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Regular Article

A potential regulatory role for mRNA secondary structures within the prothrombin 3'UTR

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ABSTRACT

The distal 3'UTR of prothrombin mRNA exhibits significant sequence heterogeneity reflecting an inexact 3′-cleavage/polyadenylation reaction. This same region encompasses a single-nucleotide polymorphism that enhances the normal post-transcriptional processing of nascent prothrombin transcripts. Both observations indicate the importance of 3'UTR structures to physiologically relevant properties of prothrombin mRNA. Using a HepG2-based model system, we mapped both the primary structures of reporter mRNAs containing the prothrombin 3'UTR, as well as the secondary structures of common, informative 3'UTR processing variants. A chromatographic method was subsequently employed to assess the effects of structural heterogeneities on the binding of candidate *trans*-acting regulatory factors. We observed that prothrombin 3'UTRs are constitutively polyadenylated at seven or more positions, and can fold into at least two distinct stem-loop conformations. These alternate structures expose/sequester a consensus binding site for hnRNP-I/PTB-1, a *trans*-acting factor with post-transcriptional regulatory properties. hnRNP-I/PTB-1 exhibits different affinities for the alternate 3'UTR secondary structures *in vitro*, predicting a corresponding regulatory role *in vivo*. These analyses demonstrate a critical link between the structure of the prothrombin 3'UTR and its normal function, providing a basis for further investigations into the molecular pathophysiology of naturally occurring polymorphisms within this region.

Introduction

The activated form of prothrombin (F.II) plays a pivotal role in physiologically important thrombotic processes. Thrombin promotes blood clotting by converting fibrinogen to fibrin; by activating coagulation factors V, VIII, and XIII; by stimulating endothelial cell release of von Willebrand factor, PAI-1, endothelin, and tissue factor; and by inducing platelet aggregation [1]. The hemostatic activities of thrombin are balanced by its participation in anticoagulant processes, including thombomodulin-dependent activation of protein C, and by its stimulated production of vasoregulatory factors such as tPA, prostacyclin, and NO.

Recent studies have suggested that post-transcriptional mechanisms play a critical–but previously unrecognized–role in regulating steady-state plasma prothrombin levels. Several groups have focused on the functional consequences of a naturally occurring G→A transition at position 20210, the nominal site at which F.II pre-mRNAs undergo 3' cleavage/polyadenylation. Two studies have demonstrated that the G20210A polymorphism enhances the efficiency of 3'-end formation for prothrombin pre-mRNAs [2,3], while a third report has concluded that the single-nucleotide exchange additionally prolongs the cytoplasmic half-life of mature F.II mRNA [4]. The clinical consequences of post-transcriptionally dysregulated prothrombin mRNA are highly significant: heterozygotes for the G20210A mutation exhibit plasma prothrombin levels that are approximately 25% higher than normal [5–7], with corresponding increases in risks for venous thromboembolism [5,8], cerebral vein thrombosis [9,10], and other vaso-occlusive events [11,12]. The incidences of thromboses in individuals with other genetic thrombophilias, including factor V Leiden and protein C deficiency, are also elevated in prothrombin G20210A heterozygotes [13].

The pathological properties of G20210A-polymorphic mRNAs are likely to be mediated through altered interactions between the terminal 3'UTR and *trans*-acting factors that are critical to normal post-transcriptional processes. We [14] and others [15] have noted that this same region of 3'UTR also exhibits significant sequence heterogeneity resulting from the utilization of several alternate 3′-cleavage/polyadenylation sites. These observations have led us to speculate that the accuracy of 3′-end processing, too, might impact the regulation of F.II mRNA. Precedent literature endorses the principle

Abbreviations: F.II, prothrombin; PTB-1, polypyrimidine tract binding protein 1.

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that structural alterations in this region can create or ablate structural determinants with subsequent, highly consequential effects on mRNA function [16,17]. A description of mechanistically relevant structures within the F.II 3'UTR, as well as *trans*-acting effector factors that bind to them, would provide important insights into constitutive processes affecting prothrombin gene expression, and enhance our understanding of the molecular pathophysiology of G20210A-mutant prothrombin mRNA.

The current manuscript formally tests for structural heterogeneities within the F.II 3'UTR that affect its post-transcriptional regulation. Using reporter genes that are stably expressed in cell-homologous HepG2 cells, we quantitate a significant variability in the accuracy of 3' cleavage/polyadenylation for 3'UTRs derived from both wild-type and G20210A prothrombin gene sequences. Subsequent mRNA mapping analyses demonstrate that the different 3'-terminal isotypes also assume distinct secondary structures. We validate the assembly of two such high-order mRNA conformations, and note that they differentially incorporate a consensus binding site for hnRNP-I/PTB-1, a *trans*-acting factor that regulates the post-transcriptional processing of other, unrelated pre-mRNAs. Finally, we show that hnRNP-I/PTB-1 exhibits different binding affinities for the two conformationally distinct prothrombin 3'UTRs. These studies demonstrate the capacity of a specific 3'UTR structure to attract functional *trans*-acting factors, and indicate how this event can be subverted by modest changes in the sequence of the prothrombin 3'UTR.

