Cyclic Nucleotide Regulation of PAI-1 mRNA Stability

IDENTIFICATION OF CYTOSOLIC PROTEINS THAT INTERACT WITH AN A-RICH SEQUENCE*

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Incubation of HTC rat hepatoma cells with the cyclic nucleotide analogue 8-bromo-cAMP results in a 3-fold increase in the rate of degradation of type-1 plasminogen activator-inhibitor (PAI-1) mRNA. Previous studies utilizing HTC cells stably transfected with β-globin: PAI-1 chimeric constructs demonstrated that at least two regions within the PAI-1 3'-untranslated region mediate the cyclic nucleotide-induced destabilization of PAI-1 mRNA; one of these regions is the 3'-most 134 nucleotides (nt) of the PAI-1 mRNA (Heaton, J. H., Tillmann-Bogush, M., Leff, N. S., and Gelehrter, T. D. (1998) J. Biol. Chem. 273, 14261–14268). In the present study, ultraviolet cross-linking analyses of this region demonstrate HTC cell cytosolic mRNA-binding proteins ranging from 38 to 76 kDa, with a major complex migrating at ~50 kDa. RNA electrophoretic mobility shift analyses demonstrate high molecular weight multiprotein complexes that specifically interact with the 134-nt cyclic nucleotide-responsive sequence. The 50, 61, and 76 kDa multiprotein complexes form with an A-rich sequence at the 3' end of the cyclic nucleotide-responsive region; a 38-kDa complex forms with a U-rich region at the 5' end of the 134 nt sequence. Mutation of the A-rich region prevents both the binding of the 50-, 61-, and 76-kDa proteins and formation of the multiprotein complexes, as well as cyclic nucleotide-regulated degradation of chimeric globin:PAI-1 transcripts in HTC cells. These data suggest that the proteins identified in this report play an important role in the cyclic nucleotide regulation of PAI-1 mRNA stability.

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§ The abbreviations used are: PA, plasminogen activator; PAI-1, type-1 plasminogen activator inhibitor; cA, 8-bromo-cAMP plus IBMX; nt, nucleotide; UTR, untranslated region; R-EMSA, RNA electrophoretic mobility shift analyses; E64, trans-epoxysuccinyl-1-leucylamido-4-guanidino butane; Pefabloc SC (AEBSF), 4-(2-aminoethyl)benzoylsulfonyl fluoride-hydrochloride; poly(A), polyadenylic acid; nt, nucleotide; UTR, untranslated region; R-EMSA, RNA electrophoretic mobility shift analyses; E64, trans-epoxysuccinyl-1-leucylamido-4-guanidino butane; Pefabloc SC (AEBSF), 4-(2-aminoethyl)benzoylsulfonyl fluoride-hydrochloride; poly(A), polyadenylic acid.

Plasminogen activators (PAs) are serine proteases that catalyze the conversion of the zymogen plasminogen to plasmin, a broad spectrum endopeptidase that is responsible for intravascular fibrinolysis (1). This protein is also known to play a major role in biological processes involving localized proteolysis of extracellular matrix, such as tissue remodeling and tumor cell invasion and metastasis (2). Type-1 plasminogen activator-inhibitor (PAI-1), a 50-kDa glycoprotein, is a major regulator of plasminogen activation (3, 4). PAI-1 is synthesized in a variety of cell types and its expression is regulated by growth factors and hormones, including agents that elevate intracellular cAMP levels (5–8).

In HTC rat hepatoma cells, the cyclic nucleotide analogue 8-bromo-cAMP, together with the phosphodiesterase inhibitor isobutylmethylxanthine (designated cA), increases tissue type PA activity more than 50-fold primarily by decreasing PAI-1 mRNA by 90% and protein by 60–70%. The decrease in PAI-1 mRNA is due to a 60% decrease in the rate of PAI-1 gene transcription and, more importantly, a 3-fold increase in the rate of PAI-1 mRNA decay (8, 9). Utilizing HTC cells stably transfected with chimeric constructs containing portions of the mouse β-globin gene and rat PAI-1 cDNA, the 1730-nucleotide (nt) PAI-1 3'-untranslated region (UTR) (nt 1531–3060) was shown to contain sequences that mediate the cA-induced destabilization (10). Furthermore, results obtained from deletion and insertion analyses using a series of β-globin coding region: PAI-1 3'-UTR chimeric constructs demonstrated that at least two regions within the PAI-1 3'-UTR mediate the cA effect. One of these regions is the 3'-most 134 nt, from position 2926 to 3060, of the PAI-1 mRNA. This 134-nt sequence includes a 75-nt U-rich region present at its 5' end and a 24-nt A-rich region at its 3' end (Fig. 1A) (10, 11).

