

Prothrombin G20210A is a Bifunctional Gene Polymorphism

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Summary

The G20210A polymorphism has been shown to alter the efficiency of prothrombin mRNA processing. Here we show that the G20210A mutation also alters prothrombin mRNA stability. Three-fold more prothrombin protein and mRNA were produced in NIH-3T3 cells transfected with the prothrombin cDNAs containing the 20210A variant compared to cells expressing the 20210G variant. mRNA stability assays using chimeric globin transcripts harboring the G or A variant of the 97 nt prothrombin 3'-UTR indicated that the 20210G variant conferred greater instability to the globin reporter transcript than the A variant in transfected HepG2 cells. Both variants of the prothrombin 3'-UTR were shown to provide binding sites for a number of cellular proteins including HuR, an RNA binding protein associated with mRNA stability. Our results indicate that the G20210A is a bifunctional polymorphism, as it not only alters the efficiency of mRNA processing, but also the decay rate of prothrombin mRNA.

Introduction

The prothrombin G20210A polymorphism is located in the 3'-untranslated region (3'UTR) of the prothrombin mRNA, proximal to the poly (A) addition site (1). The 20210A allele has been associated with increased risk for venous thrombosis in a number of studies, and is the most common genetic risk factor for venous thrombosis in the Spanish population (2). In addition, there is a significant association of G20210A with plasma levels of prothrombin activity and antigen, with the highest levels reported in individuals homozygous for the 20210A allele and the lowest levels in those homozygous for the 20210G allele. In the Leiden Thrombophilia Study, the association of the 20210A allele with thrombosis was accounted for entirely by its association with plasma prothrombin levels (1). Soria et al. (3) confirmed an association of the 20210A allele with a 3-fold increased risk of thrombosis and an association with plasma prothrombin. Furthermore, using a combined linkage/disequilibrium analysis, they demonstrated that the G20210A polymorphism accounted for the majority of variation in plasma prothrombin that can be attributed to the prothrombin gene locus, supporting the suggestion that it is a functional polymorphism

and not in linkage disequilibrium with an, as yet unidentified, polymorphism elsewhere in the prothrombin gene locus.

The location of this polymorphism at the last residue of the prothrombin 3'UTR suggests that it may be influencing prothrombin levels via a mechanism related to prothrombin mRNA stability, mRNA processing or protein translation. A recent study by Gehring et al. (4) has indicated that this mutation does in fact alter the efficiency of prothrombin mRNA processing. In this current study, we sought to determine whether the G20210A polymorphism also influences prothrombin protein secretion and mRNA stability independently of changes in prothrombin mRNA processing.

Methods

Cell Culture

Mouse NIH-3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM: Life Technologies), supplemented with 10% (v/v) heat inactivated foetal calf serum (HI-FCS), 2 mM glutamine, and streptomycin and penicillin, in a humidified atmosphere at 37° C with 5% CO₂ as previously described (5) HepG2 cells were also maintained in DMEM containing 10% fetal calf serum, but were supplemented with 1X non-essential amino acids (Sigma, MO, USA), and 1 mM sodium pyruvate.

Plasmids

To assess the influence of the prothrombin G20210A polymorphism on prothrombin secretion *in vitro*, expression vectors containing full-length wild-type (20210G) and mutant (20210A) prothrombin cDNA variants were generated. The prothrombin cDNA (6) inserted into the *Pst*I site of pBR 322 is missing part of exon 1 which includes 31 bp of 5'UTR and the ATG codon of the prothrombin lead sequence. An extended oligonucleotide including this missing sequence (5' – TTA CTA GTC GAC AAG CTT AAT TCC TCA GTG ACC CAG GAG CTG ACA CAC TAT GGC GCA CGT CCG AGG CTT GCA GCT GCC TGG CTG CC – 3') was used in conjunction with oligonucleotides directed to the 3'UTR with the relevant base substitution (underlined residue): 20210G: 5' – ATG TAT ACG CGG CCG CCG CTG AGA GTC ACT TTT ATT G – 3'; 20210A: 5' – ATG TAT ACG CGG CCG CTG CTG AGA GTC ACT TTT ATT A – 3', in order to amplify each variant of full-length prothrombin cDNA using Vent polymerase (New England Biolabs, MA, USA). The prothrombin PCR fragments (both variants) were inserted into the multiple cloning site (5' – *Sal*I; 3' – *Not*I) of the pCI neo mammalian expression vector (Promega, Madison, WI, USA) to produce vectors pCMV-PT-WT and pCMV-PT-Mut (see Fig. 1 panel A). The prothrombin cDNA inserts were verified by sequencing. These vectors express prothrombin mRNA using the SV40 polyadenylation site in the pCI-neo vector, hence the prothrombin mRNA produced contains additional sequence at the 3' end due to the use of this polyadenylation site and is also cleaved and processed downstream of this position. Using this approach, the two prothrombin mRNA variants undergo identical cleavage and polyadenylation reactions. This approach was taken to allow us to study the effect of the polymorphism on prothrombin mRNA stability independent of any additional effects this mutation may have on mRNA processing.

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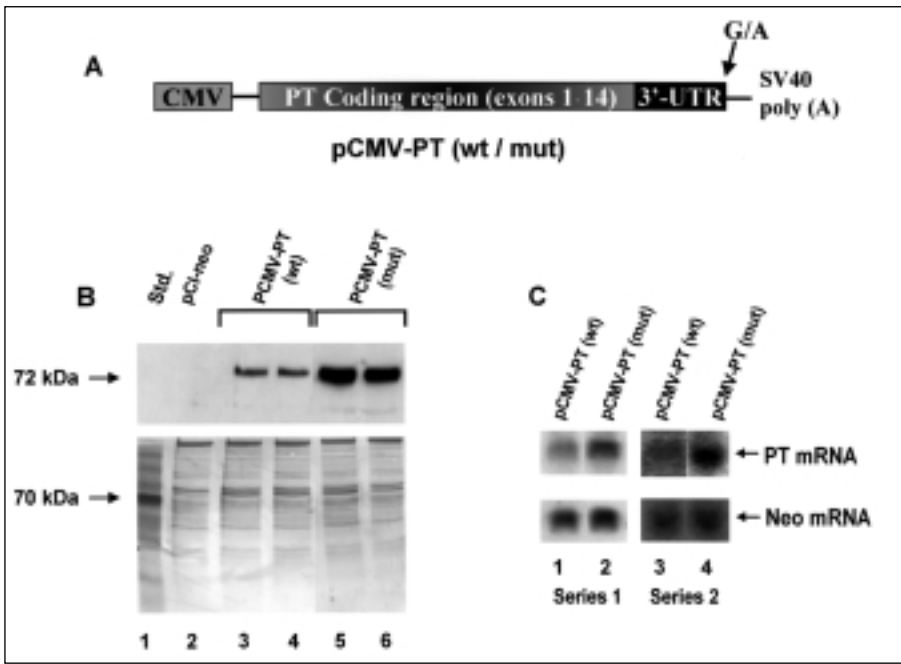


