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Species-specific differential cleavage and polyadenylation of plasminogen activator inhibitor type 1 hnRNA

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ABSTRACT

Plasminogen activator inhibitor type 1 (PAI-1) is the primary physiologic inhibitor of the naturally occurring plasminogen activators. In higher primates two forms of mature PAI-1 mRNA (3.2 kb and 2.2 kb) arise by alternative cleavage and polyadenylation of PAI-1 hnRNA which is regulated in a tissue-specific fashion in humans. In other mammals only the 3.2 kb mRNA has been detected. The putative downstream polring primate evolution we prepared plasmids in which the 3′ nontranslated region of the human PAI-1 gene or the mouse PAI-1 cDNA was inserted downstream of the neomycin gene in the plasmid pSV$_2$neo. We show that the 3′-nontranslated region of the human PAI-1 gene but not the mouse PAI-1 cDNA conferred alternative cleavage and polyadenylation to the neomycin gene in transfected human Hep G2 cells as well as mouse NIH3T3 and rat L6 cells.

INTRODUCTION

Plasminogen activator inhibitor type 1 (PAI-1) a member of the serpin family of protease inhibitors is the physiologic inhibitor of both tissue-type and urokinase-type plasminogen activators and may play an important role in the regulation of a variety of fibrinolysis-dependent biologic processes (1). Low PAI-1 activity in plasma results in unstable hemostatic plugs and leads to accelerated fibrinolysis and bleeding. Increased PAI-1 activity has been demonstrated in a variety of conditions associated with thrombotic disease such as gram negative sepsis with disseminated intravascular coagulation, the second and third trimesters of pregnancy when the risk for thrombosis increases, deep vein thrombosis, acute phase reactions after surgery and trauma and in young survivors of myocardial infarction as well as in metabolic disorders that are associated with the development of atherosclerosis and coronary artery disease such as obesity, hyperlipidemia and diabetes (2–5).

The human PAI-1 gene encodes two transcripts that are approximately 3.2 and 2.2 kb in length, are colinear from their 5′ ends and differ only in the length of their 3′ nontranslated regions (NTR) (6). The 3.2 and 2.3 kb transcripts are not the result of alternative splicing but are due to the presence of multiple polyadenylation splice sites because the 3′ NTR's of the cDNA and gene sequences are colinear (7–9). Although two transcripts were detected in orangutans and African green monkeys, only the larger form was present in cells from lower primates and other mammalian species suggesting that the proximal polyadenylation site may have been acquired recently during primate evolution (10). Although the biologic significance of multiple polyadenylation sites is, in general, unknown, the existence of alternatively processed mRNAs could provide a mechanism for post-transcriptional regulation (6). It is of interest that the two forms of PAI-1 mRNA are expressed in a tissue-specific manner in humans (11) and are regulated differently in response to specific stimuli including phorbol esters and insulin in Hep G2 cells (12,13) and low density lipoprotein in endothelial cells (14). The 3.2 kb transcript has a shorter half-life than the 2.2 kb transcript in Hep G2 cells (15) possibly due to the presence of AU-rich sequences unique to the 3′-NTR of the 3.2 kb form and similar to those which impart instability to the mRNA of transiently expressed cytokines (16,17).

In humans, the putative downstream polyadenylation signal consists of three overlapping copies of the consensus sequence at position 12,120 (AATAAAATAAATAAA) (12). No consensus sequence is present upstream at a position that could generate the shorter mRNA species. The sequence AATAAT at 11,170 is thought to serve as a polyadenylation signal for the 2.3 kb transcript (8,12).

Tissue-specific and developmentally regulated alternative RNA splicing depends on the existence of specific trans-acting factors (18). Because the two forms of PAI-1 mRNA are differentially expressed in human tissues and are regulated individually in human cell lines in response to specific stimuli we sought to determine whether differential cleavage and polyadenylation of PAI-1 mRNA is dependent on trans-acting RNA processing factors.

EXPERIMENTAL PROCEDURES

Cells and cell culture

Human hepatoma (Hep G2), mouse fibroblast (NIH-3T3), and rat myoblast (L6) cell lines were obtained from the American Type Culture Collection (ATCC #s HB8065, CRL1658, and CRL1458, respectively). Hep G2 cells were maintained in minimum essential medium (MEM) with Earle's salts, L-

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glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% Nu-Serum (Collaborative Research). NIH-3T3 cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 4500 mg/L D-glucose, L-glutamine (2 mM), penicillin (50 units/ml), streptomycin (50 μg/ml) and 10% calf serum. L6 cells were maintained in DMEM with 10% fetal calf serum.