The decay rates of many mRNAs have been shown to be regulated by a variety of external stimuli including hormones, growth factors, and agents that elevate intracellular cAMP levels (see Refs. 12 and 13 for review). Studies aimed at elucidating the mechanism(s) involved in regulating mRNA stability have identified a number of potential cis-acting sequences and/or trans-acting factors (12, 13); however, the molecular basis for the cyclic nucleotides regulation of mRNA stability remains largely undefined.

The aim of the present study was to further elucidate the mechanism(s) involved in the cyclic nucleotide-induced destabilization of PAI-1 mRNA in rat HTC cells. To this end, studies were conducted to identify the cytosolic factors that bind to the 3'-most 134 nt of the PAI-1 mRNA, to characterize the specificity and binding sites for these factors, and to determine their role in cA-induced PAI-1 mRNA destabilization. Results from ultraviolet (UV) cross-linking analyses demonstrate that specific RNA-protein complexes of ~38, 50, 53, 61, 65, and 76 kDa form with the 134-nt sequence, while RNA electrophoretic mobility shift analyses (R-EMSA) demonstrate the formation of high molecular mass multiprotein complexes. The 50-, 61-, and
76-kDa and multiprotein complexes form between the A-rich region and HTC cell cytosolic proteins that are found in both polysomal and post-ribosomal fractions, while the 38-kDa complex forms between the U-rich region and proteins found in the polysomal fraction. Mutations in the A-rich region abolished formation of the 50, 61, and 76 kDa and multiprotein complexes as well as the ability of cA to regulate the decay of transcripts from stably transfected globin:PAI-1 chimeric constructs, suggesting that these RNA-protein complexes play an important role in the CA-induced destabilization of PAI-1 mRNA.

**EXPERIMENTAL PROCEDURES**

**Materials**—8-Bromo-cAMP, isobutylmethylxanthine, benzamidine, trans-e-poxysuccinyl-l-leucylamido-(4-guanidinobutane) (E64), heparin (sodium salt), RNase T1, and tRNA (from Bakers’ yeast, type X) were purchased from Sigma. Leupeptin, 4-(2-aminoethyl)benzoyl sulfonyle fluoride-hydrochloride (Pefabloc®SC (AEBSF)), proteinase K, RNase inhibitor, and T3 RNA polymerase were obtained from Boehringer Mannheim. Coomassie® Plus Protein Assay reagent was acquired from Pierce (Rockford, IL). Eagle’s minimal essential medium, RNase A, RNase T2, T4 DNA ligase, T4 DNA polymerase, restriction enzymes, Benchmark® prestained molecular weight markers, okadaic acid, and calfintestinal phosphatase were purchased from Life Technologies (Gaithersburg, MD). Amersham® T3 transcription kit was obtained from Amersham (Madison, WI). Polyadenylic (poly(A)), polycytidylic (poly(C)), polyguanadylic (poly(G)), and polyuridylic (poly(U)) acids were acquired from Pharmacia Biotech. Centricon® concentrators were purchased from Amicon (Beverly, MA). [α-32P]UTP (800 Ci/mmol) and [γ-32P]ATP (400 Ci/mmol) were obtained from Amersham. Presynthesized SDS-polyacrylamide gel electrophoresis (PAGE) standards were from Bio-Rad.

**Cytosplasmic Extract Preparation**—Monolayer cultures of rat HTC hepatoma cells were grown and maintained as described previously (9). Cells were incubated in serum-free medium for 10 to 16 h, harvested by trypsinization, pelleted, and resuspended in 25 mM Tris (pH 7.9), 0.1 mM EDTA, 1 mM Pefabloc®SC (AEBSF), 1 mM leupeptin, 1 mM benzamidine, 1 mM E64. The cell pellets were subjected to three freeze/thaw cycles (10 min/cycle) followed by centrifugation at 4 °C at 10,000 × g for 15 min. The supernatant fraction (S10) was removed, assayed for protein concentration using Coomassie® Plus Protein Assay reagent as directed by the manufacturer, and stored at −70 °C. Isolation of polysomal and post-ribosomal (S130) extracts was performed essentially as described (14) with additional protease inhibitors added to buffer A (1 mM Pefabloc®SC (AEBSF), 1 mM leupeptin, 1 mM benzamidine, 1 mM E64). The S130 fraction was concentrated using Centricon®-3 concentrators as instructed by the manufacturer. The polysomal and S130 fractions were assayed for protein concentration and stored at −70 °C.

