

Plasminogen Activator Inhibitor Type 2 Contains mRNA Instability Elements within Exon 4 of the Coding Region

SEQUENCE HOMOLOGY TO CODING REGION INSTABILITY DETERMINANTS IN OTHER mRNAs*

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Marcus J. Tierney and Robert L. Medcalf‡

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

Plasminogen activator inhibitor type 2 (PAI-2) is a serine protease inhibitor that inhibits urokinase. Constitutive and regulated PAI-2 gene expression involves post-transcriptional events, and an AU-rich mRNA instability motif within the 3'-untranslated region of PAI-2 mRNA is required for this process (Maurer, F., Tierney, M., and Medcalf, R. L. (1999) *Nucleic Acids Res.* 27, 1664–1673). Here we show that instability determinants are present within various exons of the PAI-2 coding region, most notably within exon 4. Deletion of exon 4 from the full-length PAI-2 cDNA results in a doubling in the half-life of PAI-2 mRNA, whereas a 28-nucleotide region within exon 4 contains binding sites for cytoplasmic proteins. Inducible stabilization of PAI-2 mRNA in HT-1080 cells treated with phorbol ester and tumor necrosis factor does not alter the binding of proteins to the exon 4 instability determinant, but resulted in a transient increase in the binding of factors to the AU-rich RNA instability element. Hence, PAI-2 mRNA stability is influenced by elements located within both the coding region and the 3'-untranslated region and that cytoplasmic mRNA binding factors may influence steady state and inducible PAI-2 mRNA expression. Finally a 10-nucleotide region flanking the exon 4 protein-binding site is homologous to instability elements within five other transcripts, suggesting that a common coding region determinant may exist.

These proteases are themselves regulated by the plasminogen activator inhibitors (PAIs), PAI-1 and PAI-2. PAI-1 effectively inhibits both t-PA and u-PA; however, PAI-2 is widely considered to modulate u-PA activity in the extracellular compartments and plays a less important role in regulating t-PA. Although PAI-2 is found as a secreted glycosylated protein, a more abundant form exists within the cytosolic compartment (3). The predominant intracellular location of PAI-2 has fueled much speculation about additional functions for this inhibitor. Indeed, growing evidence has indicated a role for PAI-2 in the intracellular events associated with differentiation (4), proliferation (5), apoptosis (6), and signal transduction (7).

The gene encoding PAI-2 has generated particular interest, not only for its extracellular and presumed intracellular roles but also because of its impressive regulatory profile. PAI-2 gene transcription rates are markedly increased in response to the tumor promoter phorbol 12-myristate 13-acetate (PMA) (8) and the phosphatase inhibitor, okadaic acid (9). The PAI-2 gene is also one of the most tumor necrosis factor- (TNF) (10) and lipopolysaccharide (LPS) (11)-responsive genes described. For the latter, this has been further confirmed by serial analysis of gene expression analysis of primary human monocytes, whereby PAI-2 mRNA levels were shown to be increased 105-fold by LPS (12), being the third most LPS-induced transcript produced in these cells.

Notwithstanding the important contribution of transcriptional control of PAI-2 expression (13, 14), the role of post-transcriptional regulation of the PAI-2 gene has recently been highlighted (1, 15). These investigations stemmed from earlier results whereby treatment of HT-1080 fibrosarcoma cells with a combination of PMA and TNF produced a 50–100-fold increase in PAI-2 gene transcription but a 1500-fold increase in PAI-2 mRNA over a 24-h period (9). The discrepancy in the degree of mRNA production was suggestive of inducible stabilization of PAI-2 mRNA under these conditions. Subsequent studies have shown that the rate of PAI-2 mRNA decay can indeed be reduced by phorbol ester (15) and dioxin (16) or increased by dexamethasone (17).

Functional studies on the 3'-UTR of PAI-2 mRNA led to the identification of an AU-rich mRNA destabilizing determinant (15). This element provides a binding site for a number of cytoplasmic and nuclear proteins including HuR (1), an mRNA-stabilizing protein that can shuttle between the nucleus and the cytoplasm (18, 19). Although the degree of PAI-2 mRNA instability is influenced by the AU-rich motif in the 3'-UTR, elements within the coding region and possibly the 5'-UTR are also likely to contribute to the control of PAI-2 mRNA stability since the PAI-2 transcripts lacking the 3'-UTR are still relatively unstable (1). Indeed, functional mRNA stability determinants have been detected within the coding region of a growing list of mRNAs including the mRNAs of c-Myc

The plasminogen activator system is an important proteolytic cascade that plays a role in the removal of blood clots from the circulation and the turnover of a variety of extracellular matrix proteins (2). The effector enzyme of this system is the powerful protease plasmin, generated from its inactive precursor plasminogen by the plasminogen activators, namely urokinase- or tissue type plasminogen activator (u-PA¹ and t-PA).

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‡ To whom correspondence should be addressed: Dept. of Medicine, Monash University, Box Hill Hospital, Box Hill 3128, Victoria, Australia. Tel.: 61 3 9895 0318; Fax: 61 3 9895 0332; E-mail: robert.medcalf@med.monash.edu.au.

¹ The abbreviations used are: u-PA, urokinase-type plasminogen activator; t-PA, tissue-type plasminogen activator; uPAR, urokinase receptor; PAI-2, plasminogen activator inhibitor type 2; UTR, untranslated region; nt, nucleotide; PMA, phorbol 12-myristate 13-acetate; TNF, tumor necrosis factor; LPS, lipopolysaccharide; DMEM, Dulbecco's modified Eagle's medium; HI-FCS, heat-inactivated fetal calf serum; HGH, human growth hormone; PCR, polymerase chain reaction; REMSAs, RNA electrophoretic mobility shift assays; bp, base pair; PAGE, polyacrylamide gel electrophoresis; VEGF, vascular endothelial growth factor; CRD-BP, coding region determinant-binding protein.

TABLE I
Oligonucleotides synthesized to amplify individual or groups of exons of the PAI-2 cDNA

Bold type in uppercase represents restriction sites for *KpnI* and *SacI*. Lowercase extensions represent nucleotides added to facilitate improved digestion with restriction enzymes.

Oligonucleotide name	Sequence	Position within PAI-2 cDNA
Exon 2 sense	5' ctatga CTCGAG ATTGAAACAATGGAGGATC 3'	Position 23–41
Exon 2 antisense	5' ctatga GAGCTC CTTGGCCATCTGGTCTTC 3'	Position 182–199
Exon 3 antisense	5' ctatga GAGCTC CTGCAAATCGCATCAGG 3'	Position 302–319
Exon 4 sense	5' ctatga GGTACC GCACAAGCTGCAGATAAAAATC 3'	Position 320–340
Exon 4 antisense	5' ctatga GAGCTC TTCCCGGAAGCTCGCAG 3'	Position 432–448
Exon 5 antisense	5' ctatga GAGCTC TTTGGTTTGGAGTGTGAC 3'	Position 548–566
Exon 6 antisense	5' ctatga GAGCTC CGAGTTACACGGAAAGG 3'	Position 692–709
Exon 7 sense	5' ctatga CTCGAG GCTCAGCGCACACCTG 3'	Position 710–725
Exon 8 antisense	5' ctatga CTCGAG TTAGGGTGTAGCAAAAATCTG 3'	Position 1261–1279

TABLE II
Oligonucleotides synthesized to prepare overlapping exon 4 RNA probes

Bold type in uppercase represents restriction sites for *KpnI* and *HindIII*.

