding of cytoplasmic and nuclear proteins to the mutant sequence was substantially reduced (lanes 4 and 5) compared with the binding produced with the wild-type sequence (lanes 2 and 3). This pattern was highly reproducible with similar results observed on four separate occasions.

UV cross-linking experiments were performed to determine the size of the cytoplasmic and nuclear AU-rich binding proteins (AUBPs). Extracts prepared from HT-1080 cells were incubated with the RNA probes containing either the wild-type or mutated AU-rich sequence as described in the REMSA protocol, then UV cross-linked and treated with RNase T1. Protein-RNA complexes were analyzed by SDS-PAGE and autoradiography. As shown in Figure 4D (lane 1), a predominant complex with an apparent molecular weight of ~100 kDa and three minor species ranging between 38 and 48 kDa are produced with cytoplasmic extracts. UV cross-linking of nuclear extracts failed to generate the 100 kDa moiety, but produced abundant levels of the 38–48 kDa complexes. Consistent with the data presented in Figure 4B, no complexes were detected when using the mutant RNA sequence as a probe (lanes 3 and 4). Although the UV cross-linking experiments suggest that the size of these complexes range between 38 and 100 kDa, we note that these studies tend to overestimate the molecular weight of proteins within the complex since the size of the RNA component is unknown.

#### AU-rich binding proteins are expressed in various cell lines

To examine the distribution of the AUBPs, REMSAs were performed using cytoplasmic and nuclear proteins extracted from cells which can be induced to express high levels of PAI-2 (HT-1080 cells and endothelial cells), which express PAI-2 constitutively (human keratinocytes) and those which do not express PAI-2 at all (HeLa cells). REMSA analysis indicated that AUBPs were present in all cytoplasmic and nuclear extracts tested, but the relative distribution of the various complexes differed between some of the cell types (data not shown). Consistent with the pattern of results presented in Figure 4, nuclear extracts from all cells tested contained abundant B4-forming complexes (data not shown).

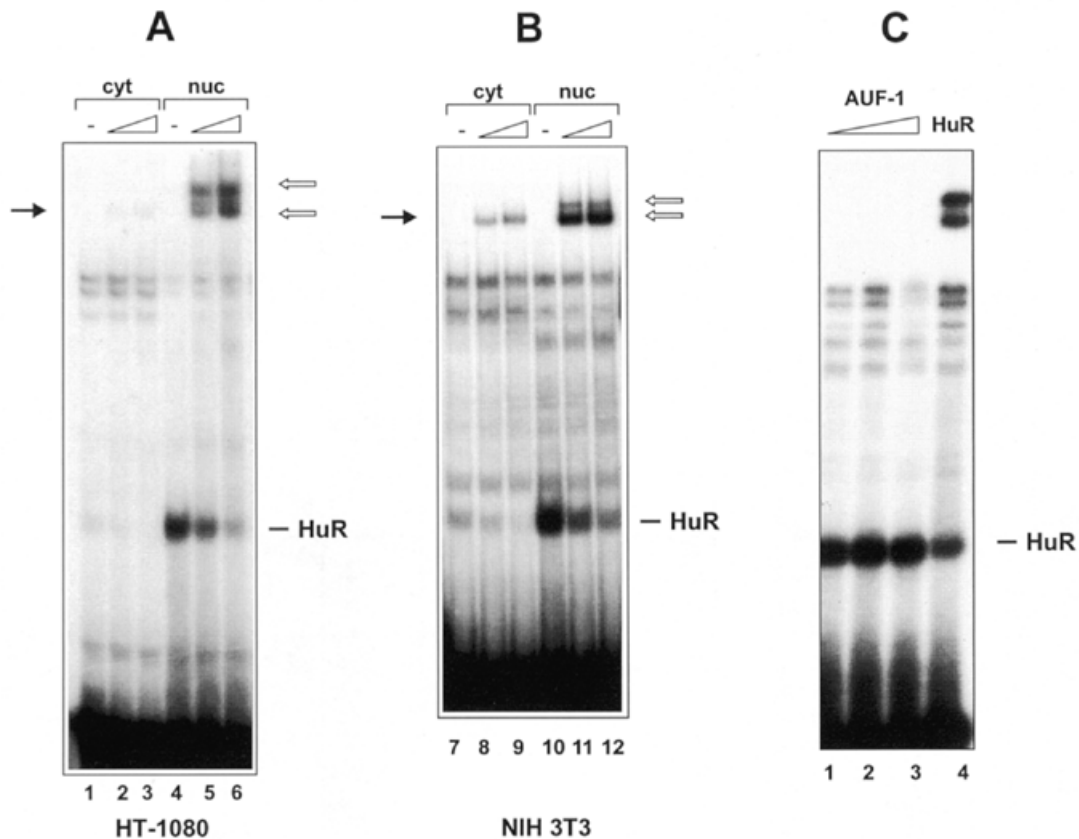
#### HuR recognizes the AU-rich element in the 3'-UTR of PAI-2 mRNA and is associated with complex B4