**Preparation and Radiolabeling of RNA Probes**—The DNA template for transcribing the PAI-1 RNA probes 2926–3060 (the full-length 134 nt region of the 3′-UTR) and 2926–2986 was prepared as follows: pSVL G/G, which contains the PAI-1 3′-UTR subclone, was digested with the XhoI site of the pBluescript KS− (pKS−) multicloning site (MCS), was digested with KpnI (pKS−: MCS) and Apal (PAI-1: bp 2926), incubated with T4 DNA polymerase to generate blunt ends, and religated using T4 ligase. PAI-1 2926–3060 or 2926–2986 template DNA was linearized with XbaI or NotI, respectively, prior to in vitro transcription. The DNA template for generating the PAI-1 probe 2926–3060 was prepared as described (15). DNA digestion with KpnI 3′-PAI with KpnI and NotI (PAI-1: bp 2926), creating blunt ends using T4 DNA polymerase, and religating using T4 ligase. The template was linearized with XbaI prior to in vitro transcription. The DNA template for PAI-1 sequence 2125–2296 was generated by deletion of the SacI (pKS−: MCS)-NcoI (PAI-1: bp 2125) fragment from pKS− 3′-PAI, blunt end formation and ligation, and linearization with NotI (PAI-1: bp 2296) prior to in vitro transcription. The DNA template for PAI-1 sequence 2528–2829 subcloned into the EcoRI and SmalI sites of the pKS− MCS was digested with BamHI (pKS−: MCS) prior to in vitro transcription.

The DNA templates used to generate PAI-1 RNA probes 2926–3024, 3010–3060, and 3002–3060 were generated by polymerase chain reaction (PCR). Sequence for the T3 RNA polymerase promoter (AATTACCCCTCACTAAAGGG) was included at the 5′ end of the forward primer and used with the appropriate reverse primer in PCR reactions with pSVL G/G (globin coding region/PAI-1 3′-UTR; Ref. 10) as template DNA. The DNA templates for 2926–3060 containing the A-rich region mutations were also generated by PCR (see Fig. 5A for description of mutations) by incorporating the mutant sequences into the reverse PCR primer. The DNA templates used to generate the wild-type and mutant 3010–3040 (Fig. 5A) were prepared by annealing complementary oligonucleotides containing the sequence for the T3 RNA polymerase promoter at the 5′ end followed by the appropriate PAI-1 sequence.

Preparation of radiolabeled RNA probes was performed as described (15) using the DNA template DNA (oligo- or PCR-generated templates: 100–200 ng, linearized plasmid templates: 0.5–1.0 μg) with 10 units of T3 RNA polymerase under the following conditions: 40 μM Tris-HCl (pH 8.0, 20 °C), 6 mM MgCl2, 2 mM spermidine, 20 mM dithiothreitol, 0.4 mM CTP, 0.4 mM GTP, 37.5 μM ATP, 37.5 μM UTP, 10 units of RNase inhibitor, 100 μCi of [α-32P]UTP (800 Ci/mmol) per μl of [γ-32P]ATP (400 Ci/mmol). To generate unlabeled competitor RNA, in vitro transcription from the template DNAs was carried out using the Ampliscribe® T3 transcription kit as directed by the manufacturer.

**R-EMSA and UV Cross-linking Analyses**—For R-EMSA, extracts and 32P-radiolabeled RNA were incubated for 20 min at 25 °C in buffer containing 5 units of RNase inhibitor, 10 μg of yeast tRNA, 10 mM Hepes (pH 7.6), 5 mM MgCl2, 40 mM KCl, 5% glycerol, and 1 mM dithiothreitol. RNase T1, (1 unit/μl) was added to each reaction and incubation continued at 25 °C for 10 min. Heparin (5 mg/ml) was added and the reactions were incubated at 25 °C for an additional 10 min. The reactions were subjected to electrophoresis through a 5% nondenaturing polyacrylamide gel (80:1 acrylamide: bisacrylamide, 40 mA, 4 °C, 3 h) followed by autoradiography. For UV cross-linking analyses, extracts and 32P-radiolabeled RNA were incubated and treated with RNase T1 and heparin as described for R-EMSA. The reactions were then exposed to a UV light source (UV Stratallinker 1800, Stratagene) at a distance of 2.5 cm from the light source for 10 min (1.8 μJ/cm2) except otherwise specified. RNase A (10 mg/ml) and RNase T2 (100 units/ml) were added and incubation continued at 25 °C for 10 min; a combination of RNases was utilized in order to maximize digestion of the unbound radiolabeled RNA probe. SDS sample buffer was added and reactions were heated at 85 °C for 2 min; RNA-protein complexes were analyzed by 0.1% SDS-10% PAGE (38:1 acrylamide: bisacrylamide, 40 mA, 25 °C, 4 h) followed by autoradiography. For competition analyses, S10 proteins were preincubated with unlabeled competitor RNA for 10 min at 25 °C prior to the addition of radiolabeled RNA. Each R-EMSA or UV cross-linking analysis figure presented is representative of at least three independent experiments.