Preparation of probes

The human PAI-1 cDNA was provided by K.Kretzmer (Monsanto, St Louis, MO) (19). The human PAI-1 probe was obtained by digestion of the cDNA with Eco RI/SalI. The mouse PAI-1 cDNA was graciously provided by Mike Cole (Princeton University) (20) and the mouse PAI-1 probe obtained by digestion of the cDNA with AvrII/StuI. The plasmid pSV_2neo (21) (ATCC #37149) was obtained from the American Type Culture Collection and the neomycin probe was obtained by digestion of the cDNA with HindIII/SmaI. DNA fragments were separated by electrophoresis on 0.7% agarose gels and purified by batch affinity adsorption with sodium iodide glass beads (GENECLEAN, Bio101, Inc.). The probes were radiolabeled with [^{32}P]dCTP to a specific activity of $1-2\times10^9$ dpm/μg by the random primer method of Feinberg and Vogelstein (22).

Preparation of plasmids

A 3950 base pair SpeI/XbaI fragment of the human PAI-1 gene (23) consisting of 265 base pairs of the eighth intron, the entire ninth exon (containing the 3'-NTR) and 1815 base pairs of intergenic sequences was inserted into the SpeI/XbaI restriction sites of pBluescript. The resultant plasmid was digested with XbaI, the ends made flush with Klenow enzyme and SmaI linkers were added. The plasmid was then digested with XmaI and the 3950 base pair XmaI-linked PAI-1 fragment harvested from a 0.7% agarose gel. This fragment was inserted in the sense orientation into the unique XmaI site at the 3' end of the coding region of the neomycin gene in the plasmid pSV_2neo resulting in the plasmid $pSV_2neoPAI13980$ (Figure 1A). Orientation and copy number of the insert was confirmed by restriction enzyme digestion and DNA sequence analysis.

The mouse PAI-1 cDNA subcloned in the EcoRI site of pBS (20) was digested at position 1534 of the 3'-NTR with BlnI and the ends were made flush with Klenow enzyme. After the addition of SmaI linkers and digestion with XmaI a 1480 base pair fragment containing the majority of the 3'-untranslated region was harvested from an agarose gel and inserted in the XmaI site of pSV_2neo in the sense orientation resulting in the plasmid $pSV_2neom1534$ (Figure 1B). Orientation and copy number of the insert was confirmed by restriction enzyme digestion and DNA sequence analysis.

Cell transfection

Cells were plated in 5 ml of their respective media in 60 mm dishes and grown to 60–90% confluence. The day of transfection the cells were refed and transfections were then performed with the use of the calcium phosphate coprecipitation method (23). Precipitates contained a total of 20 μg of plasmid DNA consisting of 10 μg of chimeric plasmid construct and 10 μg of filler DNA (pBluescript). Cells were incubated with precipitate for 4 hr, subjected to a glycerol shock for 3 min, then washed and refed with the appropriate media. Forty-eight hours after transfection the cells were harvested for preparation of RNA.