Oligonucleotide name	Sequence
Exon 4 part A sense	5' CGCACAAGCTGCAGATAAAAATCCATTATCCTTCCGCTCTCTCAGCTCTGCA 3'
Exon 4 part A antisense	5' AGCTT GACAGAGCTGAGAGAGCGGAAGGATGAATGGATTTATCTGCAGCTTGTGCC GGTAC 3'
Exon 4 part B sense	5' CTCAGCTCTGCAATCAATGCATCCACAGGGGATTATTTACTGGAAAGTGTCA 3'
Exon 4 part B antisense	5' AGCTT GACACTTTCCAGTAAATAATTCCTGTGGATGCATTGATTGCAGAGCTG AGGTAC 3'
Exon 4 part C sense	5' CGGAAAGTGTCAATAAGCTGTTTGGTGAGAAGTCTGCGAGCTTCCGGGAAA 3'
Exon 4 part C antisense	5' AGCTT TTCCCGGAAGCTCGCAGACTTCTACCAAAACAGCTTATTGACACTTTCC GGTAC 3'

(20–22), yeast Mat $\alpha 1$ (23–25), vascular endothelial growth factor (VEGF) (26), u-PAR (27) and c-Fos (28, 29).

Here, we have analyzed exons within the PAI-2 coding region for functional mRNA instability elements. Our findings indicate that the control of PAI-2 mRNA stability is controlled by *cis*-elements located throughout the coding region, most notably within exon 4, whereas a 28-nt region within this exon provides a specific binding site for cytoplasmic factors. Hence PAI-2 mRNA decay is influenced by both coding region instability elements as well as the AU-rich instability element in the 3'-UTR. Of further interest is that the region immediately adjacent to the 5' end of the exon 4-binding site bears homology to mRNA instability elements located within the coding region of five other mRNAs. This suggests that a common coding region instability motif may be involved in mRNA turnover.

EXPERIMENTAL PROCEDURES

Cell Culture—Mouse NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.), supplemented with 10% (v/v) heat-inactivated fetal calf serum (HI-FCS), 2 mM glutamine, 50 μ g/ml streptomycin, and 50 units/ml penicillin, in a humidified atmosphere at 37 °C with 5% CO₂. For mRNA decay experiments, 4 × 10⁵ NIH3T3 cells were plated onto 10-cm² dishes and grown for 24 h in 10% HI-FCS DMEM, washed twice in phosphate-buffered saline solution, serum-starved in 0.5% HI-FCS DMEM for 48 h prior to stimulation with 15% HI-FCS DMEM. Cells were then harvested at selected intervals up to 24 h (30).

Probes and Plasmids, c-fos Promoter-driven PAI-2 Constructs—Plasmid pfos-HGH (30) was kindly provided by Dr. Gregory Goodall (Hanson Center, 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 (see Fig. 1, panel A). Plasmid pfos was generated by removing the HGH insert from pfos-HGH using the restriction enzymes *HindIII* and *SacI* (1).

Individual or groups of PAI-2 exons were amplified by PCR using the pJ7 PAI-2 cDNA (8) as a template. *KpnI* and *SacI* sites were engineered into the 5' and 3' sites of the PCR products, respectively, to facilitate the ligation into the *c-fos* HGH vector. The sizes of the exons are indicated in Fig. 1, panel A. The primers used for the amplification are provided in Table I.

Generation of Plasmids for in Vitro Transcription—DNA templates for the *in vitro* transcription of labeled RNAs for the RNA electrophoretic mobility shift assays (REMSAs, see below) were prepared. The full-length exon 4 was amplified by PCR from the pJ7 PAI-2 cDNA

template and inserted into the *KpnI* and *HindIII* sites of pBluescript (Stratagene). The sequence of the exon 4 sense primer used for this is provided in Table I. The antisense exon 4 primer is also indicated in Table I but had *HindIII* restriction site added at the 5' end, rather than *SacI*. The generation of the shorter RNA probes containing the overlapping exon 4 sequences was prepared by annealing 5'-phosphorylated oligonucleotides encompassing the sense and antisense sequences of exon 4, regions 4A, 4B, and 4C, and then directly inserted into the *KpnI* and *HindIII* sites of pBluescript II KS⁺ (Stratagene). The orientation of these inserts was assessed by DNA sequencing. The sequence of the oligonucleotides used for this are provided in Table II.

Mutagenesis—The plasmid containing the full-length PAI-2 cDNA driven by the *fos* promoter (pfos-PAI-2) has been described previously (1). Removal of exon 4 from the PAI-2 cDNA using pfos-PAI-2 as a template was performed by site-directed mutagenesis using the Transformer DNA kit (CLONTECH). The mutagenic primer designed to delete exon 4 had the following sequence: PAI-2 exon 4 deletion primer, 5'-CTGATGCGATTTTGCAGGAATATATTCGACTCTGTC-3'. The selection primer used to prepare pfos-PAI-2 exon 4 mutant was designed to replace the *BamHI* site in pfos-PAI-2 with an *EcoRI* site (underlined) as follows: selection primer (pfos-HGH), 5'-CATGTCTGAATTCGGTCGACCTCG-3'. The pfos vector harboring the PAI-2 mutant cDNA was confirmed by sequencing.

Transfection Studies—Stable transfection of plasmids into NIH3T3 cells was performed by calcium phosphate precipitation procedure (31) using 5 μ g of DNA. Transfected clones were selected in medium supplemented with 600 μ g/ μ l of G-418 (Life Technologies, Inc.), and resistant colonies (>200) were pooled by trypsinization.

Northern Blot Analyses—Total RNA was purified from selected cells as described by Chomczynski and Sacchi (32). Aliquots of 5 μ g of RNA were electrophoresed through 1% agarose gels containing 20% formaldehyde and subsequently transferred to Hybond-N⁺ membranes (Amersham Pharmacia Biotech). The filters were hybridized with the ³²P-labeled DNA probes as described (33). Membranes were processed by standard techniques and exposed to Kodak BioMax film (Eastman Kodak Co.) at -80 °C with two intensifying screens. Signals were quantitated using a Fujix BAS 1000 PhosphorImager or by densitometry using a Linotype-Hell scanner.

The labeled inserts used for hybridization were obtained as follows: the 1.8-kilobase pair *EcoRI* cDNA fragment of plasmid pJ7 containing the full-length PAI-2 cDNA (8); the various labeled PAI-2 exons were prepared by PCR amplifying using plasmid pJ7 as a template; the 679-bp *BamHI/HindIII* HGH cDNA fragment of pfos-HGH containing the human growth hormone cDNA (30); the 1.2-kilobase pair *PstI* cDNA fragment of mouse β -actin (34); the 972-bp *BamHI/HindIII* cDNA fragment of neomycin from pCI-neo.

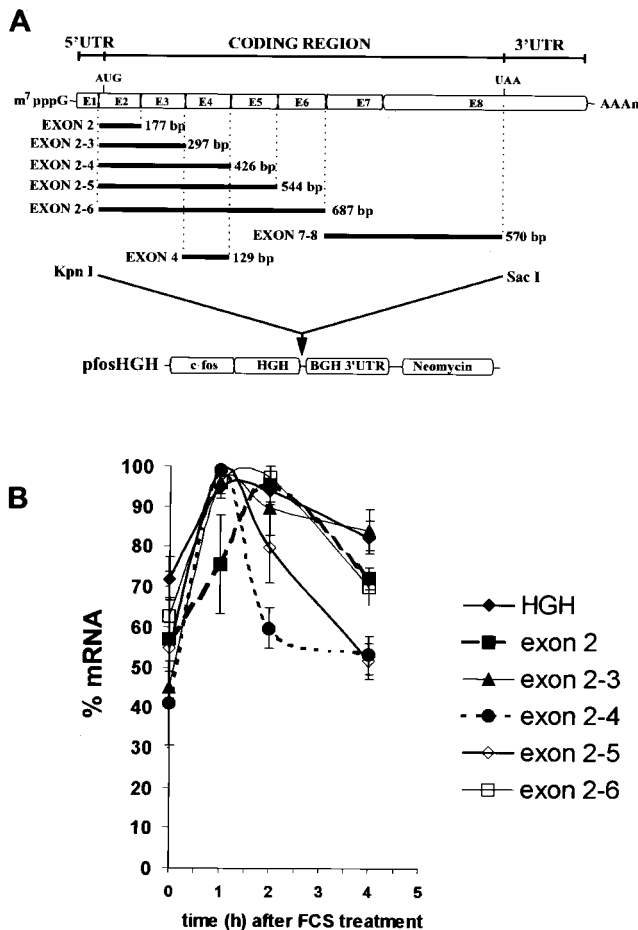


FIG. 1. Various exons within PAI-2 mRNA confer instability to the HGH reporter mRNA. Panel A, schematic representation of the PAI-2 cDNA showing the exons of PAI-2 that were amplified by PCR and inserted into the 3'-UTR of the human growth hormone (HGH) reporter gene driven by the serum-responsive chicken *c-FOS* promoter (pfos-HGH). E, exon. Panel B, mRNA decay curves of HGH-containing transcripts in NIH3T3 cells stably transfected with pfos-HGH alone (HGH) or containing the following PAI-2 exons inserted in to the 3'-UTR of the HGH gene: exon 2, 2-3, 2-4, 2-5, and 2-6 (as indicated in the figure). For these experiments, cells were treated with 15% FCS, and RNA was harvested at the indicated time points and assessed for HGH transcripts by Northern blot analysis using a cDNA probe specific for HGH. Signals were quantitated by densitometric analysis, and results were expressed relative to the maximal signal obtained. Results presented are the mean data of 3 or 4 separate experiments. Bars represent S.E.