A number of proteins have been found to interact with AU-rich sequences, including HuR (31). HuR has further been associated with AU-rich-mediated mRNA decay and can bind to AU-rich sequences in *c-fos*, *IL-3* and *c-myc* (31). To determine whether HuR associated with the nonameric motif in the 3'-UTR of PAI-2, monoclonal antibodies directed against human HuR were incubated with cytoplasmic or nuclear extracts prepared from HT-1080 and NIH 3T3 cells after addition of the single copy RNA probe and assessed by REMSA. Addition of increasing amounts of anti-HuR antibodies produced a weak supershifted complex using HT-1080 cytoplasmic extracts (Fig. 5A, lanes 2 and 3, solid arrow), but clear supershifted complexes using cytoplasmic extracts prepared from NIH 3T3 cells (Fig. 5B, lanes 8 and 9, solid arrow). Interestingly, impressive HuR supershifts were produced using nuclear extracts extracted from both cell types (Fig. 5A, lanes 5 and 6; Fig. 5B, lanes 11 and 12). In each of these samples, at least two supershifted complexes were produced (open arrows), suggesting that HuR may be associating with another RNA binding factor in the nuclear compartment. It is also evident from the figure that the intensity of the fastest migrating complex (B4) is reduced in a dose-dependent manner with increasing amounts of HuR antibody, suggesting that the supershifted complexes containing HuR are derived from complex B4.

To confirm the specificity of the HuR antibody, supershift experiments were performed with nuclear extracts using a polyclonal antibody directed against AUF-1. As shown in Fig. 5C, anti-AUF-1 antibodies did not produce a supershifted complex under these conditions. However, after prolonged exposure of the gel, a very weak supershifted complex was detected using the anti-AUF-1 antibody that migrated slower than the complexes produced with the anti-HuR antibody (data not shown). AUF-1 supershift experiments performed using cytoplasmic extracts failed to produce a supershifted complex even after prolonged exposure of the gel to X-ray film. Antibodies specific for the poly(A) binding protein and hnRNPA1 also failed to produce supershifted complexes (data not shown).

#### DISCUSSION

In this study, we have assessed the influence of the PAI-2 AU-rich motif and its flanking sequences on the expression of chimeric mRNAs as well as on the PAI-2 transcript itself. The chicken *c-fos* promoter-driven mRNA decay system was used to assess the decay characteristics of PAI-2 mRNA in the absence of global inhibitors of transcription. Results demonstrated that the full-length PAI-2 transcript is unstable displaying a half-life of ~1 h. Furthermore, the 3'-UTR of PAI-2 was shown to be capable of conferring instability to the HGH-PAI-2 chimeric reporter mRNA. Importantly, the half-life of the chimeric mRNAs containing mutations within the nonameric sequence was substantially extended, indicating that this motif is a functional destabilizing element. Interestingly, disruption of the nonameric motif did not fully abrogate the destabilizing effect of the full-length 3'-UTR on HGH mRNA. We observed a similar effect when the same variants of the 3'-UTR of PAI-2 mRNA were inserted into the rabbit  $\beta$ -globin reporter system (data not shown). It was also shown that a single copy of the nonameric motif including 10 nt



