Aminopeptidase-A. II. Genomic cloning and characterization of the rat promoter

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Jiang, Qingping, Marta Troyanovskaya, Gomathi Jayaraman, and Dennis P. Healy. Aminopeptidase-A. II. Genomic cloning and characterization of the rat promoter. Am. J. Physiol. Regulatory Integrative Comp. Physiol. 278: R425–R434, 2000.—Aminopeptidase-A (APA) has a widespread tissue distribution consistent with a role in the metabolism of circulating or locally produced ANG II or CCK-8. APA is also highly expressed in pre-B lymphocytes, but its role in lymphoid cell development is unknown. To begin to understand the basis for cell-specific regulation of APA expression, we sought to clone and characterize the rat gene promoter. Screening of a rat genomic library with a partial rat APA cDNA resulted in isolation of a 12-kb clone found to contain the first exon and >3 kb of 5′-flanking sequence. Primer extension of rat kidney mRNA indicated that the major transcription start site was 312 bp upstream of the translation start codon and 22 bp downstream from a TATA box. Constructs containing portions of the 5′-flanking region placed upstream of a chloramphenicol acetyltransferase reporter gene indicated that expression was cell specific and that high activity could be obtained with constructs containing as little as 110 bp of 5′-flanking region sequence. We further identified an upstream regulatory element between −1063 and −348 that suppressed transcription in a cell-specific manner. This element (termed upstream suppressor of APA, or USA) also suppressed transcription of a heterologous promoter. These results indicate that the organization and regulation of the rat APA is not consistent with it being a housekeeping gene and further suggest that rat APA gene transcription might be regulated through the presence of a novel strong upstream suppressor element.

gene; transcription; reporter gene; suppressor

PEPTIDASE ENZYMES ARE GENERALLY thought to be encoded by “housekeeping” genes, i.e., genes that are widely and constitutively expressed. Aminopeptidase-A (APA, glutamyl aminopeptidase, EC 3.4.11.7) selectively hydrolyzes acidic amino acid residues from oligopeptides (43). APA is highly expressed along with other proteases and peptidases in the brush border of cells active in protein and peptide degradation, including enterocytes lining the small intestine and proximal tubule cells (23, 40). Lower levels of APA are seen widely throughout the body concentrated along vascular elements and sinusoids (40). In these locations, APA might be involved in metabolism of circulating or locally formed bioactive peptides. There are only two biologically active peptides that are known to be substrates for APA, namely [Asp1]ANG II and [Asp1]CCK-8 (43). We have speculated that APA might be regulated by ANG II because APA expression in the kidney is altered under conditions where ANG II levels are changed (16, 38). Likewise, APA is identical to a well-characterized pre-B cell differentiation antigen, i.e., BP-1/6C3 (45). Whereas it does not appear that the enzyme activity of BP-1/6C3 (i.e., APA) is essential for B cell development (24), the expression pattern of this antigen is tightly regulated developmentally. Thus these lines of evidence would suggest that expression of the APA might be more highly regulated than expected if it were simply a housekeeping enzyme.

In a companion article (40), we reported on the cloning and expression of the rat APA cDNA. Here, we used the 5′ portion of the cDNA to isolate and clone the first exon and 5′-flanking region of the rat APA gene. We further report that the 5′-flanking region contains a strong negative element that may be essential for regulation of the rat APA gene.