Fig. 1 Cells overexpressing full length mutant prothrombin cDNA produce 3-fold more protein and mRNA than cells expressing the wild-type prothrombin counterpart. **Panel A:** Schematic representation of the prothrombin expression vectors (pCI-PT WT/ Mut) generated for overexpression of either wild-type or mutant prothrombin in NIH-3T3 cells. **Panel B:** Cells transfected with pCI-PT-Mut secrete higher levels of 72 kDa prothrombin (PT) protein than cells transfected with CI-PT-WT. Western blot analysis of conditioned medium obtained from pooled populations of NIH-3T3 cells transfected with either pCI-neo (control; lane 2), pCI-PT WT (lanes 3 and 4) or pCI-PT Mut (lanes 5 and 6). The lower panel shows a coomassie stain of the same filter used for the Western blot analysis. Lane 1: Molecular weight markers. **Panel C:** Cells transfected with pCI-PT-Mut express higher levels of prothrombin mRNA than cells expressing pCI-PT-WT. Northern blot analysis of two series of NIH-3T3 cells stably transfected with either pCI-PT-WT (series 1 and 3) or pCI-PT-Mut (series 2 and 4) was performed using a full length-prothrombin cDNA probe. The filter was rehybridized for neomycin phosphotransferase (neo) mRNA to assess changes in transfection efficiency

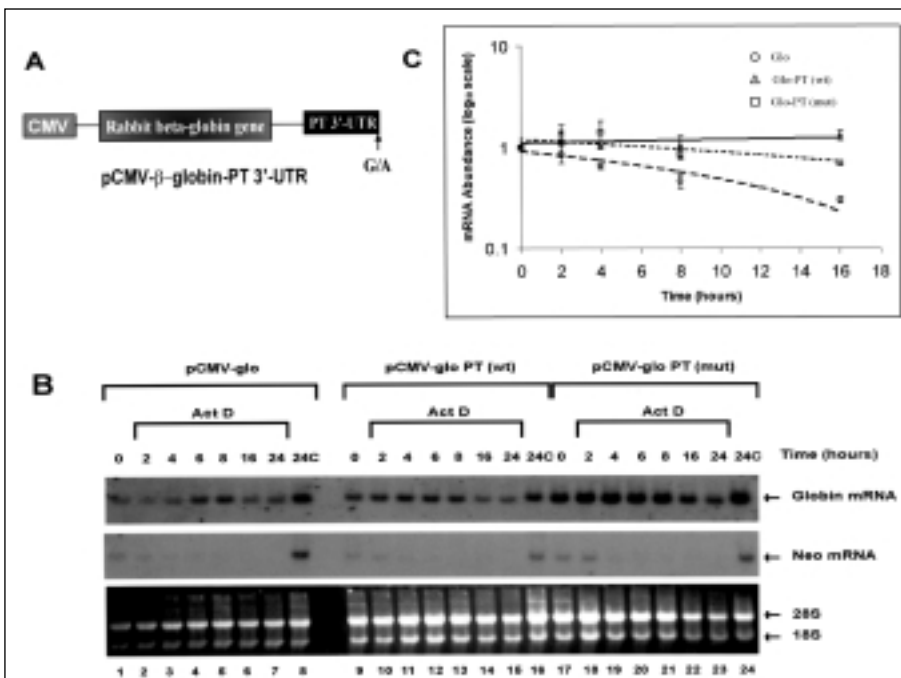


Fig. 2 The G allele variant of the prothrombin 3'-UTR confers greater instability to globin mRNA than the A allele variant in transfected HepG2 cells. **Panel A:** Schematic representation of the chimeric globin-prothrombin vectors (pCMVglo-PT WT and pCMVglo-PT-Mut) prepared for analysis of the decay rate of globin mRNA in stably transfected HepG2 cells. **Panel B:** The wild-type prothrombin 3'-UTR variant confers greater mRNA instability to a globin reporter transcript than the wild-type counterpart. HepG2 cells expressing pCMVglo, pCMVglo-PT-WT or pCMVglo-PT-Mut were treated with actinomycin D (Act-D) for up to 24 h as indicated. Globin and neomycin phosphotransferase (Neo) mRNA were assessed by Northern blot analysis. The lower panel shows the ethidium staining of the agarose gel showing the relative intensities of the 28S and 18S ribosomal RNA markers. **Panel C:** Quantitation of the decay rate of β -globin and chimeric β -globin-prothrombin mRNA by real time PCR and regression analysis. Globin mRNA containing the wild-type prothrombin 3'-UTR (diamond symbol) decays at a faster rate (half-life 10 h) than globin transcripts harbouring the mutant prothrombin 3'-UTR (solid circles; half-life approximately 20 h). The graph represents an average of two independent actinomycin-D time course experiments. Error bars indicate standard error of the mean

The pCMVglo mRNA stability system was used to assess the influence of each variant of the prothrombin 3'UTR on reporter gene mRNA stability (7). This method assesses the ability of the 97 nt prothrombin 3'-UTR variants to destabilise the normally stable rabbit β -globin (glo) mRNA under the control of the CMV promoter during actinomycin-D induced transcriptional blockade. As with the pCI-neo expression vector described above, this vector also utilises the SV40 polyadenylation site. Hence, the globin-PT mRNA variants were both subjected to identical cleavage and polyadenylation processes. The two variants of the prothrombin 3'-UTR were amplified using oligonucleotides encompassing this region with the relevant base substitution (bold type): forward: 5' – ACT CTA GAC TCG AGG GGG CCA CTC ATA TTC TG – 3'; reverse 20210G: 5' – ATG AAT TCC TCG AGC GCT GAG AGT CAC TTT TAT TG – 3'; reverse 20210A: 5' – ATG AAT TCC TCG AGT GCT GAG AGT CAC TTT TAT TG – 3'. Products were inserted into the *Xho* I site of plasmid pCMVglo (7) producing pCMVglo PT-WT (containing the prothrombin wild-type 3'-UTR) and pCMVglo PT-Mut (containing the prothrombin mutant 3'-UTR) (see Fig. 2 panel A).