**Figure 5.** HuR is part of complex B4. RNA supershift experiments were performed to determine the identity of some of the factors associating with the nonameric AU-rich motif in the PAI-2 3'-UTR. Cytoplasmic (cyt) and nuclear (nuc) extracts (2  $\mu$ g) prepared from HT-1080 (A) and NIH 3T3 cells (B) were incubated with *in vitro* transcribed RNA containing a single copy of the AU-rich nonameric motif. Increasing concentrations of anti-HuR monoclonal antibody (0, 1 or 5  $\mu$ l of 1:2 diluted antibody) were added and the samples were incubated for 1 h on ice before being subjected to electrophoresis. The solid arrows to the left of (A) and (B) indicate the position of the supershifted complexes. (C) Nuclear extracts prepared from HT-1080 cells were incubated with increasing concentrations of a polyclonal antibody specific for AUF-1 (5  $\mu$ l of 1:1000, 1:100 or 1:10 dilution of antisera; lanes 1–3, respectively) or with HuR (3  $\mu$ l of 1:2 diluted antibody; lane 4).

of 5' and 3' flanking sequences inserted into the 3'-UTR of rabbit  $\beta$ -globin mRNA was capable of independently reducing constitutive  $\beta$ -globin mRNA expression, but was less effective than the full-length 3'-UTR (data not shown). Clearly, other uncharacterized elements in the 3'-end of PAI-2 mRNA may act separately or cooperate with the nonamer to control stability. These motifs are likely to reside within the last 367 nt sequence, since this region is as effective as the full-length 3'-UTR in reducing constitutive  $\beta$ -globin expression (12). It is interesting to note that the pentameric AUUUA motif within this region (position 1503) is conserved in the 3'-UTR of murine PAI-2 mRNA, suggesting that this site may have a functional role.

Although our data provide little doubt that the 3'-UTR of PAI-2 mRNA and the AU-rich motif destabilize chimeric transcripts, we conducted experiments to verify that this form of regulation can occur within the context of PAI-2 mRNA itself. A potential limitation in using chimeric mRNAs to study the effects of a defined sequence is that its contribution to the overall post-transcriptional regulation of the full-length mRNA can only be inferred. Structural features and/or stability determinants located outside the PAI-2 3'-UTR may potentiate or negate its intrinsic effects, hence an assessment of this region in isolation may not

necessarily reflect the stability of the endogenous mRNA. To address this point the full-length wild-type PAI-2 mRNA and a mutant PAI-2 mRNA harboring a 4 nt substitution within the AU-rich motif were stably expressed in HT-1080 cells. Northern and western blot data indicated that mutation of the AU-rich sequence resulted in an increase in steady-state levels of PAI-2 mRNA and protein. These data provide another example of post-transcriptional influence of a defined AU-rich motif using an approach independent of chimeric reporter mRNA systems. Although previous demonstrations of an effect of the 3'-UTR on the post-transcriptional control of non-chimeric mRNAs have been reported [e.g. for erythropoietin (48), IL-3 (49) and c-fos (50)], relatively large, even complete, 3'-UTR deletions were created which made the assessment of individual motifs unclear. In our system, a 4 nt substitution targeted to a single AU-rich motif was sufficient to significantly alter the post-transcriptional features of the full-length PAI-2 mRNA.