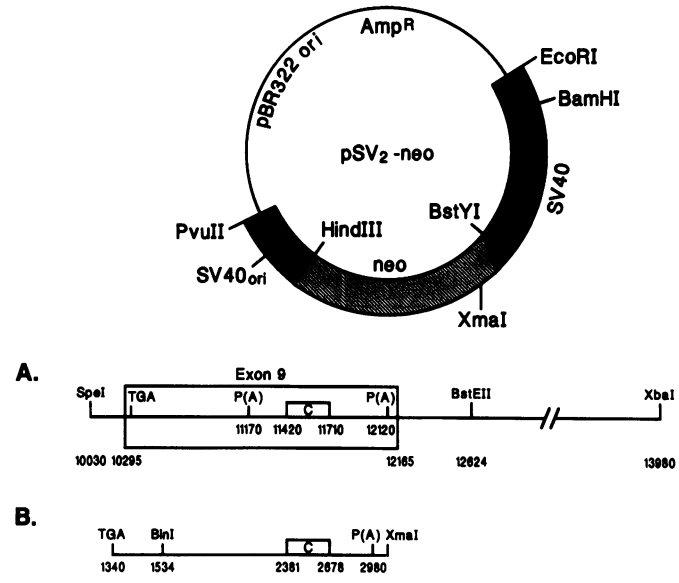

Figure 1. Construction of pSV_2neo/PAI-1 reporter plasmids. **A.** A 3950 base pair SpeI/XbaI fragment of the human PAI-1 gene was inserted into the unique XmaI site at the 3' end of the coding region of the neomycin gene in the plasmid pSV_2neo (top panel (21)) with the use of SmaI linkers to form $pSV_2neoPAI13980$. The translation stop site (TGA), the polyadenylation signal sequences P(A), a highly conserved region (when compared to the mouse sequence as noted by Pendergast, et al (20)) within the 3'-NTR (C) and select unique restriction sites are shown. The coordinates of the PAI-1 gene are from Bosma, et al (7). **B.** The 3'-NTR of the mouse PAI-1 cDNA is shown with coordinates from Prendergast, et al (20). A 1480 base pair fragment was released after digestion at the unique BlnI site and the XmaI site in the polylinker of pBS and inserted into the XmaI site of pSV_2neo with the use of SmaI linkers to form the plasmid $pSV_2neom1534$. The translation stop site (TGA), non-consensus (AATATA) putative polyadenylation signal sequence P(A) and highly conserved region (see above) (C) are shown.

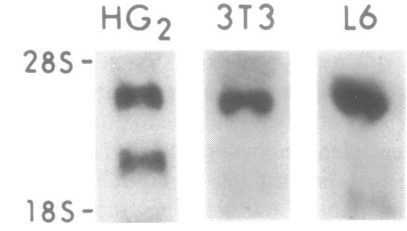

Figure 2. PAI-1 mRNA expression in human and rodent cell lines. RNA extracted from Hep G2 (HG_2), NIH-3T3 (3T3), and L6 cells was probed with a human (HG_2 cells) or mouse (3T3 and L6 cells) PAI-1 cDNA as described in the Methods. The position of migration of 28S and 18S rRNA is shown to the left of the Figure.

Preparation of RNA and Northern blot analysis

The cells were washed twice with phosphate buffered saline and harvested in RNAzol B, (Cinna Biotecx) (15) with a cell scraper. Total cellular RNA was purified by an additional chloroform extraction, and precipitated with isopropyl alcohol. RNA samples were resuspended in 1 mM EDTA and quantified by absorbance at 260 nm. Northern blots were prepared as previously described (11) with GeneScreen (New England Nuclear) membranes with 10 μg of RNA. Hybridization was performed with either human

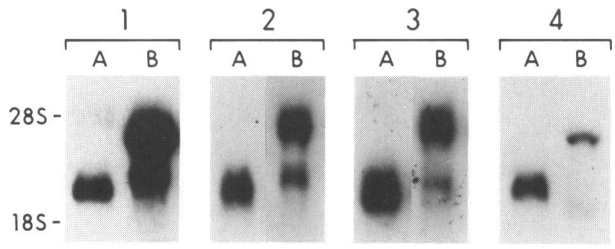

Figure 3. Northern blot analysis of cells transfected with neomycin reporter plasmids. Representative Northern blots of total cellular RNA extracted from transfected cells and probed with a neomycin coding region probe are shown. Panel 1: Hep G2 cells transfected with pSV_2neo (A) or $pSV_2neoPAI13980$ (B). Panel 2: NIH3T3 cells transfected with pSV_2neo (A) or $pSV_2neoPAI13980$ (B). Panel 3: L6 cells transfected with pSV_2neo (A) or $pSV_2neoPAI13980$ (B). Panel 4: Hep G2 cells transfected with pSV_2neo (A) or $pSV_2neom1534$ (B).

or mouse PAI-1 or neomycin cDNA probes (600,000 dpm/ml) for 18−24 hrs at 42°C with agitation. Membranes were washed three times in a solution of 2×SSC (0.3 M NaCl, 0.03 M Na citrate) with 1% SDS for 5 min at room temperature, and once in a solution of 0.2×SSC and 0.1% SDS for 15 min at 65°C. Autoradiograms were prepared with Kodak XAR-5 film and Cronex intensifying screens at −70°C.