Cell Transfection

NIH-3T3 fibroblasts were stably transfected with pCI-neo, pCMV-PT-WT and pCMV-PT-Mut by calcium phosphate precipitation followed by G418 selection (600 μ g/ml). HepG2 hepatoma cells were stably co-transfected with pCI-neo (to confer neomycin resistance) and pCMVglo, pCMVglo PT-WT or pCMVglo PT-Mut by the Effectine transfection system (QIAGEN, Clifton Hill, Australia) using a DNA:Effectine ratio of 1:10, followed by G418 selection (1000 μ g/ml). Pooled clones (at least 200 from each transfection) were used for all subsequent experiments.

Western Blot Analyses for Secreted Prothrombin Protein

NIH-3T3 cells transfected with pCI-neo, pCMV-PT-WT and pCMV-PT-Mut were seeded into 60 \times 15 mm dishes at a density of 1.0×10^6 cells/dish and cells incubated at 37° C for 16 h in 10% HI-FCS DMEM. Following two washes with PBS, cells were grown for a further 48 h in 1.5 ml of serum-free DMEM, and the conditioned medium removed. Conditioned medium (25 μ l) was subjected to 10% SDS-PAGE and the samples electroblotted on to PVDF membranes. Prothrombin antigen was assessed by Western Blot analysis using a prothrombin monoclonal antibody (United States Biological, MA, USA) at a 1:2500 dilution. Secondary antibodies directed against mouse IgG coupled to Horseradish peroxidase (Silenus, Australia) were used at a 1:10,000 dilution. Signals were revealed by chemiluminescence (Amersham, Australia).

Northern Blot Analyses

Total RNA was extracted from selected cells as described by (8). Aliquots of 5 to 10 μ g of RNA were electrophoresed through 1% agarose gels containing 20% formaldehyde and subsequently transferred to Hybond-N⁺ membranes (Amersham, Australia). The filters were hybridized with ³²P-labeled DNA probes as previously described (9). Membranes were processed by standard techniques and exposed to Kodak BioMax film (Eastman Kodak) at –80° C with an intensifying screen. Signals were quantified by densitometry using a Linotype-Hell scanner.

The labelled inserts used for hybridisation were obtained as follows: the full-length *Eco* RI cDNA fragment of prothrombin and the 972 bp *Bam* HI/*Hind* III cDNA fragment of neomycin from pCI-neo, and the 380 bp *Bam* HI/*Hind* III fragment of pGEMglo containing exon 2 of rabbit β -globin (7).

mRNA Stability Systems

HepG2 cells stably transfected with pCMVglo, pCMVglo PT-WT and pCMVglo PT-Mut were seeded into 60 \times 15 mm dishes at 1.0×10^6 cells/dish. Transcription was blocked by the addition of 5 μ g/ml actinomycin D (Sigma, MO, USA) and cells were maintained in culture for 4, 8, 16, and 24 h. Zero and 24 h non-treated control dishes were also included. At the appropriate time

point, medium was removed and cells were washed in PBS. Total RNA was extracted from cells and processed as described above. Filters were probed with ³²P-labelled globin cDNA probes using Rapid-Hyb hybridisation buffer following manufacturers instructions (Amersham, Australia) or as previously described (9). Washed membranes were exposed to Kodak BioMax film (Eastman Kodak, Australia) with intensifying screens. In order to assess transfection efficiency and loading, filters were stripped and re-probed for neomycin mRNA. Signals were quantified using densitometry or by real-time PCR, after adjustment for rRNA.

Fluorogenic 5' Nuclease Assay

Globin mRNA levels in HepG2 cells were also quantified by fluorogenic 5' nuclease assay (TaqMan assay, real-time PCR). Primers were designed to amplify a 99 bp product of globin mRNA using Primer Express software (Applied Biosystems): forward primer: 5'-AGT GAA CTG CAC TGT GAC AAG CT-3'; reverse primer: 5'-TTC TTT GCC AAA ATG ATG AGA CA-3'; probe: 5'-CCA GGA GCC TGA AGT TCT CAG GAT CCA-3' (labelled at the 5' end with FAM as the reporter dye and at the 3' end with TAMRA as the quencher dye). These primers span exons 2 and 3 and were designed to discount genomic DNA contamination. 18S rRNA was evaluated as the endogenous control (Applied Biosystems pre-developed assay reagents). Total RNA was reverse transcribed using reagents from Applied Biosystems as follows: 2 μ g RNA was incubated with 1 μ l random hexamer primer, 4 μ l dNTP in a total volume of 10 μ l at 65° C for 5 min and then placed on ice. Sample mix and reverse transcriptase mix (containing 2 μ l X10 buffer, 4.4 μ l magnesium, 0.4 μ l RNase inhibitor and 0.5 μ l reverse transcriptase in a total volume of 10 μ l/sample) were then heated to 25° C for 2 min. 10 μ l transcriptase mix was added to each sample and incubated at 25° C for 10 min, 48° C for 50 min and 95° C for 5 min, and samples diluted 1:2 with nuclease free water.

1 μ l cDNA (50 ng total RNA concentration) was added to each well of a 96 well plate and 24 μ l of master mix added (12.5 μ l TaqMan Universal 2X Master Mix, 300 nM forward primer, 900 nM reverse primer, 150 nM probe, 1.25 μ l 18S 20X primer/probe mix, 8.9 μ l nuclease free water). Samples were amplified using an ABI Prism 7700 SDS (Applied Biosystems) with standard conditions of 50° C for 2 min, 95° C for 10 min followed by 40 cycles of 95° C for 15 s, 60° C for 1 min. Relative globin expression was determined by the comparative cycle threshold (CT) method (10). The half-life of β -globin and chimeric β -globin-prothrombin transcripts was assessed by regression analysis using Microsoft Excel (version 97).

RNA Electrophoretic Mobility Shift Assays

RNA electrophoretic mobility shift assays (REMSAs) were performed as previously described (11) to determine whether cytoplasmic or nuclear proteins could recognise either variant of the prothrombin 3'-UTR. Two different RNA probes containing the prothrombin variant were synthesised: one containing the entire 97 nt 3'-UTR, and another containing the last 30 nt of the prothrombin transcript plus 16 adenine residues (designed to constitute a short poly(A) tail).