REMSAs performed using HT-1080 cytoplasmic extracts and *in vitro* transcribed RNA probes possessing one copy of the AU-rich region produced up to four specific RNA-protein complexes (B1–B4). Proteins associated with complexes B1–B3 were also present in the nuclear fraction but to a lesser extent than

observed in the cytoplasm. Interestingly, nuclear proteins produced a substantial increase in complex B4. Our supershift assays identified HuR as being the major factor associated with this complex. Furthermore, two supershifted complexes were also observed using nuclear extracts prepared from both HT-1080 and NIH 3T3 cells, suggesting that B4 comprises HuR associated with other factors.

UV cross-linking experiments demonstrated that the molecular weights of the proteins associating with the AU-rich motif ranged between 38 and 48 kDa. A 100 kDa complex was also found in the cytoplasmic fraction. The proteins comprising the 38–48 kDa complexes were abundantly expressed in the nuclear compartment and we believe it likely that HuR is associated with these complexes. At this stage, the identity of the 100 kDa complex found in the cytoplasmic compartment is unknown.

Although HuR is one of the factors associating with the AU-rich motif, the identity of the other binding factors remains to be determined. It is interesting to note that the UUAUUUAUU motif alone has recently been shown to provide a binding site for an LPS-inducible 55 kDa cytoplasmic factor in mouse macrophages (51). Although we did not perform any REMSA or UV cross-linking experiments using extracts prepared from LPS-treated cells, it is unlikely that this factor was present in the extracts prepared from the cells used in our study due to differences in molecular weight.

HuR, a 36 kDa protein and a distant member of the Hu (or Elav-like) family of proteins, is ubiquitously expressed in mammals (31) and has recently been associated with AUUUA-mediated mRNA decay events (34). It has been shown to interact with AU-rich sequences in *c-fos*, *IL-3* and *c-myc* mRNAs (31). Recent studies have indicated that HuR is part of the mRNA stabilization machinery. For example, hypoxia-mediated stabilization of VEGF mRNA has been shown to be associated with the binding of HuR to AU-rich sequences within its 3'-UTR (52). Expression of antisense HuR was further shown to abrogate the stabilization of VEGF during hypoxia (52). From these studies and others, a picture is now emerging which suggests that HuR may play a broad role in implementing RNA stability. The preferential localization of HuR to the nuclear compartment has also been confirmed in two recent reports (53,54). In addition, these studies demonstrated that HuR could shuttle between the nucleus and the cytoplasm and may act as a stabilizing factor escorting AU-rich-containing mRNAs from the nucleus to the cytoplasm, providing protection from nucleases until binding activity is reversed (55). Other reports suggest that HuR may also play a role as a destabilizing factor since a direct correlation exists between the *in vitro* affinity of AU-rich sequences for HuR and their ability to direct *in vivo* degradation of a reporter mRNA (34). This latter scenario is consistent with a role for HuR in promoting PAI-2 mRNA decay since a positive correlation was also found between the ability of the AU-rich motif to promote PAI-2 mRNA instability, and HuR binding. The binding of HuR to the AU-rich motif was markedly reduced when a 4 nt substitution was introduced into the core of the AU-rich motif, while the same mutation was shown to reduce the decay rate of chimeric reporter mRNAs and to increase constitutively expressed PAI-2. If HuR plays a role in the post-transcriptional regulation of PAI-2, then it would appear to perform this task within the nuclear compartment, since only relatively low levels of HuR binding activity were found in the cytoplasm.

In summary, our study has focused on the role of the AU-rich region within the PAI-2 3'-UTR in the control of PAI-2 gene expression. We have shown that this region is involved in the mRNA decaying properties of the PAI-2 3'-UTR and that it provides a target for ubiquitously expressed cytoplasmic and nuclear RNA binding factors which include HuR. Further studies are needed to determine the functional role of these AU binding proteins, including HuR, in the post-transcriptional control of PAI-2 gene expression. The fact that disruption of the UUAUUUAUU motif within a constitutively expressed PAI-2 cDNA results in an increase in PAI-2 mRNA and protein expression highlights the potential relevance of this site in the control of PAI-2 expression *in vivo*.

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