METHODS

Genomic library screening. A rat genomic phage library (Lambda Dash II, Stratagene) was screened with a 5′ cDNA clone of rat APA (40). The bacterial strain P2392 was grown in LB media (per liter: 10 g NaCl, 10 g Bacto-Tryptone, 5 g yeast extract) containing 0.2% maltose and 10 mM MgSO4 for 5–6 h at 37°C; then 200 µl bacteria (absorbance at 600 nM = 0.22) was added to 4 ml melted (48°C) top agarose solution (LB medium containing 7.5 g/l agarose, and 300 mg MgSO4) plus diluted phage in SM buffer (per liter: 5.8 g NaCl, 2 g MgSO4, 50 ml of 1 M Tris·HCl, pH 7.5, 5 ml 2% gelatin) and poured onto LB plus MgSO4 (1.2 g/l) plates. Phage were diluted to a density of ~4 × 105 phage per 7-mm plate. Phage were transferred in duplicate to nitrocellulose membranes and screened with a partial clone from the 5′ cDNA as previously described for the screening of a rat kidney cDNA library (40). Briefly, nitrocellulose membranes were prehybridized at 59°C for 1 h in hybridization buffer minus probe and then transferred to hybridization buffer (6× NET, 5× Denhardt’s, and 0.1% SDS) (20× NET: 3 M NaCl, 20 mM EDTA, 0.3 M Tris·HCl, pH 8.0) and labeled DNA. Filters were incubated at 59°C overnight with agitation. Washes were then at room temperature for 1 h with four changes of 2× sodium chloride-sodium citrate (SSC) and 0.1% SDS followed by a 2-h wash at 59°C in 2× SSC and 0.1% SDS. The filters were then dried.
and exposed to X-ray film. Positive plaques were picked up and placed in SM buffer and titrated. Additional screenings with greater and greater dilution were done until positive plaques were pure. Phage DNA was prepared using standard procedures (34), and the genomic clone was excised from the phage with EcoRI and separated on a 1% agarose gel and viewed under ultraviolet illumination to verify the size. The DNA was transferred to nitrocellulose membranes as described previously and hybridized with the labeled probe. Positive bands were subcloned into the EcoRI site of pBluescript (Stratagene) and sequenced.

The transcription start site for the APA gene as expressed in kidney was determined using primer extension as previously described (13). A oligonucleotide primer (APAAAN14, 5′-GTCCTGCACGTCTCCGCCGACCTCTGGG-3′) was designed to be complementary to the 5′ untranslated region of the rat kidney cDNA sequence. The targeted sequence was found in the previously characterized genomic clone (see below). The oligonucleotide was end labeled with γ[32P]-ATP and T4 polynucleotide kinase and hybridized to 36 µg of rat kidney total RNA at 65°C for 2 h in 4 µl total volume. Thirty microliters of reaction buffer containing (in µl) 0.9 of 1 M Tris-HCl, pH 8.3, 9 of 50 mM MgCl2, 2.5 of 1 M dithiothreitol, 3.38 of a 2-mg/ml solution of actinomycin D, 0.7 of 10 mM dNTPs, 11.65 diethyl pyrocarbonate-treated water, and 2 of 2.5 U/µl of AMV RT was then added and incubated for 1 h at 42°C. Control samples were handled identically, except that RNA was omitted. The reactions were stopped by adding 105 µl of RNase mixture (20 µg/ml DNase-free RNase A, 100 µg/ml salmon sperm DNA in Tris-EDTA buffer, pH 7.5, containing 100 mM NaCl) and incubating for 15 min at 37°C. The DNA was then precipitated and loaded onto a 9% acrylamide-7 M urea sequencing gel. The length of the product was determined by comparison to the DNA sequence of genomic DNA using the same primer and the dideoxy sequencing method and run in parallel lanes simultaneously. The gel was then dried and exposed to X-ray film.

Plasmid construction. The rat APA promoter was characterized by transfecing successively truncated portions of the 5′-flanking region of the APA gene annealed to a reporter gene in various mammalian cells (see below). The reporter gene that we used was the chloramphenicol acetyltransferase (CAT). Fragments of the 5′-flanking region were placed into the promoterless pCAT-Basic construct (Promega). Sequence analysis of the 5′-flanking region from the isolated genomic clones indicated the presence of a number of unique restriction sites. The genomic clone (g1–7) was digested with EcoRI and PvuII (both within the multiple cloning site). The resultant construct was termed pCAT-1063.

Gene transfer experiments were performed in various mammalian cells using Lipofectamine reagent (GIBCO-BRL). Lipofectamine transfections were performed in serum-free and antibiotic-free medium according to the manufacturer’s protocol. Cells were grown to 60% confluency in six-well plates and transfected with plasmid DNA (2.0 µg) and Lipofectamine diluted according to the manufacturer’s protocol at room temperature for 10–15 min. Cells were harvested 24 h later. CAT enzyme constructs were co-transfected with a pSV-β-galactosidase expression vector (0.25 µg/well; Promega) as a positive control for transfection efficiency. β-Galactosidase and CAT enzyme activity were measured using standard procedures (Promega).