For the generation of RNA probes containing the entire 3'-UTR, DNA templates were produced by inserting each prothrombin 3'UTR variant (same fragments used to create pCMVglo-PT-WT/Mut; see above) into the *Xho* I site of pBluescript II KS+, generating pPT-WT and pPT-Mut. The orientation of the inserts was determined by DNA sequencing. pPT-WT and pPT-Mut were linearised with *Sal* I. *In vitro* transcription was carried out by incubating 0.5 μ g of each template with 50 μ Ci [α -³²P] UTP, 10 μ M UTP, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 20 U RNase inhibitor, 1X polymerase buffer and 50 U T3 RNA polymerase at 37° C for 2 h. Prothrombin RNA transcripts generated by this approach contained 29 nt of vector sequence at the 5' end and 6 nt of vector sequence at the 3'-end. The total length of the transcribed RNA was 132 nt of which 97 nt comprised the entire prothrombin 3'-UTR (either G or A allele variant). Samples were electrophoresed through denaturing 6% polyacrylamide gels containing 4M urea. Probes were excised and eluted in elution buffer (500 mM NH₄CH₃COO, 1 mM EDTA) overnight at room temperature with shaking, ethanol precipitated at –80° C and finally resuspended in water (100 cps/ μ l).

To generate the RNA probes containing the last 30 nt of prothrombin mRNA plus 16 adenine residues, complementary oligonucleotides spanning this region of the prothrombin transcript (wild-type and mutant variants) were synthesised and annealed. The oligonucleotides were designed to incorporate *Sac* I and *Hind* III sites at the 5' and 3' ends, respectively, to enable direct subcloning into pBluescriptKS+ that had been digested with *Sac*-I/*Hind* III. The *Hind* III site was selected at the 3'-end as linearization of the plasmids with this restriction enzyme produces an adenine residue as an overhang. The sequences of oligonucleotides that were synthesised and annealed are below. Residues in lower case represent *Sac*-I or *Hind* III overhangs, while the residues in bold-type represent the polymorphic variants:

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5' - cTATGGTCCCAATAAAAAGTACTCTCAGCG/
AAAAAAAAAAAAAAAAA - 3'
5' - agctTTTTTTTTTTTTTTTC/
TGCTGAGAGTCACTTTTATTGGGAACCATAgagct - 3'
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In vitro transcription of RNA probes containing this shorter prothrombin probe were produced using T7 polymerase and ³²P-UTP as described above following linearization with *Hind* III. Prothrombin RNA probes (containing either the full length 3'-UTR or the end region and short poly (A) tail) were incubated with 4 µg HepG2 cytoplasmic or nuclear protein extracts [extraction procedure as previously described (11)]. Samples were electrophoresed through native 6% polyacrylamide gels, fixed, dried and exposed to X-ray film. Where indicated, REMSA supershift experiments were performed using antibodies specific for HuR and AUF-1 (kind gifts from Dr. Henry Furneaux and Dr. Gary Brewer, respectively). As we have previously shown these antibodies specifically recognize their target antigen (11). 2 and 4 µl of 1:2 diluted antibody solution was used in the supershift experiments.

Generation of Unlabelled RNA Competitors

Unlabelled RNA sequences were produced as previously described (11). Competition for binding to the RNA probes containing the full-length prothrombin 3'-UTR was performed using a 74 nt sequence of exon 4 of the plasminogen activator inhibitor type 2 (PAI-2) transcript (11). For competition for binding of proteins to the 60 nt prothrombin transcript containing the short poly (A) tail (46 nt prothrombin sequence plus 14 residues of 5' lead sequence), was performed using RNA sequences generated from the empty pBluescript vector that had been linearized with *Hind* III. RNA (80 nt) was transcribed using T7 polymerase and the RNA isolated as described (11). Competition titration experiments were performed using 100-1000-fold excess of unlabelled competitors.

Results

Cells Expressing the Prothrombin Mutant cDNA Variant Produced 3-fold more Prothrombin Protein and mRNA than Cells Expressing the Wild-type Counterpart

Conditioned medium of NIH-3T3 cells stably expressing either the full-length wild-type (G allele) or mutant (A allele) prothrombin cDNAs (Fig. 1, panel A) were assessed for secreted prothrombin protein by Western blotting using a monoclonal anti-human prothrombin antibody. As shown in Figure 1 (panel B), cells transfected with the mutant variant produced more prothrombin antigen than cells transfected with the wild-type variant. Cells transfected with the pCI-neo expression vector alone did not produce any detectable prothrombin antigen. To more accurately assess this data, the same experiment was performed using conditioned medium obtained from two further sets of stably transfected NIH3T3 cells. The degree of prothrombin protein released into the conditioned medium was assessed by Western blotting and quantitated by densitometry. These combined data revealed that the

mutant prothrombin variant produced 3.1 ± 0.21 -fold more prothrombin protein than the wild-type counterpart.

Northern blot experiments were performed to determine whether the increase in prothrombin antigen in cells transfected with the mutant variant was reflected at the level of prothrombin mRNA. As shown in Figure 1 (panel C), cells transfected with the mutant variant produced higher steady state levels of prothrombin mRNA than cells transfected with the wild-type counterpart. As a control for transfection efficiency, the filters were probed for the neomycin phosphotransferase mRNA produced by the pCI-neo expression vector. Densitometric quantitation of the levels in mRNA revealed that prothrombin mRNA levels were increased 3.1 ± 0.06 -fold in cells expressing the mutant variant. This data is the average of two independent experiments and calculated after corrected for changes in neomycin phosphotransferase mRNA. When these data were assessed by real-time PCR, the fold-increase produced in cells expressing the mutant prothrombin variant was 2.8-fold (relative to ribosomal RNA) compared to the wild-type prothrombin counterpart. It can therefore be concluded from these studies that the higher levels of prothrombin protein produced from cells transfected with the mutant prothrombin cDNA (20210A) are due to higher levels of prothrombin mRNA produced by this variant compared to the wild-type (20210G) variant.