**Analysis of Chimeric Globin:PAI-1 mRNA Stability in HTC Cells**—Cell culture and maintenance, stable transfections, construction of pSVL G/G + 2925/3054, riboprobe preparation, and ribonucleic acid protection analyses were conducted as previously reported (15). To prepare the chimeric construct pSVL G/G + 2925/3054 double mutant, the PAI-1 sequence from nt 2925–3054 was amplified by PCR, digested with BglII, and ligated into the BglII site of pSVL G/G (10). The forward primer contained BglII sequences at its 5′ end, while the reverse primer contained the two mutations in the A-rich region (nt 3023–3028; AAAAAA changed to cccccc and nt 3030–3035 AUAAA changed to cccgcc) and BglII sequences at its 5′ end.

**RESULTS**

**Interaction of HTC Cell Cytosolic Proteins with the CA-responsive 3′-most 134 nt of the PAI-1 3′-UTR**—To identify potential mRNA-binding proteins in HTC cell cytosolic extracts that interact with the 3′-most 134 nt of the PAI-1 3′-UTR (nt 2926–3060), UV cross-linking analyses were conducted. Using radiolabeled nt 2926–3060 and HTC cytoplasmic extracts, a major ribonucleoprotein complex with an Mr of 50,000 (doublet) and minor complexes of 38,000, 53,000, 61,000, 65,000, 76,000, and 86,000 were detected (Fig. 1B, lane 1). Formation of the RNA:protein complexes was dependent on protein concentration and on the time of exposure to UV light; no additional interactions were detected with greater than 50 μg of extract or after 5 min of UV exposure (data not shown). A time course for the formation of RNA-protein interactions, all subsequent reactions were exposed to UV light for 10 min, 1.8 μJ/cm2. Ribonucleoprotein complex formation was abolished when the samples were treated with protease K or when S10 proteins were denatured by heating the extract prior to incubation with radiolabeled RNA (Fig. 1B, lanes 2 and 4, respectively). No complexes were detected when the RNA alone was subjected to UV cross-linking analysis. These results confirm that the com-
plexes are comprised of RNA and protein. Heating the RNA prior to incubation with protein had no effect on complex formation (Fig. 1C, lane 3).

**Subcellular Distribution of Cytoplasmic RNA-binding Proteins**—To determine the subcellular distribution of the cytoplasmic proteins that interact with PAI-1 sequence 2926–3060, UV cross-linking analyses were performed using S10, polysomal, or S130 fractions as the source of proteins. The 38-kDa complex was formed with polysome-associated proteins, but not with S130 proteins; the remainder of the RNA-protein complexes formed using proteins found in both the polysomal and S130 fractions. Using equal amounts of protein from the polysomal and S130 fractions, the majority of the mRNA binding activity was in the polysomal fraction. Likewise, the multiprotein complexes detected by R-EMSA formed with proteins found in both the polysomal and S130 fractions (data not shown).

**Specificity of RNA-Protein Complex Formation**—The specificity of the interactions detected between HTC cell cytoplasmic proteins and the radiolabeled PAI-1 sequence 2926–3060 was demonstrated by competition UV cross-linking analyses and R-EMSA (Fig. 2). Extract was preincubated with unlabeled competitor RNA prior to the addition of radiolabeled PAI-1 probe. Unlabeled PAI-1 sequence 2926–3060 as competitor reduced formation of the RNA-protein complexes detected by both UV cross-linking (Fig. 2A, lanes 1–4) and R-EMSA (Fig. 2B, lanes 1–5) in a concentration-dependent manner, indicating that these interactions are specific. A portion of the PAI-1 3′-UTR (nt 2125–2296), which when inserted into the 3′-UTR of the β-globin gene fails to confer CA-responsiveness (10), was not able to compete with labeled 2926–3060 for complex formation (Fig. 2, A, lane 6; B, lane 7). Likewise, there was no competition for most of the complexes when CAT RNA was used as a nonspecific competitor (Fig. 2, A, lane 5; B, lane 6); however, the 86-kDa complex may be nonspecific as CAT RNA did compete for its binding. Furthermore, no RNA-protein interactions were detected when HTC cell S10 extract was incubated with radiolabeled PAI-1 sequence 2125–2296 or CAT RNA (data not shown), supporting the conclusion that the observed interactions are specific for the 134-nt CA-responsive sequence.