RESULTS AND DISCUSSION

Expression of PAI-1 mRNA in cell lines

To assess the species-specific differences in expression of PAI-1 mRNA transcripts, human Hep G2, mouse NIH-3T3, and rat L6 cells were grown to confluence and total cellular RNA was extracted for Northern blot analysis. Hep G2 mRNA was probed with the human PAI-1 cDNA, and NIH-3T3 and L6 mRNA was probed with the mouse PAI-1 cDNA. As shown in Figure 2, Hep G2 cells express two forms of PAI-1 mRNA approximately 3.2 and 2.2 kb in size as shown previously (23). NIH-3T3 and L6 cells express one PAI-1 mRNA transcript, approximately 3.2 kb in size (24).

Expression of neomycin and neomycin-PAI-1 chimeric mRNA in transfected cells

To assess the effect of the 3′ NTR of the human PAI-1 gene on processing of a heterologous mRNA, the plasmid $pSV_2neoPAI13980$ was transfected into Hep G2 cells and control cells were transfected with pSV_2neo. Total cellular RNA was harvested from the transfected cells and Northern blot membranes prepared and probed with a neomycin coding region cDNA probe. Transfection with pSV_2neo resulted in one form of neomycin mRNA, approximately 1.8 kb in size. In contrast, transfection with the neomycin/PAI-1 chimeric plasmid $pSV_2neoPAI13980$ resulted in two forms of mRNA, approximately 3.0 and 2.0 kb in size (Figure 3, Panel 1). Because the coding region of the PAI-1 mRNA (1354 base pairs) and the neomycin mRNA (1319 base pairs) are approximately the same size, the two forms of PAI-1 mRNA and the two forms of mRNA transcribed from $pSV_2neoPAI13980$ are of similar size.

To determine whether the human pattern of PAI-1 mRNA cleavage and polyadenylation would also be conferred to chimeric neomycin-PAI-1 mRNA by rodent cell lines that express only one form of endogenous PAI-1 mRNA, $pSV_2neoPAI13980$ was transfected into NIH-3T3 and L6 cells. Control cells were transfected with pSV_2neo. In both rodent cell lines transfection with pSV_2neo resulted in one form of neomycin mRNA while transfection with $pSV_2neoPAI13980$ resulted in two forms of neomycin-PAI-1 chimeric mRNA (Figure 3, Panels 2 and 3).

To determine whether the 3′-untranslated region of the mouse PAI-1 cDNA would confer the murine pattern of PAI-1 cleavage and polyadenylation to the neomycin gene in a human cell line that expresses two forms of endogenous PAI-1 mRNA the plasmid $pSV_2neom1534$ was transfected into Hep G2 cells. In contrast to the results seen after transfection with $pSV_2neoPAI13980$, transfection with the mouse PAI-1/neomycin chimeric plasmid resulted in only one form of neomycin mRNA, approximately 3.0 kb in size (Figure 3, Panel 4).

Our results show that the 3′ untranslated region of the human PAI-1 gene conferred the human pattern of PAI-1 alternative cleavage and polyadenylation to a heterologous mRNA in rodent cell lines that express only a single form of endogenous PAI-1 mRNA while the 3′ untranslated region of the mouse PAI-1 cDNA conferred the murine pattern of PAI-1 cleavage and polyadenylation to the same heterologous mRNA in a human cell line. Therefore, the species-specificity of PAI-1 cleavage and polyadenylation is dependent on cis-acting sequence differences and not on differences in trans-acting factors. Mechanisms involved in the selection of one poly(A) site over another remain poorly understood. Conserved sequences downstream of the poly(A) addition site also shown to be important for 3′ processing of polyadenylated mRNAs can often be fitted to the consensus sequence YGTGTTYY and many genes show an overrepresentation of the tri-nucleotide TGT in conjunction with oligo-T stretches (G/T clusters), immediately downstream of the cleavage site at which poly(A) is added (25). These downstream flanking sequences, may influence selection of different polyadenylation signals as shown for the adenovirus major late transcription unit (26).

Genes that have been shown to display tissue-specific and developmentally regulated differential cleavage and polyadenylation of their mRNA transcripts include the human liver aspartate aminotransferase gene which gives rise to two mRNAs with different half lives whose ratio varies among species and in humans during development (27) and the rabbit angiotensin-converting enzyme gene which encodes two tissue-specific mRNAs that differ only in their 3′-nontranslated regions (28). The molecular mechanisms that result in developmental and tissue-specific differential cleavage and polyadenylation of these mRNAs and the biologic significance, if any, of this process is not known. The difference in half-life of the two PAI-1 mRNA transcripts and their differential regulation in response to specific mediators suggests the existence of a potentially important locus of post-transcriptional modulation of the PAI-1 gene that is unique to higher primates.

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