In Vitro Transcription and RNA Electrophoretic Mobility Shift Assays (REMSAs)—The pBluescript DNA templates used to transcribe the PAI-2 exon 4 RNA probes were first linearized with *EcoRI*. For *in vitro* transcription, 500 ng of template was incubated for 2 h at 37 °C in the presence of 50 μ Ci of [α - 32 P]UTP (DuPont), 10 μ M UTP, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 20 units RNase inhibitor (Promega), and 50 units of T3 RNA polymerase. Templates harboring the 29-nt AU-rich element in the 3'-UTR were linearized with *XbaI*, and labeled RNA was transcribed *in vitro* as described above, but using 50 units of T7 polymerase. RNA probes were purified on a 6% polyacrylamide-urea denaturing gel, eluted in 500 mM $\text{NH}_4\text{CH}_3\text{COO}$, 1 mM EDTA solution overnight at room temperature, ethanol-precipitated at -80 °C, and resuspended in water (500 cps/ μ l).

Unlabeled RNA competitors were also prepared by *in vitro* transcription, but using 3 μ g of template. The relative concentrations of the cold RNAs were estimated by ethidium bromide staining on agarose gels. When used in the binding assays, cold competitors were preincubated 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 50–1200-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.

To prepare protein extracts for the REMSAs, confluent cells were collected by trypsinization, washed three times with phosphate-buffered saline, and then lysed for 5 min on ice in 100 μ l/10⁶ cells of cytoplasmic extraction buffer (CEB: 10 mM HEPES, pH 7.1, 3 mM MgCl_2 , 14 mM KCl, 0.2% Nonidet P-40, 1 mM dithiothreitol, 2 μ g/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μ g/ml leupeptin). The nuclei were pelleted for 1 min at 1,000 $\times g$ 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 described previously (35). Protein concentrations were determined by using the Bio-Rad protein dye reagent.

For the binding assays, 2–4 μ g (see figure legends) of protein extracts were preincubated with 5 μ g/ μ l of heparin (Sigma) in a total volume of 20 μ l, for 10 min at room temperature before addition of the RNA probe (500 cps). The probe was heated to 65 °C for 5 min and then cooled on ice before adding to the sample. After a 30-min incubation at room temperature, samples were treated with 1 unit of RNase T1 (Roche Molecular Biochemicals) for 10 min at room temperature and then subjected to electrophoresis through a 5% native polyacrylamide gel, and protein-RNA complexes were visualized by autoradiography.

REMSA supershift experiments were performed as described (1). Antibodies (1 μ l of 1:2 diluted material) were added to the samples immediately following the 30-min incubation of the extract with the labeled RNA and left for 1 h on ice. For supershift experiments, the RNase T1 step was omitted.

Antisense DNA Masking Experiments—This procedure was performed as described by Coulis *et al.* (36). Four overlapping antisense DNA oligonucleotides were prepared and annealed to the exon 4A RNA probe that includes the first 50 bp of PAI-2 exon 4 (see Table II). Oligonucleotides 1–3 are 15 nt in length, and oligonucleotide 4 is 16 nt in length (see Fig. 6, panel A). The sequence of these oligomers is as follows: oligo 1, 5'-GAATGGATTTTATCT-3'; oligo 2, 5'-GAGCGGAAGATGAA-3'; oligo 3, 5'-TGCAGAGCTGAGAGAG-3'; and oligo 4, 5'-GTCATCACAGGGTCCTGA-3'. An unrelated DNA oligonucleotide 5'-GTCATCACAGGGTCCTGA-3' was used as a negative control. The DNA oligonucleotides were added to give a final concentration of either 0.1, 1.0, or 10 pmol. Following annealing of the oligomers to the RNA template, cytoplasmic extracts and heparin were added as described in the REMSA protocol.

UV-Cross-linking Assays—UV-cross-linking of RNA cellular proteins to RNA probes followed by SDS-PAGE was performed as described (1) with slight modifications. Briefly, following the binding reaction (REMSA protocol) using 15–20 μ g of cytoplasmic extract, samples were digested with 1.0 units of RNase T1 for 10 min at room temperature and then transferred to microtiter plate wells and placed on ice. Samples were placed 7 cm from a UV source (Ultra LUM modeUVB-20) and cross-linked for 15 min. RNase A (Roche Molecular Biochemicals) was added directly to the wells (final concentration of 100 μ g/ml) and left at 37 °C for 15 min. Samples were transferred to Eppendorf tubes and denatured at 100 °C for 5 min in the presence of 6 \times SDS-PAGE loading buffer containing dithiothreitol before being resolved on 10% SDS-PAGE gels under reducing conditions. Gels were dried and labeled RNA-protein complexes detected by autoradiography. For competition experiments, higher concentrations of unlabeled RNA were included in these experiments compared with the REMSAs due to the higher concentration of protein extract.

Western Blot Assays—Western blot analysis for PAI-2 antigen was performed as described previously (15). 50 μ g of cytoplasmic extract was subjected to 10% SDS-polyacrylamide gel electrophoresis under reducing conditions and blotted onto a polyvinylidene difluoride membrane. Membranes were initially blocked with TBS-T buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 2 h at room temperature. Membranes were then washed and incubated with a primary anti-PAI-2 antibody (American Diagnostics) at a final dilution of 1:4000 and incubated overnight at 4 °C. Finally, membranes were washed in TBS-T and incubated with the appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 1 h at room temperature. Immunoreactive proteins were detected by the enhanced chemiluminescence system (ECL reagents, PerkinElmer Life Sciences).

RESULTS

PAI-2 mRNA Coding Region Contains Instability Motifs—The pfos-HGH mRNA decay system was used to identify mRNA instability determinants within the PAI-2 coding re-

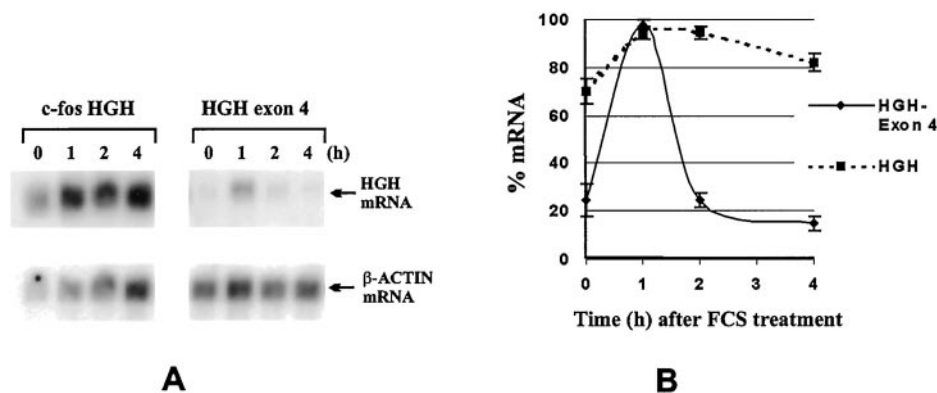


FIG. 2. **Exon 4 of PAI-2 mRNA confers marked instability to the HGH-reporter transcript.** *Panel A*, NIH3T3 cells stably transfected with *c-fos*-HGH alone or the same plasmid containing the complete exon 4 sequence inserted into the 3'-UTR of the HGH gene (*HGH exon 4*) were subjected to serum treatment, and HGH transcripts were assessed by Northern blot analysis. The same filter was stripped and re-hybridized for β -actin mRNA. *Panel B*, mRNA decay curves of HGH-containing transcripts in NIH3T3 cells stably transfected with *pfos*-HGH alone (*HGH*, dotted line) or containing exon 4 inserted into the 3'-UTR of the HGH gene (*HGH-exon 4*, solid line). Results presented are the mean data of four separate experiments. Signals were quantitated by densitometric analysis, and results were expressed relative to the maximal signal obtained. Bars represent S.E.