Design and methods

Cell culture

Parental hepatocellular HepG2 cells were maintained in MEM supplemented with Earle's salts, 10% FBS, 1.0 mM pyruvate, 1.4 g/L bicarbonate, 0.1 mM nonessential amino acids, 4 mM L-glutamine, and penicillin/streptomycin. Subclones expressing the tetracycline trans-activator (tTA) fusion protein [18] were generated by G418 selection of cells transfected with pTetOff (Clontech). Clonal tTA activity was established by Dual-luciferase analysis (Promega) using cells that had been transiently cotransfected with a TRE-linked firefly luciferase gene (pTRE-luc; Clontech) and a tTA-indifferent control renilla luciferase gene (pRL-SV40; Promega). A single clone exhibiting high tTA activity was used for subsequent subcloning.

Plasmid construction

All plasmids were derived from parental TRE-β^{WT}, comprising a 3.3-kb fragment of DNA, containing the intact human (h) β-globin gene and contiguous 3'-flanking region, inserted into the polylinker *Sac*II-*Cla*I site of pTRE2 (Clontech) [19]. TRE-βF.IIG was generated from TRE-β^{WT} by a two-step splice-overlap-extension/polymerase-chain-reaction [20,21] using DNA fragments corresponding to (i) the native 31-nt F.II 5'UTR, (ii) the native hβ-globin coding region and included introns, and (iii) the native 100-nt F.II 3'UTR and 63 nts of contiguous 3'-flanking region. TRE-βF.IIA was similarly constructed to contain a G→A substitution at 3'UTR position 20210. Plasmids used for stable transfections were modified to contain a hygromycin-resistance gene derived from the 1.5-kb *Xho*I fragment of pTRE2hyg (Clontech). Double-stable cells were generated by selecting TRE-βFIIG and -βF.IIA-transfected tTA-expressing HepG2 cells with 0.6 μg/ml hygromycin. Subclones selected for study encompassed a two-log range of steady-state βF.II mRNA expression levels, allowing artifacts of saturation kinetics, if present, to be recognized.

3′-cleavage site

Approximately 750 ng total RNA purified from βF.II-expressing HepG2 cells (Pure-link, Invitrogen) was RT-PCR amplified (One-Step, Invitrogen) in the presence of reverse (5'GGATCCGAGCTCT$_{20}$3') and forward oligomers (5'GTGCTGGTCTGTGTGCTGG3'). The forward oligomer is positioned within the β-globin coding region and does not amplify endogenous, full-length prothrombin mRNAs. Reaction products were ligated into pCR4-Topo, and chemically competent cells transformed (Top-10, Invitrogen). Plasmid DNAs prepared from individual colonies were sequenced by the Nucleic Acid/Protein Core Facility at The Children's Hospital of Philadelphia.

RNA secondary-structure mapping

DNA templates for *in vitro* transcription of F.IIA, F.IIG, or F.IIG^{+2} 3'UTRs, each containing an 18-nt poly(A) tail, were directionally inserted into the *Xho*I-*Bgl*II polylinker site of pSP72. *Bgl*II-linearized plasmids were transcribed, and RNAs subsequently 5'-end labeled with [γ-^{32}P]ATP using Maxiscript and Kinase Max kits, respectively (Ambion). [^{32}P]-labeled RNAs were digested with RNase A (100, 10, 1.0, and 0.1 ng/ml), RNase T1 (100, 10, 1.0, and 0.1 mU/μl), or RNase V1 (10, 1.0, 0.1, and 0.01 mU/μl) as directed by the supplier (Ambion). RNases A and T1 exhibit cleavage specificities for pyrimidine bases and guanosines within single-stranded regions of RNA, respectively, while RNase V1 cleaves 3' to bases within double-stranded regions of RNA. Reaction products were resolved on a 33×40 cm 6%:8 M acrylamide: urea gel, in parallel with a migration-control 'ladder' generated by alkaline hydrolysis of [^{32}P]-labeled transcripts.

Protein identification

Affinity-enrichment

Molar equivalents of 5'-biotinylated ssDNAs were liganded to streptavidin-coated agarose beads and incubated with HepG2 cell extract as previously described [19]. Retained factors were resolved on a precast 4-12% gradient Bis-tris SDS-PAGE gel (Invitrogen).

Mass spectroscopy

Tryptic digests of excised gel slices were resolved by TOF-TOF analysis (University of Pennsylvania Proteomics Core Facility); protein identities were deduced from MS-Fit analysis of peptide fragments using the NCBInr database.

Data analyses

Individual panels are derived from single autoradiographs; irrelevant lanes have been omitted to preserve clarity. Gamma levels were adjusted, when required, to facilitate analysis.

Results

Generation of a cell-homologous model system

Post-transcriptional events that affect eukaryotic mRNAs can be highly cell-type specific [22,23]. Consequently, we elected to characterize naturally occurring features of the F.II 3'UTR in hepatocellular carcinoma HepG2 cells, which express liver-restricted factors [24] and recapitulate characteristic hepatocellular processes [25]. Derivative HepG2 cells were engineered to constitutively express β-globin reporter genes containing the complete prothrombin 3'UTR and contiguous 3'-flanking region (Fig. 1A). The reporter genes contain either a G or an A at F.II position 20210, reproducing the 3'UTR sequences of wild-type and G20210A-polymorphic sequences, respectively. Steady-state levels of the encoded βF.IIG and βF.IIA mRNAs were determined in 17 and 18 subclones, respectively, using an RNase protection method (Fig. 1B), and representative βF.IIG and βF.IIA clones selected for further analyses.