mRNA Stability Studies

To address the possibility that the increase in prothrombin mRNA produced by the A allele variant was due to enhanced prothrombin mRNA stability, we assessed the ability of the prothrombin 3'-UTR variants to destabilise the rabbit β-globin reporter mRNA during actinomycin-D induced transcriptional blockade in HepG2 cells. Each variant of the 97 nt 3'-UTR of PT mRNA was cloned into the 3'-UTR of the rabbit β-globin gene under the control of the CMV promoter (pCMVglo), creating plasmids pCMVglo-PT-WT and pCMVglo-PT-Mut (Fig. 2 panel A) and stably transfected into HepG2 cells. The decay rate of the β-globin reporter transcript was determined by Northern blotting following treatment with 5 µg/ml actinomycin-D (Fig. 2 panel B). Quantitation of the β-globin transcripts was assessed by real-time PCR after correcting for 18S rRNA (Panel C). As shown in Figure 2 (panels B and C), control β-globin mRNA levels remained stable for the 24 h actinomycin D time course. However, introduction of either variant of the prothrombin 3'-UTR decreased the half-life of the reporter transcript but to differing degrees. Interestingly, the presence of the wild-type (G allele) prothrombin 3'-UTR reduced globin mRNA half-life to approximately 10 h, whereas the mutant (A allele) variant of the PT 3'-UTR was less effective, reducing globin mRNA half-life to approximately 20 h. Hence, the wild-type variant of the prothrombin 3'-UTR confers greater mRNA instability than the mutant variant. To verify that actinomycin D had indeed blocked transcription, the membranes were hybridized for the neomycin phosphotransferase (neo) mRNA. As shown in panel B, levels of the neomycin transcript were rapidly reduced in each of the three HepG2 cell lines following actinomycin-D treatment.

RNA Electrophoretic Mobility Shift Assay

RNA electrophoretic mobility shift assays (REMSAs) were performed to determine whether the full length 97 nt 3'-UTR of prothrombin mRNA could provide a binding site for cellular factors. Results indicated that up to three retarded RNA-protein complexes were produced with both cytoplasmic and nuclear extracts (complexes C1-C3;

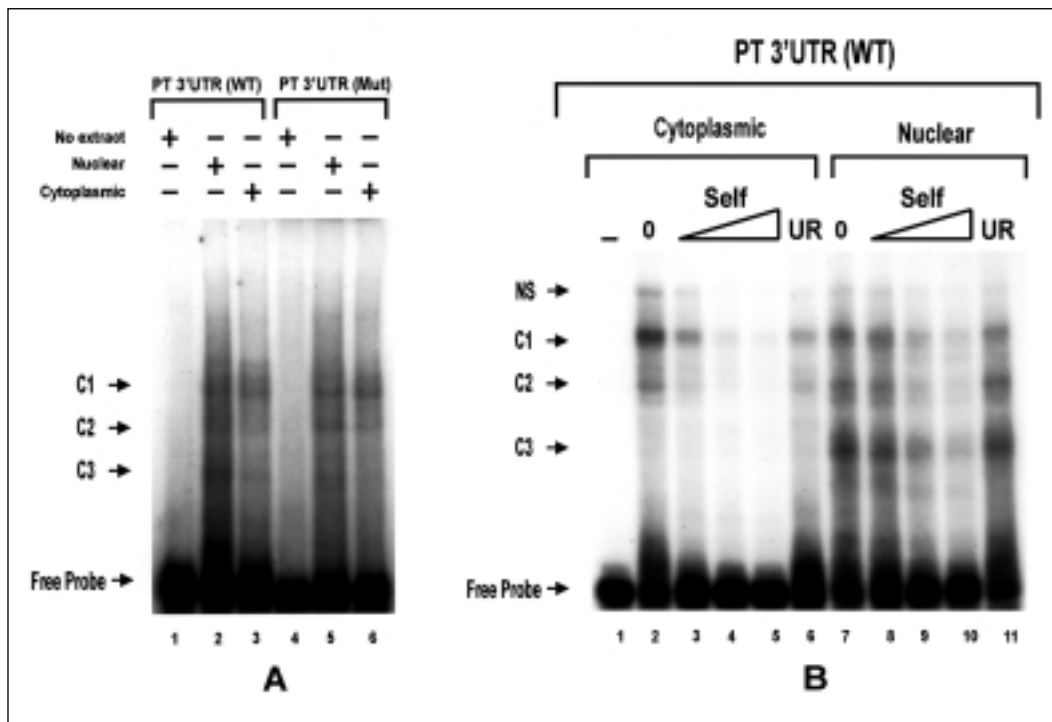


Fig. 3 The 3'-UTR of prothrombin mRNA provides a binding site for cytoplasmic and nuclear factors. *Panel A:* REMSAs were performed using RNA probes containing the wild-type (lanes 1-3) or mutant (lanes 4-6) prothrombin 3'-UTR incubated with either nuclear or cytoplasmic extracts from HepG2 cells. Lanes 1 and 4: no extract added. Three protein-RNA complexes were generated (C1, C2, C3) as indicated by the arrows to the left of the figure. *Panel B:* Binding of proteins to the wild-type prothrombin 3'-UTR is specific. HepG2 cytoplasmic (lanes 2-6) or nuclear (lanes 7-11) extracts were incubated with 32 P-labelled prothrombin 3'-UTR (wild-type variant) in the absence or presence of unlabelled prothrombin competitor RNA. Increasing concentrations of unlabelled wild-type prothrombin 3'-UTR (lanes 3-5 and lanes 8-10) competed for binding. Binding was not competed by addition of an unlabelled RNA of unrelated (UR) sequence (containing the instability element in the 3'-UTR of PAI-2 mRNA [11]) (lanes 6 and 11). The positions of protein-RNA complexes C1, C2 and C3 are indicated. NS: non-specific complex

Fig. 3 panel A), with nuclear extracts producing more abundant C3 complex. The fainter slower migrating complexes were not always seen. Binding specificity was determined by competition titration experiments using the wild-type prothrombin 3'-UTR as a probe. As shown in Figure 3 (panel B), binding of cytoplasmic (lanes 2-5) or nuclear (lanes 7-10) extracts was specific as inclusion of unlabelled RNA of the same sequence (i.e. unlabelled prothrombin 3'-UTR) effectively competed for binding whereas unlabelled RNA of an unrelated sequence failed to compete (lanes 5 and 11). These data indicate that sequences within the prothrombin 3'-UTR provide binding sites for cellular factors. Comparison of the binding profiles obtained using the wild-type and mutant prothrombin 3'-UTR variants did not provide any convincing evidence for a change in the binding pattern.

HuR is one of the Nuclear Factors that Recognises the 3'-UTR of Prothrombin

To identify the proteins interacting with the 3'-UTR of prothrombin, we performed supershift assays using antibodies directed against known RNA binding proteins, specifically HuR and AUF-1. As shown in Figure 4, addition of increasing amounts of anti-HuR antibodies generated two clear supershifted complexes, predominantly in the nuclear fraction (lanes 3-4 and 9-10). Antibodies specific against AUF-1 did not produce any supershifted complex with either nuclear or cytoplasmic proteins bound to the prothrombin 3'-UTR (data not shown). Complexes C1-C3 are also indicated by the arrows in the Figure. The additional complex that migrates just above complex C1

was occasionally observed. The presence of two supershifted complexes suggests that HuR may also be interacting with an additional factor while bound to the prothrombin mRNA. Although there appears to be slight difference in the intensity of the anti-HuR supershifted complexes when using the mutant and wild-type prothrombin 3'-UTR probes, this was not a consistent observation. Based on our repeated experiments, we did not see any significant change in the binding of HuR to either prothrombin 3'-UTR variant.