gion. To this end, a series of individual exons or groups of consecutive exons of the PAI-2 coding region were amplified by PCR and introduced into the 3'-UTR of the HGH gene (see Fig. 1, *panel A*). NIH3T3 cells stably transfected with these constructs were subjected to serum treatment, and the decay rate of HGH containing transcripts was determined by Northern blotting and quantitated by densitometric analyses.

As shown in Fig. 1, *panel B*, serum treatment of cells transfected with the parent *pfos*-HGH plasmid produced a stable HGH transcript that displayed a half-life in excess of 3 h. Insertion of exon 2 alone into the 3'-UTR of HGH mRNA slightly increased the decay rate of the HGH reporter transcript (half-life 2½ h). However, insertion of a fragment containing exon 2 + 3 together did not alter the decay rate of the chimeric HGH mRNA. These results suggest that exon 2 possesses instability determinants that are counteracted by sequences present within exon 3. Interestingly, HGH transcripts containing PAI-2 exons 2 + 3 + 4 were particularly unstable, with the half-life of the chimeric transcript reduced to less than 1 h. These data suggests that exon 4 possesses particularly powerful destabilizing elements. Curiously, longer chimeric transcripts containing exons 2-5 or exons 2-6, although promoting destabilization of the reporter transcript (mRNA half-lives: 1½ and 2½ h, respectively), were not as effective as exons 2-4 alone. This also suggests that sequences within exon 5 or 6 contained motifs that counteract the instability elements present in exon 4. Insertion of fragments containing exons 7 and 8 into the 3'-UTR of the *pfos*-HGH plasmid conferred a destabilizing effect upon the reporter transcript to an extent similar to that produced by exons 2-4 (data not shown). Taken together, these results suggest that instability and stability determinants are located throughout the PAI-2 coding region, with powerful instability elements associated with the presence of exon 4 and also within exons 7 and 8. In this study, we focused our efforts to assess the role of exon 4 in the control of PAI-2 mRNA stability. The instability elements within exons 7 and 8 will be investigated in a separate study.

To assess the stability of exon 4 in isolation, the entire exon 4 sequence was introduced into the 3'-UTR of HGH and the mRNA half-life determined. As shown in the Northern blot experiment presented in Fig. 2 (*panel A*), HGH-exon 4 chimeric transcripts were induced after 1 h of serum treatment, but decayed very rapidly with the signal barely detectable after 2 h. Quantitation of the mRNA signals indicated that the half-life of the exon 4 containing transcript was ~30 min (*panel B*). The observation that exon 4 in isolation produced greater instabil-

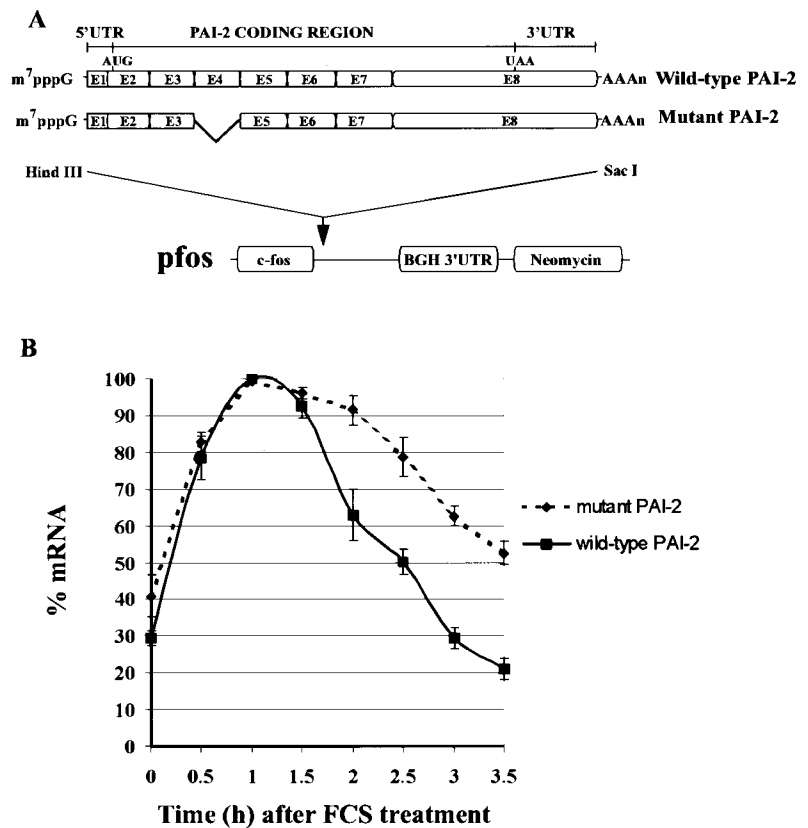
ity to the reporter transcript than seen in the context of other exons further supports the notion that mRNA stability determinants exist within neighboring exons to counteract partially the effects of the destabilizing elements in exon 4.

Removal of Exon 4 from the PAI-2 cDNA Results in a Doubling of PAI-2 mRNA Stability—To provide more evidence that sequences within exon 4 play a role in PAI-2 mRNA stability, the 129-bp exon 4 was deleted in-frame from the full-length PAI-2 cDNA using plasmid *pfos*-PAI-2 as a template (Fig. 3, *panel A*). The resulting construct (*pfos*-PAI-2 Δ 4) as well as the construct containing the full-length wild-type PAI-2 cDNA were stably transfected into NIH3T3 cells. Two independent series of transfection experiments were performed. The collective results of two individual serum time courses and Northern blot experiments of both series of transfected cells indicated that the mRNA half-life of the wild-type PAI-2 transcript to be ~1 h, which is in agreement with previous results (1). However, the half-life of the mutant PAI-2 transcript was extended to 2 h, double that of its wild-type counterpart. These data indicate that sequences within exon 4 promote PAI-2 mRNA instability.

PAI-2 Exon 4 Provides a Specific Binding Site for Cytoplasmic Proteins—Experiments were conducted to determine whether regions within exon 4 provided binding sites for cellular factors. To this end, RNA probes containing the full-length (129 nt) exon 4 sequence were incubated with cytoplasmic extracts prepared from both NIH3T3 cells and HT-1080 fibrosarcoma cells, and binding activity was assessed by REMSA analysis. HT-1080 cells were utilized in these experiments because these cells express the endogenous PAI-2 gene and are widely used to study PAI-2 gene regulation. As shown in Fig. 4, numerous protein-RNA complexes were formed on the exon 4 probe that displayed different migration profiles on the native gel (*lane 1*). To determine the specificity of these interactions, competition experiments were performed whereby increasing concentrations of unlabeled RNA of identical (*lanes 2-4*) or unrelated sequence (*lanes 5-7*) were added to the samples at the same time as the exon 4 probe. Results indicated that one of the faster migrating complexes was indeed specific (indicated with arrow) as formation of this complex was competed by increasing concentrations of unlabeled exon 4 RNA (*lanes 2-4*) but not by unrelated RNA (*lanes 5-7*). Similar experiments performed using NIH3T3 cells produced essentially identical results (data not shown).