Since PAI-1 sequence 2926–3060 contains a 75-nt U-rich region and a 24-nt A-rich region (Fig. 1A), competition UV cross-linking studies (Fig. 3A), formation of the 38-kDa complex was inhibited by the presence of poly(U), but not poly(A). In contrast, formation of the 50-, 61-, and 76-kDa complexes was inhibited by the presence of poly(A), but not poly(U); the 38-, 53-, and 65-kDa complexes remained after competition with poly(A). Poly(C) and poly(G) did not inhibit formation of any of the observed complexes. These data suggest that the 38-kDa complexes form with PAI-1 sequences that are CA-responsive and that the 50-, 61-, and 76-kDa complexes are formed when the 134-nt CA-responsive sequence is located between the 38- and 53-kDa complexes. These results suggest that the multiprotein complexes and the major complexes observed by UV cross-linking require the same A-rich sequence.

**Identification of the Sequences Involved in RNA-Protein Complex Formation**—To further define the nucleotide sequences to which the cytoplasmic proteins bind, radiolabeled RNA probes corresponding to those diagrammed in Fig. 4A were generated and used in UV cross-linking analyses. In each
case, molar equivalents of each radiolabeled RNA were used (Fig. 4B). S10 proteins and nt 2966–3060 formed complexes with the same migration as those observed using nt 2926–3060. Cytoplasmic proteins and nt 3010–3060 or 3024–3060, containing the A-rich region, also formed complexes essentially the same as those observed using 2926–3060, except that the 38-kDa complex was absent. When S10 proteins were incubated with 3010–3040, the abundant complexes migrating at 50 kDa were present as well as faint complexes at 53, 61, 65, and 76 kDa; the 38-kDa complex was absent.

Incubation of cytoplasmic proteins with nt 2926–3024 (Fig. 4B, lane 6), which contains only the U-rich region, resulted in formation primarily of the 38-kDa complex; only faint bands appeared at 50, 53, and 65 kDa. This pattern of RNA-protein interactions was also observed using another 3′-UTR U-rich sequence located upstream at nt 2790–2911 as probe (data not shown). S10 incubated with nt 2926–2966 resulted in no RNA-protein complex formation (Fig. 4B, lane 7).

As a correlate to these studies, radiolabeled 2926–3024 or 3010–3040 were used as probes in R-EMSA to determine if the A-rich region was also important for formation of the multiprotein complexes detected by native PAGE. As shown in Fig. 4C, the complexes detected using nt 2926–3060 also form with nt 3010–3040, but not with nt 2926–3024 (compare lanes 2 and 3 with lane 1). These data indicate that the multiprotein complexes form using the same A-rich region as those ribonucleoprotein complexes detected by UV cross-linking analyses. Together with results from homoribopolymer competition assays, these studies suggest that (i) the majority of the RNA-protein complexes detected by both R-EMSA and UV cross-linking analysis are the result of an interaction of cytoplasmic proteins with a sequence containing the A-rich region (nt 3023–3046) and (ii) only formation of the 38-kDa RNA-protein complex detected by UV cross-linking analysis results from specific interactions between S10 proteins and a sequence containing the U-rich region (nt 2948–3022).

Mutational Analysis of the A-rich Region—Since the U-rich region of the 134-nt sequence alone does not confer CA-responsiveness onto the otherwise non-responsive globin mRNA (10) and the majority of the RNA-protein interactions occur within the A-rich region, mutational analyses were performed on the A-rich region. Mutations were generated in the context of the 134-nt PAI-1 sequence (nt 2926–3060), as illustrated in Fig. 5A. Mutation of the A-rich region (mutant I: nt 3023–3028, AAAAA to cccecc and mutant II: nt 3030–3035, AAAUAA to ccgccc) results in loss of RNA binding activity when the mutations were present in either RNA context (Fig. 5B), suggesting...
that the A-rich region located between nt 3023–3035 is necessary for formation of the 50-, 61-, 65-, and 76-kDa complexes. In addition, formation of the multiprotein complexes detected by R-EMSA also requires this region as indicated by the lack of complex formation using radiolabeled 2926–3060 containing either mutation (Fig. 5C). Mutations in the A-rich region made in the context of the 134-nt sequence also decreased formation of the 38-kDa complex (Fig. 5B, lanes 2 and 3).