Sequences Surrounding the G20210A Polymorphism Provide Protein-binding Sites

To determine whether the sequences in the immediate vicinity of the G20210A polymorphism provided protein-binding sites for HepG2 cytoplasmic proteins, RNA probes harbouring the terminal 30 nt of the prothrombin 3'-UTR as well as 16 adenine residues were used as probes in a REMSA. As shown in Figure 5, HepG2 cell-derived cytoplasmic proteins specifically interacted with this prothrombin RNA probe containing the wild-type sequence (lane 2). The two faster migrating protein/RNA complexes were specific (arrows) as determined by competition with self (lanes 3-6) or unrelated (lanes 7-10) competitor RNA. The two slower migrating complexes seen on the gel are non-specific. REMSAs performed using the mutant prothrombin variant produced an identical pattern (data not shown). Hence, although the sequences surrounding the polymorphic region produce a specific protein-binding site, we have not observed any obvious change in the binding characteristics as a consequence of the polymorphism.

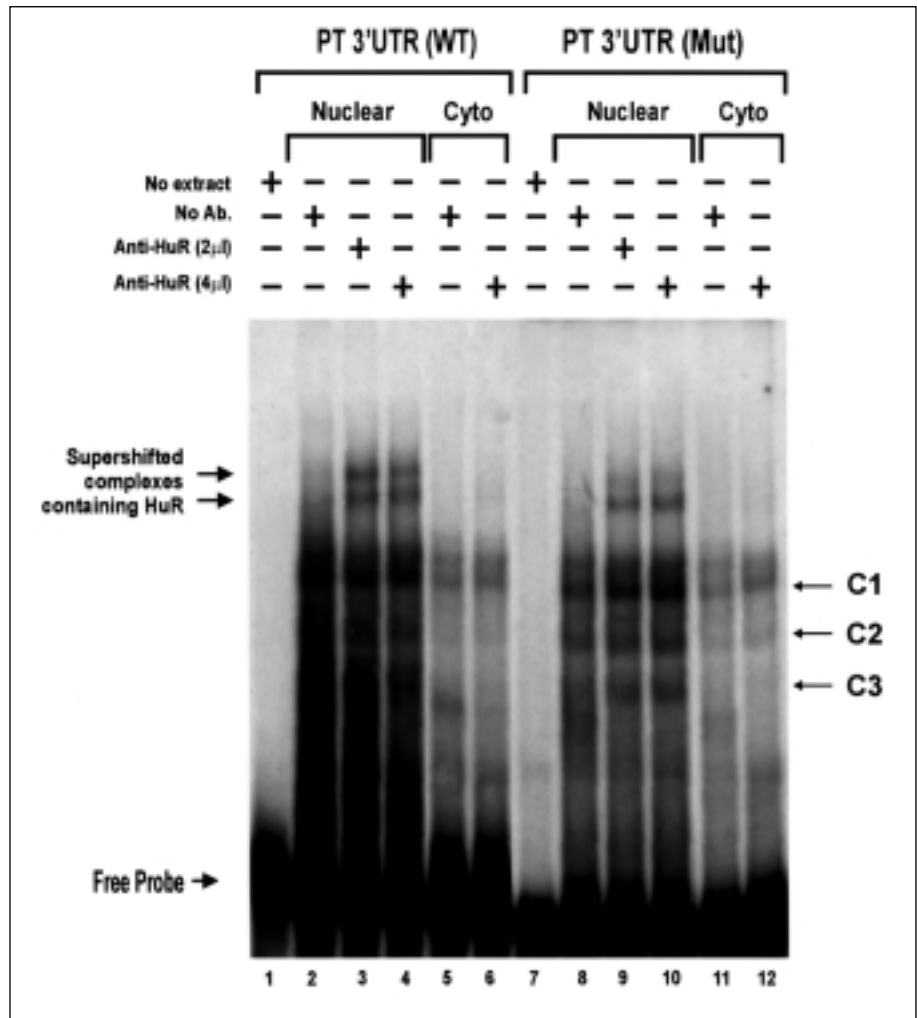


Fig. 4 HuR is part of the complex of proteins that recognise the prothrombin 3'-UTR. REMSAs were performed using ³²P-labelled RNA probes harbouring either the wild-type or mutant prothrombin 97 nt 3'-UTR incubated with either nuclear or cytoplasmic extracts prepared from HepG2 cells. Increasing amounts of the anti-HuR antibody produced two supershifted complexes when using nuclear extracts with both the wild-type sequence (lanes 3 and 4) and the mutant prothrombin 3'-UTR (lanes 9 and 10) as indicated with arrows to the left of the figure. Anti-HuR supershifted complexes using cytoplasmic extracts were only visible after prolonged exposure of the gel to X-ray film