The First 52 nt of Exon 4 Provide Protein-binding Sites for Cytoplasmic Proteins—To localize further the protein binding

FIG. 3. Deletion of exon 4 from the full-length PAI-2 transcript doubles the half-life of PAI-2 mRNA. *Panel A*, schematic representation of the full-length PAI-2 construct (*wild-type PAI-2*) and the mutant construct containing an in-frame deletion of exon 4 (*Mutant PAI-2*). The PAI-2 constructs were introduced into plasmid *pfos* as described under "Experimental Procedures." *E*, exon. *Panel B*, the decay rate of PAI-2 mRNA in NIH3T3 cells transfected with either *pfos*-PAI-2 (*wild-type PAI-2*, *solid line*) or PAI-2 Δ exon 4 (*Mutant PAI-2*, *dotted line*) was assessed by Northern blot analysis following a serum time course up to 3.5 h. The intensity of the signals was quantitated by densitometric analysis. The data shown were derived from two independent transfection experiments with each series of transfected cells being assessed at least two times ($n = 4$). Data for each experiment was expressed as a percentage of the maximal mRNA signal obtained following serum treatment. The half-life of the wild-type PAI-2 transcript was calculated to be 1 h, whereas the half-life of the exon 4-deleted transcript was extended to ~ 2 h.



region within the 129-nt exon 4 sequence, a series of REMSAs was performed using shorter RNA probes. Three overlapping RNA probes spanning the exon 4 sequence were generated (regions A (52 nt), B (52 nt), and C (51 nt); see Fig. 5, *panel A*) and used in a REMSA assay using cytoplasmic proteins prepared from HT-1080 cells.

As shown in Fig. 5, *panel B*, the first 52 nt of exon 4 (exon 4A) produced three protein RNA complexes (*lane 2*). Competition experiments confirmed that the two fastest migrating complexes (referred to as complexes I and II) were specific as only the unlabeled 4A RNA sequence competed for binding (*lanes 3–5*). Binding was not competed by cold RNA sequences harboring the 4B and 4C region of exon 4 (*lanes 6–11*) or by RNA of unrelated (97-nt prothrombin 3'-UTR) sequence (*lanes 12–14*). No specific binding of cytoplasmic proteins was observed to either the 4B or 4C probes (data not shown). To confirm further that the first 52 nt of exon 4 contained the protein-binding elements, competition REMSA experiments were performed using the full-length exon 4 sequence as a probe. Complexes produced using the full-length exon 4 probe were competed by the unlabeled 4A RNA but not at all by unlabeled 4B or 4C competitors (data not shown).

Protein-binding Sites Reside within a 28-nt Region of Exon 4—To define more closely the binding sites for the proteins that bind to the first 52 nt of exon 4, an antisense DNA oligonucleotide masking experiment was performed as described by Coulis *et al.* (36). In this approach, antisense DNA oligomers directed against overlapping sequences within the 4A RNA probe (see Fig. 6, *panel A*) were allowed to anneal to the RNA probe before the addition of the cell extracts. As shown in Fig. 6 (*panel B*), the formation of complexes I and II was not at all inhibited by antisense oligomers complementary to the first 15 nt (*oligo 1*; *lanes 3–5*). Antisense oligo 2 caused inhibition only at the highest concentration used (10 pmol) (*lane 8*), whereas antisense oligo 3 completely inhibited complex formation (*lanes 9–11*), even at the lowest concentration used (0.1 pmol). Anti-

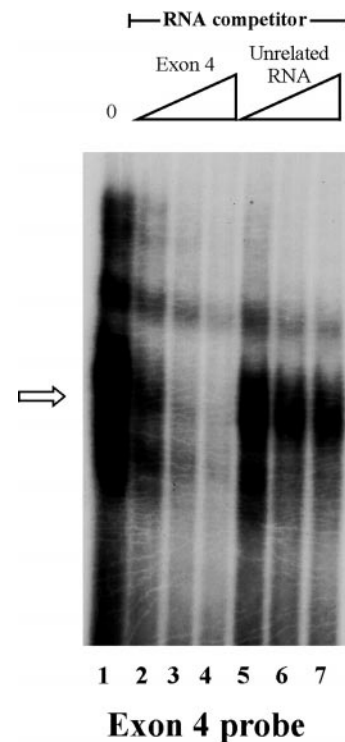


FIG. 4. Cytoplasmic proteins specifically recognize sequences within exon 4 of PAI-2 mRNA. Cytoplasmic extracts prepared from HT-1080 cells ($4 \mu\text{g}$) were incubated with RNA probes containing the full-length 129-nt exon 4 sequence, and binding was assessed by REMSA. Up to three complexes are produced in the absence of competitor (0) (*lane 1*), but only the central migrating complex (*arrow*) is specific as this was competed by addition of increasing concentrations (~ 50 -, 250 -, and 500 -fold molar excess) of unlabeled exon 4 RNA (*lanes 2–4*, respectively) but not by addition of similar concentrations of an unrelated RNA (*lanes 5–7*).

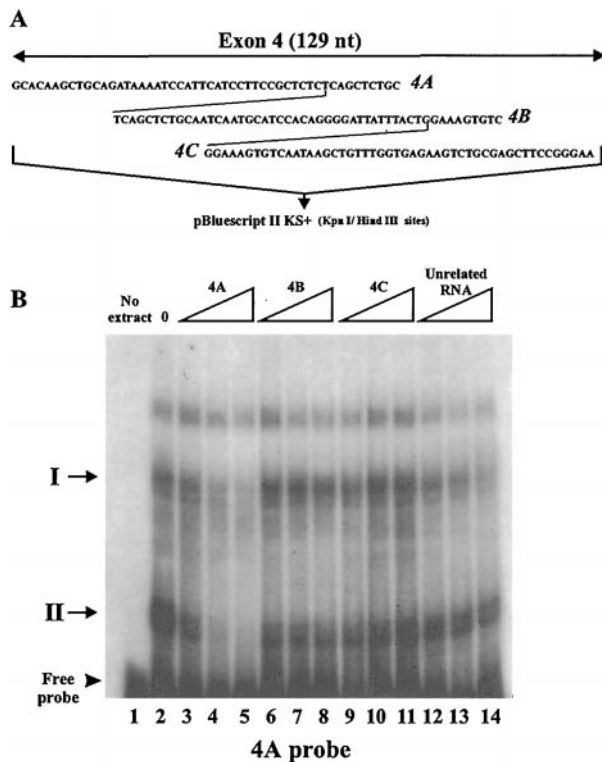


FIG. 5. The first 52 nt of exon 4 provides binding sites for cellular factors. *Panel A*, schematic representation of the overlapping RNA probes (4A, 4B, and 4C) used to localize the protein-binding site(s) within the 129-nt PAI-2 exon 4 sequence. These sequences were introduced into pBluescript, and RNA from linearized plasmids was transcribed *in vitro* using T3 polymerase. *Panel B*, REMSA experiments were performed using the RNA probe harboring the first 52 nt of exon 4 (4A probe). Incubation of cytoplasmic extracts with the 4A probe produced two specific complexes (I and II, indicated with arrows). The formation of these complexes was competed with increasing concentrations (~50-, 250-, and 500-fold molar excess) of unlabeled 4A RNA (lanes 3–5, respectively), but not with similar concentrations of unlabeled 4B (lanes 6–8), 4C (lanes 9–11), or an unrelated RNA (lanes 12–14). No specific complex formation was produced when using the 4B or 4C RNA sequences as probes (data not shown).

sense oligo 4 also substantially inhibited complex formation (lanes 12–14) and was essentially as effective as oligo 3 at inhibiting complex formation. As a negative control, antisense oligomers of unrelated sequence had no effect on binding activity (lane 15) at a concentration of 10 pmol. Since oligonucleotides 3 and 4 correspond to the last 28 nt of the 4A probe, these data indicate that these 28 residues contain sequences necessary for the formation of complexes I and II. This corresponds to residues 344–371 of the PAI-2 J7 cDNA (numbering based on the sequence published by Schleuning *et al.* (8)).