**Role of the A-rich Region in cA-regulated PAI-1 mRNA Stability**—HTC cells were stably transfected with a chimeric construct containing the wild-type cA-responsive PAI-1 fragment inserted into the 3′ UTR of the murine β-globin gene (pSVL G/G+2925/3054 (10)) or pSVL G/G+2925/3054 containing both A-rich region mutations (pSVL G/G+2925–3054 double mutant). HTC cells were incubated in the absence or presence of cA and the decay rates of the chimeric mRNAs were determined as described previously (10). The top panel of Fig. 6 shows the gel from an RNase protection assay of one such
experiment and the bottom panel shows graphically the pooled data from two experiments. The wild-type 130-nt fragment mediated cA-induced destabilization of the globin:PAI-1 chimeric mRNA; in contrast, the mutant 130-nt fragment failed to confer cA-responsiveness onto the β-globin gene. Consistent with previous reports (10), control constructs pSVL G/G (globin coding region/globin 3'-UTR) and pSVL G/P (globin coding region/PAI-1 3'-UTR) showed no cA responsiveness and a 2-fold increase in mRNA turnover in response to cA, respectively (data not shown). These results strongly suggest that the same A-rich region that interacts with HTC cell cytoplasmic proteins mediates cyclic nucleotide-induced destabilization of mRNA in HTC cells.

**DISCUSSION**

In HTC rat hepatoma cells, cA causes a 3-fold increase in the rate of degradation of PAI-1 mRNA; the 3'-most 134 nt of the PAI-1 mRNA is sufficient to mediate the major part of this effect (10). To better understand the mechanism by which cA induces destabilization of PAI-1 mRNA, studies were conducted to identify trans-acting factors that interact with the 134-nt cA-responsive sequence, to characterize the specificity and binding sites of these interactions, and to determine their role in the regulation of mRNA stability. UV cross-linking analysis demonstrated HTC cytoplasmic mRNA-binding proteins of approximately 38, 50, 53, 61, 65, and 76 kDa, and R-EMSA demonstrated multiprotein complexes of ~175 and 140 kDa that interact with the cA-responsive 134-nt sequence. The 38-kDa mRNA-binding protein appears to interact with the U-rich region. Its binding is competed by poly(U), and the 38-kDa complex forms with PAI-1 mRNA containing U-rich sequences between nt 2966 and 3024, but not with the A-rich region (nt 3024–3060). However, complex formation is markedly decreased by mutations in the A-rich region, suggesting that conformational changes or protein-protein interactions with other mRNA-binding proteins can influence formation of the 38-kDa complex. The binding site for the majority of the mRNA-binding proteins (50, 53, 61, 65, and 76 kDa) and the multiprotein complexes was limited to a 30-nt sequence containing the A-rich region. Finally, and most importantly, through mutational analyses the A-rich region was determined...
to be necessary for both RNA-protein interaction and for regulation of mRNA stability in HTC cells. Thus, these studies link the binding of cytoplasmic proteins to an A-rich region in the PAI-1 3′-UTR with the cyclic nucleotide regulation of PAI-1 mRNA stability in HTC cells.

Neither the mobility nor the abundance of the complexes formed between the 134-nt sequence and HTC cytosolic proteins, however, appears to be regulated by cA. The mRNA binding activity of cytosolic proteins isolated from HTC cells incubated with cA for 30, 60, 90, 120, 180, or 240 min was essentially the same as that of controls. In addition, the distribution of complexes between the polysome and S130 fractions did not appear to be altered after incubation with cA (data not shown). Several hypotheses may explain the observed lack of cA-regulated ribonucleoprotein complex formation. First, there may be subtle changes in complex formation that are not detectable within the limits of the assays performed in this study. Second, the protein components of the complexes may be modified in response to cA; for example, protein-protein interactions within the multiprotein complexes might be altered by cA without affecting their migration through a nondenaturing gel. Third, additional proteins that are induced or repressed by cA, but do not alter RNA-protein complex formation, may be necessary for the regulation of mRNA stability. Detection of these co-factors by R-EMSA may be difficult if their interaction with the multiprotein complexes is weak. Finally, the presence of cA may alter the function of a protein(s) by allosteric or active site modifications without affecting its ability to form ribonucleoprotein complexes. This modification would be reminiscent of the regulation of cAMP-responsive element (CRE)-binding protein (CREB) by protein kinase A (16). Phosphorylation of CREB at Ser-133 by protein kinase A enhances the transactivating potential of CREB; however, it has no effect on the binding of CREB to a consensus CRE suggesting separate regulation of CREB binding and transcriptional activity.