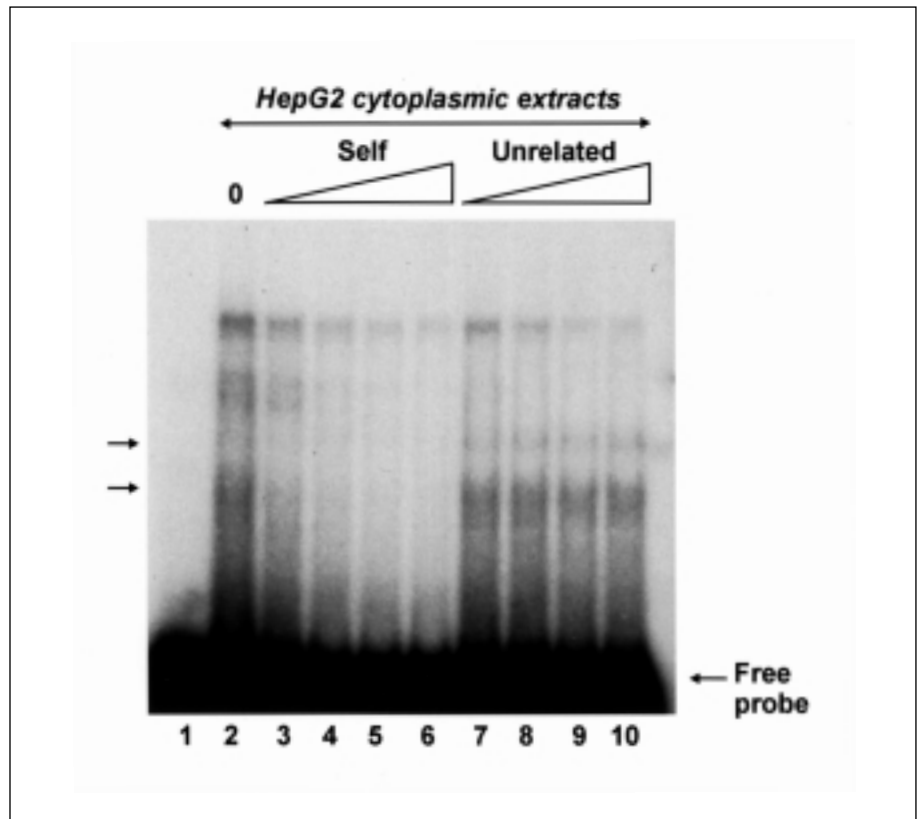


Fig. 5 The terminal sequences of the prothrombin mRNA provides a protein binding site. REMSAs were performed using a probe containing the last 30 nt of the prothrombin 3'-UTR transcript as well as 16 adenine residues to constitute a short poly(A) tail. As shown, this region of the prothrombin transcript provided a binding site for cytoplasmic extracts prepared from HepG2 cells (arrows to left of figure). Addition of 100, 250, 500 and 750 ng of unlabelled RNA of the same sequence as the probe ("Self") competed for binding (lanes 3, 4, 5 and 6, respectively), whereas no competition was seen when the same concentrations of unlabelled RNA derived from the multiple cloning site of pBluescript was used ("Unrelated"; lanes 7, 8, 9 and 10, respectively). Lane 1: probe alone

Discussion

In this study, we have addressed the molecular basis for the increase in plasma levels of prothrombin in individuals carrying the A allele variant of the prothrombin G20210A polymorphism. Genetic studies have provided strong evidence to indicate that this particular polymorphism is indeed functional (3). The position of this polymorphism in the 3'-UTR led to the suggestion that this CG to CA substitution may alter either prothrombin mRNA processing, mRNA stability or translation. It has now become clear that these processes are interlinked, for example, mRNA cleavage and polyadenylation can directly and indirectly influence mRNA stability (12–14) and translation initiation (15). Given the position of this polymorphism, it is conceptually possible that this single nucleotide substitution influences more than one parameter of mRNA metabolism. Indeed, a recent study has indicated that this polymorphism increases the efficiency of mRNA processing (4). We directed our study to address the possible influence of this polymorphism on prothrombin mRNA stability in a manner independent of mRNA processing. To allow this, we inserted the full-length prothrombin cDNAs [wild-type (G allele) and mutant (A allele)] variants into the pCI-neo expression vector. This vector processes prothrombin mRNA using the SV40 poly (A) signal contained in the plasmid. Indeed, the transcribed prothrombin transcripts both contained this additional sequence as assessed by RT-PCR (data not shown). This strategy was followed to eliminate possible effects of the G20210A on mRNA processing and therefore permitted an assessment of this mutation on prothrombin mRNA stability in isolation.

Our results have demonstrated that overexpression of the mutant variant of the prothrombin cDNA in NIH-3T3 cells results in an increase in prothrombin protein secretion and mRNA accumulation, compared to cells expressing the wild-type counterpart. Since the relative increase in mRNA was similar to that seen at the level of protein secretion, it is unlikely that the polymorphism influences the translation rate. Furthermore, since the wild-type and mutant prothrombin expression vectors are using the same SV40 polyadenylation signal, the efficiency of mRNA processing of both transcripts is essentially identical. We speculated therefore that the reason for the increase in prothrombin expression in cells transfected with the mutant variant under these conditions was associated with an increase in mRNA stability. To address this possibility, we inserted the two 97 nt prothrombin 3'-UTR variants into the pCMVglo expression vector and stably introduced these constructs into HepG2 cells. HepG2 cells were selected for this purpose because they are a liver-derived cell line and synthesise endogenous prothrombin (16) and are likely to contain factors that may recognise regions within the prothrombin 3'-UTR and influence its decay rate. Actinomycin D transcriptional blockade experiments revealed that the decay rate of the β -globin transcript containing the mutant 3'-UTR variant decayed at a slower rate than the reporter transcript containing the wild-type 3'-UTR variant. This was quantitated by the TaqMan assay (Real-time PCR). Taken together, this suggests that the increase in prothrombin mRNA and protein production in cells containing the mutant variant arise as a consequence of slower prothrombin mRNA decay compared with the wild-type variant.

Our experiments were designed to explore the effect of the G20210A polymorphism on prothrombin mRNA stability under conditions that did not permit normal prothrombin mRNA processing to occur. Since we have shown that this polymorphism does indeed increase the stability of prothrombin mRNA, we conclude that the G20210A polymorphism is a bifunctional polymorphism: it increases mRNA processing and can also increase the half-life of the prothrombin transcript.

The observed influence of the G20210A polymorphism on mRNA stability prompted us to investigate whether the prothrombin 3'-UTR provided binding sites for cellular factors, and if so, whether there was any alteration in the binding activity due to the G/A substitution. We performed a series of REMSA experiments using RNA probes containing either the full length wild-type or mutant prothrombin 3'-UTRs and cellular proteins extracted from HepG2 cells. Results of REMSAs demonstrated that the full length prothrombin 3'-UTR did indeed provide binding sites for a number of proteins. Up to 3 protein-RNA complexes were identified by this approach and these were specific as judged by REMSA competition experiments. However, we saw no clear difference in the migration profile or binding intensity between the two 3'-UTR probes. Whether the lack of detectable change is a reflection of assay sensitivity remains to be determined.

To allow us to visualise the binding of proteins to the prothrombin transcript in the vicinity of the polymorphic region, we undertook another series of REMSAs using RNA probes containing the last 30 nt of the prothrombin 3'-UTR. This probe was generated to produce the terminal end of the prothrombin transcript as it would appear *in vivo*. The relatively short adenine [Poly (A)] tract was designed to limit the binding of the poly (A) binding protein (17), as this would otherwise interfere with the REMSA analysis. These experiments also revealed that this region of the prothrombin transcript contains sequences that provide protein-binding sites. However, we could find no obvious difference in the binding pattern of proteins to wild-type or mutant variants of this RNA sequence. We suspect however, that this is more likely to be a limitation of the REMSA assay and more substantial biochemical measures need to be undertaken to more carefully assess the influence of the G20210A polymorphism on protein binding activity to this region of the prothrombin transcript.