Proteins of 50–52 kDa Recognize Exon 4—To assess the molecular weight of the proteins binding to exon 4, UV cross-linking and SDS-PAGE studies were performed using the 52-nt 4A RNA probe. HT-1080 cytoplasmic extracts were incubated with the labeled 4A RNA and run on a 10% SDS-PAGE along with molecular weight markers (Life Technologies, Inc.). As shown in Fig. 7, two distinct closely spaced proteins (indicated by arrows) were detected that migrated with an apparent molecular mass of ~50–52 kDa (lane 2). To assess the specificity of the complexes binding to the probe, unlabeled RNA of the identical sequence to the labeled probe and unlabeled RNA identical to the last 51 nt of exon 4 (*i.e.* exon 4C RNA) were used as competitors. As indicated in the figure, the formation of the 50–52-kDa proteins was competed by increasing concentrations of the unlabeled 4A sequence (lanes 3–5) but not by the 4C sequence (lanes 6–8).

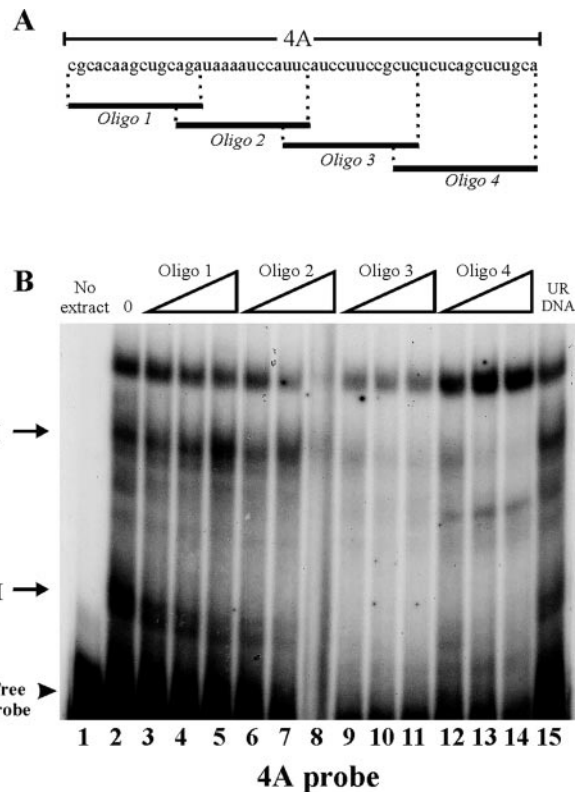


FIG. 6. Fine map localization of the protein-binding sites within exon 4 of PAI-2 mRNA. *Panel A*, schematic representation of the relative position of the four antisense oligonucleotides (Oligo 1–4), complementary to the sequences within region 4A of PAI-2 exon 4, used to perform the oligonucleotide masking experiments. *Panel B*, RNA probes containing the 4A region of exon 4 were incubated with increasing concentrations of cytoplasmic extracts were added, and binding activity was assessed by REMSA analysis. Formation of complex I and II was not inhibited by inclusion of increasing concentrations (0.1, 1.0, or 10 pmol) of antisense oligo 1 (lanes 3–5, respectively). Antisense oligo 2 did not inhibit complex formation at the lower concentrations (0.1 and 1.0 pmol; lanes 6 and 7) but did inhibit when included at the highest concentration (10 pmol, lane 8). Antisense oligo 3 (lanes 9–11) and oligo 4 (lanes 12–14) inhibited complex formation even at the lowest concentrations used. Addition of 10 pmol of antisense oligonucleotide of unrelated sequence (UR) did not inhibit complex formation (lane 15).

Effect of PMA and TNF Treatment on the Binding Activity of Proteins Binding to Exon 4 and the AU-rich Motif in the 3'-UTR—PAI-2 mRNA stability in HT-1080 cells is regulated by PMA and TNF treatment. To confirm and extend these findings, HT-1080 cells were treated with PMA + TNF for various times up to 24 h, and changes in PAI-2 mRNA and antigen levels were assessed by Northern and Western blotting, respectively. As shown in Fig. 8, *panel A*, PAI-2 mRNA levels were markedly increased by PMA + TNF treatment, with an increase in levels apparent after 2 h and maximal levels after 24 h treatment. Western blot analysis of cytoplasmic extracts prepared from the same cells further demonstrated the marked increase in endogenous PAI-2 antigen as a result of this treatment.

Experiments were then performed to determine whether the time course of PAI-2 mRNA induction by PMA + TNF could be correlated with changes in the binding profiles or activities of proteins associating with PAI-2 mRNA instability sequences. To this end, REMSAs were performed using labeled RNA probes specific for the 4A sequence and the 29-nt region within the PAI-2 3'-UTR that contains the nonameric AU-rich instability motif and cytoplasmic extracts prepared from cells treated with PMA + TNF for 1, 2, 4, 8 or 24 h. The AU-rich

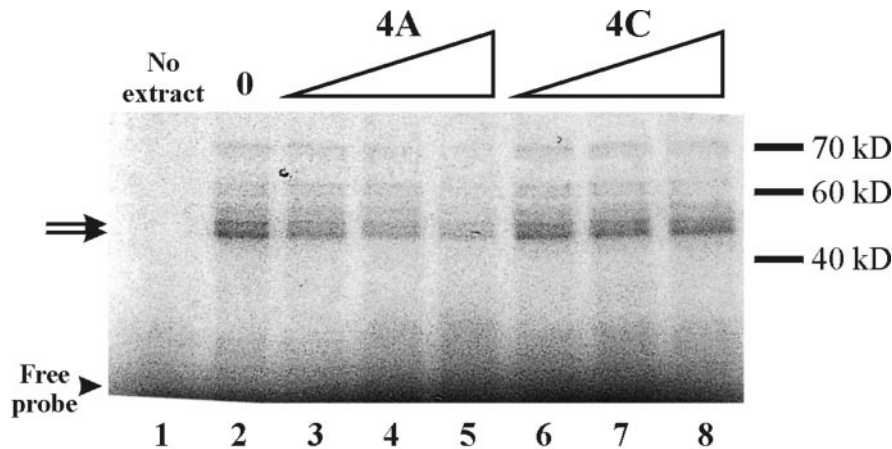


FIG. 7. **Proteins with an apparent molecular mass of 50–52 kDa associate with exon 4 of PAI-2 mRNA.** Cytoplasmic extracts (15 μ g) prepared from HT-1080 cells were incubated with an RNA probe harboring the first 52 nt of exon 4 (probe 4A). Samples were UV cross-linked and then subjected to SDS-PAGE, and labeled protein-RNA complexes were visualized after exposure to x-ray film. Protein-RNA complexes with apparent molecular masses of 50–52 kDa were produced with cytoplasmic extracts in the absence of competitor (0, lane 2). The formation of the cross-linked proteins was reduced in the presence of increasing concentrations (~100-, 400-, and 1200-fold molar excess) of unlabeled 4A RNA (lanes 3–5, respectively) but not by similar concentrations of unlabeled 4C RNA (lanes 6–8).