The stability of a variety of mRNAs is subject to regulation by intracellular cAMP levels. For example, lactate dehydrogenase A subunit (18), tyrosine aminotransferase (19), renin (20), α2-adrenergic receptor (21), osteocalcin (22), the glucose transporter, GLUT1 (23), chorionic gonadotropin (24), phosphoenolpyruvate carboxykinase (25), and the RIIβ subunit of protein kinase A (26) mRNAs are stabilized in the presence of cAMP, cAMP analogues, and/or cAMP elevating agents. Conversely, the messages for PAI-1 (9), adrenodoxin reductase (27), and tyrosine hydroxylase (28) are destabilized in the presence of cAMP, cAMP analogues, and/or cAMP elevating agents. A number of receptor mRNAs are also destabilized by elevated intracellular cAMP levels (29–37). Despite the importance of cAMP as a regulator of mRNA stability, the mechanism by which increases in intracellular cAMP levels induce changes in mRNA turnover rates remains undefined in most systems described to date.

Limited studies, however, have identified potential cis- and trans-acting mediators of cAMP-regulated mRNA stability. In hamster smooth muscle cells, the cAMP elevating agent isoprotenerol or the cAMP analogue CPT-cAMP destabilizes β2-adrenergic receptor (β2-AR) mRNA and induces the binding of the M9 50,000 β2-AR binding protein (β-ARB), to a 20-nt region in the 3′-UTR (34). The 20-nt AU-rich sequence, which contains an AUUUUA motif flanked by U-rich regions, was shown to mediate the agonist and cAMP-induced destabilization of β2-AR mRNA. A nonconsensus AU-rich nonamer (UAUUAUAUU) in the human β-AR 3′-UTR that binds the hamster β-ARB in vitro was shown to be a critical determinant for the isoproterenol-induced destabilization of β2-AR transcripts in transfected human embryonic kidney cells (35).

In contrast, a cytosine-rich region in the coding region of the luteinizing hormone/human chorionic gonadotropin receptor mRNA, which is destabilized during hCG-induced down-regulation, was found to contain an M9 50,000 ribonucleoprotein complex with rat ovary cytosolic proteins; complex formation was enhanced in the down-regulated state (37). Finally, phosphoenolpyruvate carboxykinase mRNA is stabilized by dibutyryl-cAMP, CPT-cAMP, or 8-bromo-cAMP in FTO-2B rat hepatoma cells (25, 38). CPT-cAMP also decreases the binding of a 100-kDa cytosolic protein to a region in the 3′-UTR that contains alternating purine/pyrimidine bases, numerous repeats and palindromes; binding was shown to be sequence-independent, requiring RNA secondary structure for complex formation.

The cis-acting sequences described in this report are unlike those described for other systems in which mRNA stability is regulated by intracellular cAMP levels. First, the sequence that mediates the cA-induced destabilization of PAI-1 mRNA involves a predominantly A-rich region located at the extreme 3′ end of the PAI-1 3′-UTR. This is in contrast to the C-rich region implicated in the cA regulation of leutinizing hormone/human chorionic gonadotropin receptor mRNA (37) and the primarily U-rich region containing AUUUUA or nonconsensus AU-rich motifs that has been implicated in the regulation of β-AR mRNA stability (34–36) and a number of other regulated mRNAs (39–45). Second, RNA-protein complex formation between the cA-responsive PAI-1 sequence (nt 2926–3060) or a truncated sequence containing the A-rich region (nt 3010–3040, 3010–3060, or 3024–3060) is not abolished by heating the RNA at 85 °C for 15 min, followed by rapid cooling (Fig. 1, B and C; and data not shown). This is in contrast to the cis-acting sequences involved in cAMP-regulated phosphoenolpyruvate carboxykinase mRNA stability (38); in this system, heating the RNA probe prior to UV cross-linking significantly reduced binding. However, because RNA can rapidly regain secondary structure after being heated, these experiments cannot rule out a role for RNA secondary structure in the binding we observe.

The identity of the mRNA-binding proteins described in this report is unknown. Because the target sequence of most of these proteins is A-rich, the possibility that poly(A)-binding protein is involved must be considered. Poly(A)-binding protein, which is highly conserved across species, has a molecular mass of about 70,000 (46); in contrast, the major protein binding to the PAI-1 3′-UTR has a mass of about 50,000.