It has become increasingly clear that alteration in mRNA stability provides a critical control point in the regulation of gene expression. The 3'-UTR of many unstable transcripts contain AU-rich elements that play a role in influencing the decay process (18). A number of proteins have been identified that recognise these AU-rich sequences, but only two of these (HuR and hnRNP D) have actually been shown to influence mRNA stability *in vivo* (19). HuR is a ubiquitously expressed member of the Hu family of RNA binding proteins (20) and recognises a number of transcripts, including VEGF, PAI-2 and various cytokine transcripts (19) and has been shown to stabilise VEGF and GM-CSF mRNAs (21, 22). Our REMSA supershift experiments also identified HuR as one of the factors that recognise the prothrombin 3'-UTR. The known relationship between HuR and mRNA stability suggests that HuR plays a role in prolonging prothrombin mRNA half-life. Whether HuR differentially binds to variant prothrombin mRNAs is unlikely since we saw no convincing evidence for a change in HuR binding to prothrombin RNA probes containing the two sequence variants. It seems likely that the binding of HuR to the prothrombin 3'-UTR does not involve the G20210A polymorphism but rather involves other elements located elsewhere in the prothrombin 3'-UTR. The role of HuR in the post-transcriptional regulation of the prothrombin gene remains to be determined.

In summary, we have shown that the prothrombin mutant allele, that gives rise to an increase in prothrombin protein *in vivo*, gives rise to increased prothrombin mRNA and protein *in vitro*, confirming that this is indeed a functional polymorphism. We have provided evidence that this is related to an influence on mRNA stability, although the mechanism underlying the increase in mRNA half-life remains to be determined. We have also shown that both variants of the prothrombin 3'-UTR provide a binding site for a number of cellular factors, in-

cluding the mRNA stabilising protein, HuR. Since the G20210A polymorphism has been shown to enhance prothrombin 3' end formation (4), our observation that the G/A substitution also has the potential to increase mRNA stability suggests that this polymorphism is bifunctional, being able to influence two aspects of prothrombin mRNA metabolism.

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References

- Poort SR, Rosendaal FR, Reitsma PH, Bertina RM. A common genetic variation in the 3'-untranslated region of the prothrombin gene is associated with elevated plasma prothrombin levels and an increase in venous thrombosis. *Blood* 1996; 88: 3698-703.
- Souto JC, Coll I, Llobet D, del Rio E, Oliver A, Mateo J, Borrell M, Fontcuberta J. The prothrombin 20210A allele is the most prevalent genetic risk factor for venous thromboembolism in the Spanish population. *Thromb Haemost* 1988; 80: 366-9.
- Soria JM, Almasy L, Souto JC, Tirado I, Borell M, Mateo J, Slifer S, Stone W, Blangero J, Fontcuberta J. Linkage analysis demonstrates that the prothrombin G20210A mutation jointly influences plasma prothrombin levels and risk of thrombosis. *Blood* 2000; 95: 2780-5.
- Gehring NH, Frede U, Neu-Yilik G, Hundsdoerfer P, Vetter B, Hentze MW, Kulozik AE. Increased efficiency of mRNA 3' end formation: a new genetic mechanism contributing to hereditary thrombophilia. *Nat Genet* 2001; 28: 389-92.
- Tierney MJ, Medcalf RL. 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. *J Biol Chem* 2001; 276: 13675-84.
- Degen SJ, MacGillivray RT, Davie EW. Characterization of the complementary deoxyribonucleic acid and gene coding for human prothrombin. *Biochemistry* 1983; 22: 2087-97.
- Maurer F, Medcalf RL. Plasminogen activator inhibitor type 2 gene induction by tumor necrosis factor and phorbol ester involves transcriptional and post-transcriptional events. Identification of a functional nonameric AU-rich motif in the 3'-untranslated region. *J Biol Chem* 1996; 271: 26074-80.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; 162: 156-9.
- Medcalf RL, Ruegg M, Schleuning WD. A DNA motif related to the cAMP-responsive element and an exon-located activator protein-2 binding site in the human tissue-type plasminogen activator gene promoter cooperate in basal expression and convey activation by phorbol ester and cAMP. *J Biol Chem* 1990; 265: 14618-26.
- Haugland RA, Vesper SJ, Wymer LJ. Quantitative measurement of *Stachybotrys chartarum* conidia using real time detection of PCR products with the TaqMan(TM) fluorogenic probe system. *Mol Cell Probes* 1999; 13: 329-40.
- Maurer F, Tierney M, Medcalf RL. 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. *Nucleic Acids Res* 1999; 27: 1664-73.
- Bernstein P, Peltz SW, Ross J. The poly(A)-poly(A)-binding protein complex is a major determinant of mRNA stability in vitro. *Mol Cell Biol* 1989; 9: 659-70.
- Ford LP, Bagga PS, Wilusz J. The poly(A) tail inhibits the assembly of a 3'-to-5' exonuclease in an in vitro RNA stability system. *Mol Cell Biol* 1997; 17: 398-406.
- Gao M, Fritz DT, Ford LP, Wilusz J. Interaction between a poly(A)-specific ribonuclease and the 5' cap influences mRNA deadenylation rates in vitro. *Mol Cell* 2000; 5: 479-88.
- Gallie DR. A tale of two termini: a functional interaction between the termini of an mRNA is a prerequisite for efficient translation initiation. *Gene* 1998; 216: 1-11.
- Degen SJ. The prothrombin gene and its liver-specific expression. *Semin Thromb Haemost* 1992; 18: 230-42.
- Gorlach M, Burd CG, Dreyfuss G. The mRNA Poly(A)-binding protein: Localization, abundance and RNA binding specificity. *Exp Cell Res* 1994; 211: 400-7.
- Ross J. mRNA stability in mammalian cells. *Microbiol Rev* 1995; 59: 423-50.
- Brennan CM, Steitz JA. HuR and mRNA stability. *Cell Mol Life Sci* 2001; 58: 266-77.
- Ma WJ, Cheng S, Campbell C, Wright A, Furneaux H. Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J Biol Chem* 1996; 271: 8144-51.
- Levy NS, Chung S, Furneaux H, Levy AP. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J Biol Chem* 1998; 273: 6417-23.
- Fan XC, Steitz JA. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J* 1998; 17: 3448-60.

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