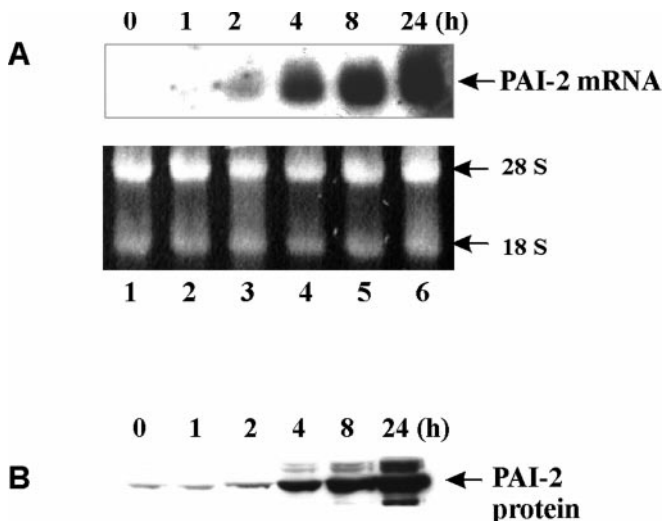


FIG. 8. **Treatment of HT-1080 cells with PMA + TNF causes a marked increase in PAI-2 mRNA and antigen.** Panel A, HT-1080 cells were treated with PMA + TNF for 0, 1, 2, 4, 8, or 24 h. Cells were harvested, and PAI-2 mRNA levels were assessed by Northern blot analysis using a PAI-2 cDNA probe. Ethidium bromide staining of the agarose gel is shown below the image. Panel B, cytoplasmic extracts (50 μ g) prepared from HT-1080 cells treated with PMA+TNF were subjected to SDS-PAGE. PAI-2 antigen levels were assessed by Western blot analysis using an anti-PAI-2 monoclonal antibody.

motif in the 3'-UTR is known to promote PAI-2 mRNA instability and to provide a binding site for cellular factors (1). As shown in Fig. 9 (panel A), PMA + TNF treatment did not alter the intensity of any of the complexes associating with the 4A sequence (lanes 2–7). In contrast, a transient increase in the intensity of one of the complexes formed with the AU-rich motif was observed following PMA + TNF treatment (lanes 9–14). This complex was only weakly detected under nontreated conditions but was clearly increased after 1 h and decreased to basal levels after 8 h of treatment (indicated with arrow). This was observed on three independent occasions using extracts prepared from different preparations of HT-1080 cells treated with PMA + TNF.

The mRNA-stabilizing protein, HuR, is known to recognize the AU-rich instability element in the 3'-UTR. As confirmed in this figure, HuR is associated with the fastest migrating complex formed with the AU-rich RNA probe, as the inclusion of anti-HuR antibodies fully displaced the fastest migrating com-

plex (lane 16, indicated with arrow). The intensity of the HuR containing complex did, however, appear to be slightly increased by treatment with these agents, but this was not a consistent observation. Taken together, inducible stabilization of PAI-2 mRNA by PMA + TNF is associated with a transient increase in the binding activity of cytoplasmic proteins to the AU-rich instability element in the 3'-UTR of PAI-2 mRNA.

Sequences Flanking the Exon 4-Binding Site Are Homologous to Coding Region Instability Elements in Other mRNAs—The protein(s) interacting with exon 4 remain to be identified. However, it is intriguing to point out that a significant degree of sequence homology exists between a 10-nt A-rich region (GATAAAATCC) that partially overlaps (3 nt) and the protein-binding site in exon 4, with sequences within the mRNAs of yeast MAT α 1 (23–25), c-Myc (20–22), uPAR (27), VEGF (26), and TFIIIA (41) (Fig. 10). For MAT α 1, c-Myc, and uPAR, this area of homology also occurs within known or suspected binding sites for proteins associated with mRNA instability. The region of homology with MAT α 1 is found within a 19-nt region of MAT α 1 mRNA instability determinant that is postulated to provide a protein-binding site (25) (boxed sequence in Fig. 10). The region of similarity with c-Myc mRNA also partially overlaps a 15-nt protein-binding site for the coding region determinant binding protein (CRD-BP) (36) (Fig. 10). Significant homology exists within the uPAR, and part of this sequence also overlaps with the beginning of the 51-nt instability region of the uPAR transcript that provides a binding site for a 50-kDa protein (27, 37). Although VEGF mRNA possesses instability determinants throughout the transcript (26), no specific protein-binding sites have been identified. Nonetheless, the region of homology of the PAI-2 exon 4 motif with the VEGF mRNA occurs within the splice site for exons 5 and 7 of the VEGF 164 gene (38) (indicated with arrow in Fig. 10).

The area of homology found within the TFIIIA transcript (position 480–489) is found within a 528-nt sequence of the coding region (position 342–870) that is associated with TFIIIA mRNA instability (41). Interestingly, two additional related motifs are also found in this region; positions 529–538 (CACAAAATCA) and 638–647 (CTGAAAATCC).

Finally, it is interesting to note that where there is sequence divergence in some of these mRNAs to the PAI-2 sequence, the substituted residue is highly conserved. The presence of a homologous motif within the coding region of six transcripts, each of which possess coding region instability determinants,

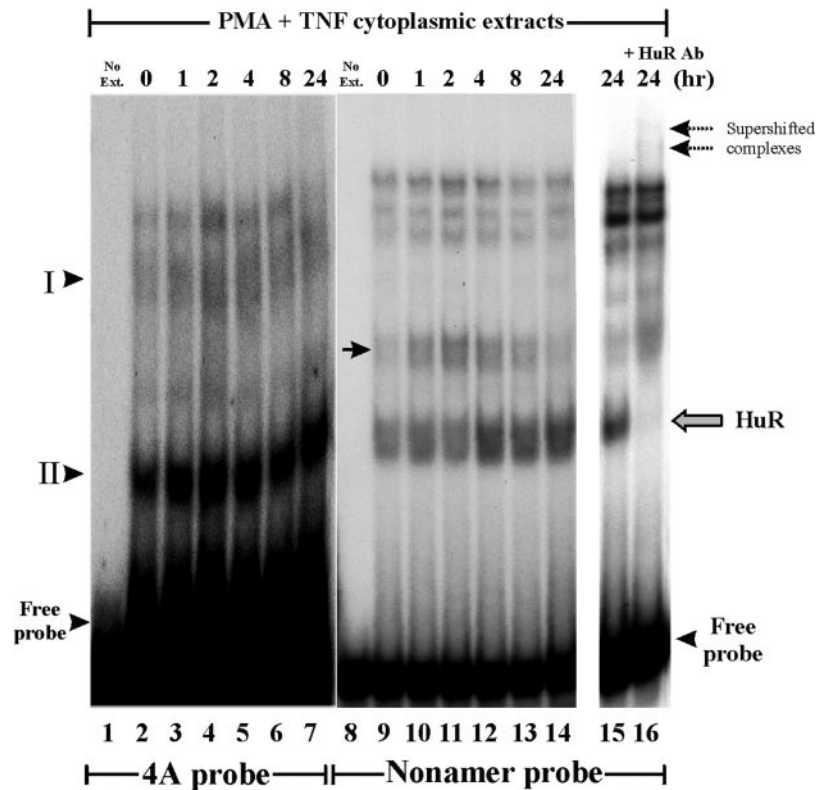


FIG. 9. Treatment of HT-1080 cells with PMA and TNF does not alter the formation of complexes on PAI-2 exon 4 but causes a transient increase in the binding of proteins to the AU-rich instability motif in the 3'-UTR. Cytoplasmic extracts were prepared from HT-1080 cells treated with PMA + TNF for 0, 1, 2, 4, 8, and 24 h (as indicated), and protein binding activity to labeled PAI-2 exon 4 (probe 4A) and the 29-nt AU-rich mRNA instability sequence present within the 3'-UTR of PAI-2 mRNA were assessed by EMSA. No change in the migration pattern or intensity of complexes associating with the exon 4 sequence was observed at any time point (lanes 2-7). However, a transient increase in the intensity of a complex formed with the AU-rich probe is seen following PMA + TNF treatment (lanes 9-14). The level of intensity of this transiently induced complex is maximal after 2 h of treatment with PMA + TNF (lane 11). The position of the induced complex is shown by the arrow to the left of lane 9. No Ext, no extract added. Lanes 15 and 16, extracts prepared from 24-h nontreated HT-1080 cells were used in a REMSA supershift using an antibody directed against HuR. The position of the HuR containing complex is indicated by the solid arrow next to lane 16. The supershifted complexes are visible as faint slow migrating complexes (indicated with dotted arrows at the top of lane 16). The increase in intensity of the complexes shown in lanes 15 and 16 is due to the omission of RNase T1 (see "Experimental Procedures"). Ab, antibody.