Degradation of mature mRNAs is a regulated process that can have a significant, and rapid, impact on gene expression. The half-lives of mRNAs in eukaryotic cells can range from minutes for highly regulated gene products such as protooncogenes, growth factors, and cytokines to many hours for very stable species such as globin (see Refs. 12 and 13 for review). Regulation of mRNA stability, often but not necessarily in conjunction with changes in transcription rates, allows the level of a particular mRNA to be increased rapidly and/or transiently in response to various stimuli (13). In most cases, however, the mechanism(s) by which these stimuli exert their effects are not clear; it is not known whether these stimuli act directly or indirectly to cause an increase or decrease in mRNA decay rates. The studies presented here provide further insight into the mechanism by which cyclic nucleotides regulate PAI-1 mRNA stability in rat HTC hepatoma cells. The minimal sequence that interacts with HTC cytosplasmic proteins was limited to a 30-nt sequence that contains an A-rich region. This same A-rich region, by mutational analysis, was shown to be critical for cA-regulated PAI-1 mRNA destabilization in HTC cells. Studies are currently directed at (i) isolating, identifying,
Cis- and Trans-acting Mediators of PAI-1 mRNA Stability

and characterizing the mRNA-binding proteins and the protein components of the multiprotein complexes and (ii) determining how the cytoplasmic proteins interact with the A-rich region to elicit the cA-induced destabilization of PAI-1 mRNA. The ability to link RNA-protein complex formation with the regulation of mRNA stability by cyclic nucleotides in HTC cells provides a valuable system in which to study cis- and trans-acting mediators of regulated mRNA stability.

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REFERENCES
1. Vassalli, J.-D., Sappino, A.-P., and Belin, D. (1991) J. Clin. Invest. 88, 1067–1072
2. Vassalli, J.-D., and Pepper, M. S. (1994) Nature 370, 14
3. Krutihof, E. K. O. (1988) Enzyme 40, 113–121
4. van Meijer, M., and Pannekoek, H. (1995) Fibrinolysis 9, 263–276
5. Andreasen, P. A., Georg, B., Lund, L. R., Riccio, A., and Stacey, S. N. (1990) Mol. Cell. Endocrinol. 88, 1–19
6. Heaton, J. H., and Gelehrter, T. D. (1989) Mol. Endocrinol. 3, 171–178
7. Heaton, J. H., Kathju, S., and Gelehrter, T. D. (1990) Mol. Endocrinol. 4, 171–178
8. Heaton, J. H., Kathju, S., and Gelehrter, T. D. (1990) Mol. Endocrinol. 6, 53–60
9. Heaton, J. H., Tillmann-Bogush, M., Leff, N. S., and Gelehrter, T. D. (1998) J. Biol. Chem. 273, 14261–14268
10. Zeheb, R., and Gelehrter, T. D. (1996) Gene 181, 202–209
11. Aussel, P. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1989) Current Protocols in Molecular Biology, pp. 4.7.1–4.7.8, Greene and Wiley Interscience, NY
12. Deleted in proofs
13. Huang, D., Hubbard, C. J., and Jungmann, R. A. (1995) Mol. Endocrinol. 9, 3711–3716
14. Chen, M., Schnerrmann, J., Smart, A. M., Brosius, F. C., Killen, P. D., and Briggs, J. P. (1993) J. Biol. Chem. 268, 24138–24144
15. Sakaue, M., and Hoffman, B. B. (1991) J. Biol. Chem. 266, 5743–5749
16. Nachaliel, N., Jain, D., and Hod, Y. (1993) J. Biol. Chem. 268, 24203–24209
17. Zaidi, S. H. E., and Malter, J. S. (1994) J. Biol. Chem. 269, 8493–8501
18. Zaidi, S. H. E., Denman, R., and Malter, J. S. (1994) J. Biol. Chem. 269, 24007–24006
19. Zaidi, S. H. E., and Malter, J. S. (1994) J. Biol. Chem. 269, 24037–24013
20. Irwin, N., Baekelandt, V., Goritchenko, L., and Benowitz, L. I. (1997) Nucleic Acids Res. 25, 1281–1288
21. Chung, S., Eckrich, M., Perrone-Bizzozero, N., Kohn, D. T., and Furneaux, H. (1997) J. Biol. Chem. 272, 6593–6598
22. Stephens, J. M., Carter, B. Z., Pekala, P. H., and Malter, J. S. (1992) J. Biol. Chem. 267, 8336–8341
23. Jain, R. G., Andrews, L. G., McGowan, K. M., Pekala, P. H., and Keene, J. D. (1997) Mol. Cell. Biol. 17, 954–962
24. Laterza, O. F., Hansen, W. R., Taylor, L., and Curthoys, N. P. (1997) J. Biol. Chem. 272, 22481–22488
25. Gdula, M., Burd, C. G., and Dreyfuss, G. (1994) Exp. Cell. Res. 211, 400–407