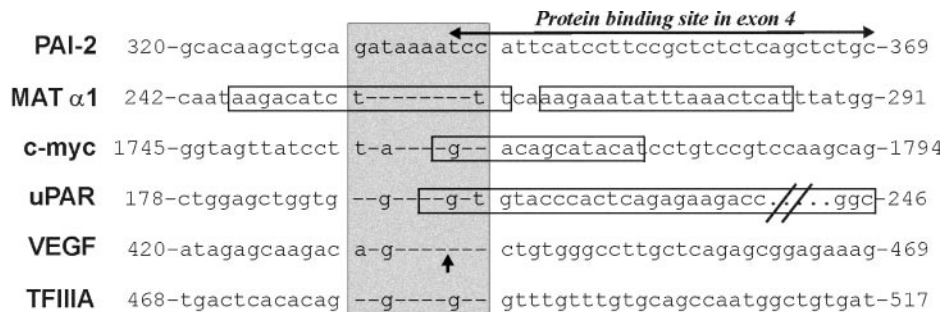


FIG. 10. A region flanking the exon 4-binding site bears homology with sequences present within instability determinants of other transcripts. Alignment of sequences present within exon 4 of PAI-2 mRNA, with known or suspected instability determinants with yeast MAT α 1, c-Myc, uPAR, VEGF and TFIIIA mRNA. A 10-nt region immediately upstream of the PAI-2 exon 4-binding site with homology to sequences in other transcripts is shown within the shaded area. Residues with identity to PAI-2 exon 4 are indicated with dashes. The protein-binding site within exon 4 is indicated with the double-headed arrow above the exon 4 sequence. The tandem 19-nt near-repeat sequences within MAT α 1 that are thought to provide a binding site for proteins are boxed. Similarly, known protein-binding sites within the coding region instability determinants of c-Myc and uPAR are also boxed. The arrow within the shaded area below the VEGF sequence indicates the splice site between exons 5 and 7 of the VEGF gene.

suggests that a common coding region element plays a role in the regulation of mRNA decay.

DISCUSSION

PAI-2 is an impressively regulated serine protease inhibitor. Numerous *in vitro* systems have demonstrated that the PAI-2 gene is increased in many cell types by inflammatory mediators including TNF and LPS. Although many earlier studies focused on the transcriptional mechanisms underlying the induction of

the PAI-2 gene, it is now well established that the post-transcriptional events controlling PAI-2 expression are very important. Indeed, post-transcriptional mechanisms account for most of the increase in PAI-2 expression in response to TNF and PMA treatment in HT-1080 cells (1, 15). PAI-2 mRNA is inherently unstable displaying a half-life of 1 h, and part of this instability is attributed to an AU-rich element located within the 3'-UTR that provides a binding site for HuR which is mainly nuclear in location.

We have extended our study on the post-transcriptional regulation of the PAI-2 gene and evaluated the potential mRNA-destabilizing properties of sequences within the coding region of PAI-2 mRNA. Instability elements elsewhere within the PAI-2 transcript were suspected to exist since the PAI-2 transcript was still relatively unstable in the absence of the 3'-UTR (1).

By using the *c-fos*-HGH mRNA stability system, we have been able to demonstrate that individual exons or groups of exons of the PAI-2 gene could confer varying degrees of instability to the HGH reporter transcript, suggesting that multiple instability elements reside within PAI-2 mRNA, including exons 4, 7, and 8. We focused our efforts on the role of exon 4 in the regulation of PAI-2 mRNA stability as this exon conferred the most potent destabilizing effects upon the HGH reporter transcript, reducing the half-life of the chimeric HGH transcript to ~30 min. Instability elements within exons 7 and 8 were not pursued further in this present study.

To provide further evidence for a role of sequences within exon 4 in the regulation of PAI-2 mRNA stability, an in-frame deletion of exon 4 resulted in a doubling of the PAI-2 mRNA half-life. Although this evidence is reasonably strong, we cannot exclude the possibility that the removal of an entire exon disrupts the secondary structure of PAI-2 mRNA in such a way as to extend the half-life of the PAI-2 transcript. Since fine mapping REMSA and DNA masking studies have mapped the protein-binding site to a 28-nt region within exon 4 (residues 344–371 of the PAI-2 transcript), it would be interesting to assess the decay rate of the PAI-2 transcript that contained a series of point mutations within this particular region.

UV cross-linking and SDS-PAGE experiments revealed that protein-RNA complexes with an apparent molecular mass of 50–52 kDa are formed when cytoplasmic extracts are incubated with RNA probes containing the first 52 nt of exon 4. The identity of this protein(s) and their role in the implementation of mRNA instability remain to be determined.

PAI-2 mRNA is markedly induced in HT-1080 cells treated with either TNF or PMA. Transcriptional increase accounts for about 10–20% of this induction suggesting that the major level of PAI-2 induction under these conditions is post-transcriptional. However, we could find no evidence to indicate that the binding of factors to the exon 4 instability element was altered by treatment with these agents. In contrast, we observed a transient increase in the binding activity of an inducible subset of cytoplasmic protein(s) that recognized the AU-rich motif in the 3'-UTR. This provides circumstantial evidence that these transiently induced proteins play a role in stabilizing PAI-2 mRNA during induction with PMA + TNF. The identity of these protein(s) and a more detailed assessment of their role in inducible regulation of PAI-2 mRNA requires further study. To date, the only known protein to recognize the AU-rich element in the 3'-UTR is HuR. Although HuR resides mainly in the nuclear compartment, a significant amount of HuR is present in the cytoplasm of HT-1080 cells, yet we saw no convincing evidence that the binding activity of HuR altered during treatment of cells with PMA + TNF.

The literature describing the functional coding region mRNA instability determinants in post-transcriptional regulation is rapidly expanding. Indeed the mRNAs for β -tubulin (39, 40), TFIIIA (41), c-Myc (20–22), yeast Mat α 1 (23–25), vascular endothelial growth factor (VEGF) (26), urokinase receptor (27, 37) and *c-fos* (28, 29) all contain coding region instability sequences. Furthermore, some of these contain binding sites for cellular factors. The mechanism by which these coding region mRNA stability/instability determinants influence mRNA decay is obscure. However, one of the best-studied coding region

instability determinants (CRD) is found in the c-Myc transcript (20–22, 42). In this example, a 70-kDa CRD-binding protein (CRD-BP) binds to a region within the last 180 bp of the c-Myc coding region (36, 42, 43) that is proposed to shield the coding region determinant from a ribosome-associated endonuclease (44, 45). Binding of the CRD-BP to this 180 nt was inhibited by a 15-nt DNA oligonucleotide that corresponded to positions 1763–1777 of the c-Myc transcript. It was noted, however, that the binding region may extend beyond these positions (23). The yeast gene MAT α 1 also contains a 65-nt instability determinant within its coding region (23, 24), and two near-identical 19-nt regions within this determinant have been postulated to provide a recognition site(s) for an RNA-binding protein(s) (25). The urokinase receptor (uPAR) mRNA contains a 51-nt instability determinant with the coding region (27) that provides a binding site for a 50-kDa protein (37).

We observed a significant degree of sequence homology between a 10-nt region within exon 4 with the sequence of CRDs in five other transcripts as follows: Mat α 1, c-Myc, uPAR, VEGF, and TFIIIA (Fig. 10). This 10-nt region is also perfectly conserved in the mouse PAI-2 mRNA sequence. This suggests a common coding region motif may play a broad role in the control of mRNA turnover. Whether this region provides a binding site for a common factor and/or facilitates binding of proteins to neighboring sites remains to be determined. We are presently performing competition experiments to determine whether the homologous sequences within the above-mentioned transcripts influence the binding of proteins to the PAI-2 exon 4-binding site.

In summary, we describe for the first time an mRNA instability determinant within exon 4 of the PAI-2 mRNA that provides a binding site for cellular factors with an apparent molecular mass of 50–52 kDa. Based on these results, we suggest that both the PAI-2 exon 4 instability element and the AU-rich element in the 3'-UTR play a role in destabilizing PAI-2 mRNA under constitutive conditions. However, a change in the assembly of proteins binding to the AU-rich motif occurs during treatment with PMA + TNF suggesting that these proteins play a role during inducible PAI-2 mRNA stabilization.

Finally, the region immediately upstream and partially overlapping the exon 4-binding site is homologous to coding region instability determinants of five other transcripts, suggesting the presence of a common element that may play a broad role in the post-transcriptional regulation of mRNA.

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