Cell culture. Porcine LLC-PK1 cells were grown in DMEM supplemented with 10% fetal bovine serum, 4.5 g/l glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.25 mg/ml amphotericin B (GIBCO-BRL). Cells were passaged by trypsinization with 0.25% trypsin and 1.0 mM EDTA. Cells were grown in 80-cm² plastic flasks at 37°C under an atmosphere of 95% air-5% CO2 and passed twice a week. The mouse fibroblast cell line NIH3T3 and the human hepatic cell line HepG2 were grown identically. A SV40-transformed mouse mesangial cell line (SV40MOe13, kindly provided by Dr. M. Lipkowitz, Mt. Sinai School of Medicine) was grown similarly except in DMEM containing 5% fetal bovine serum.

RESULTS

A rat genomic library was screened using a rat kidney cDNA clone containing the 5′ portion of the APA coding region and the 5′-untranslated region (RC-11). Excision of the genomic DNA insert from one positive clone (g1) resulted in two EcoRI fragments of ~5 and ~348 region had activator or suppressor activity in a heterologous promoter setting, the −1063 to −348 fragment (also termed USA 348 region) was essentially deleted from the pCAT-1404 construct by digestion of pCAT-1404 with HindIII (site in multiple donor region and at −1063), blunting, and insertion into a blunt end PstI site of pCAT-348. Plasmids containing the −1404/−1063 region in the correct orientation upstream of −348 were determined by sequence analysis. The resultant plasmid was termed pCAT-1404 (−1063/−348).

Transfection and CAT assays. Mammalian cells were transfected with plasmid constructs using Lipofectamine reagent (GIBCO-BRL). Lipofectamine transfections were performed in serum-free and antibiotic-free medium according to the manufacturer’s protocol. Cells were grown to ~60% confluency in six-well plates and transfected with plasmid DNA (2.0 µg) and Lipofectamine diluted according to the manufacturer’s protocol at room temperature for 10–15 min. Cells were incubated for 5 h and then overnight after addition of 0.1 ml of fetal bovine serum. The next day, the DNA-containing medium was removed and replaced with medium containing 10% fetal bovine serum and antibiotics. The cells were harvested 24 h later. CAT enzyme constructs were cotransfected with a pSV-β-galactosidase expression vector (0.25 µg/well; Promega) as a positive control for transfection efficiency. β-Galactosidase and CAT enzyme activity were measured using standard procedures (Promega).

Results. A rat genomic library was screened using a rat kidney cDNA clone containing the 5′ portion of the APA coding region and the 5′-untranslated region (RC-11). Excision of the genomic DNA insert from one positive clone (g1) resulted in two EcoRI fragments of ~5 and ~348 region had activator or suppressor activity in a heterologous promoter setting, the −1063 to −348 fragment (also termed USA 348 region) was essentially deleted from the pCAT-1404 construct by digestion of pCAT-1404 with HindIII (site in multiple donor region and at −1063), blunting, and insertion into a blunt end PstI site of pCAT-348. Plasmids containing the −1404/−1063 region in the correct orientation upstream of −348 were determined by sequence analysis. The resultant plasmid was termed pCAT-1404 (−1063/−348).
The upper band positively hybridized with the RC-11 probe and was subcloned for sequence analysis. This clone (g1–7) contained the previously characterized 5′-untranslated region (5′-UTR) as found in the RC-11 cDNA clone as well as the 5′-RACE product (40) and 621 bp of coding sequence. Because the lower band failed to hybridize with any other APA cDNA sequence (data not shown), presumably this DNA was composed entirely of intron sequence. The g1–7 clone therefore contained the first exon, which is comprised of 621 bp of coding sequence and the entire 5′-UTR, bordered by 5′-flanking sequence (Fig. 1) and intron 1. The exon 1-intron 1 splice site sequence was...GCACACCCAGTACGTTG...

The 5′ end of a previously characterized 5′-RACE product from rat kidney RNA as well as the 5′ end of a partial APA cDNA from a rat kidney library (RC-11) was located within the g1–7 genomic clone (Figs. 1 and 2). To determine directly the transcription initiation site (presumably 5′ to or coinciding with the 5′ end of the APA cDNA clone RC-11), we designed an oligonucleotide primer (APAAN14) antisense to the known 5′-UTR sequence and downstream from the 5′ ends of either the 5′-RACE product or the partial cDNA RC-11 (Fig. 2). The antisense primer APAAN14 (+153 to +124) was hybridized to rat kidney total RNA and extended with RT. The single-stranded cDNA was run on a denaturing acrylamide gel, and the size was compared with DNA sequence obtained with the same primer and conventional dideoxynucleotide sequencing (Fig. 3). A single major product and a slightly shorter (8 nucleotides) minor product were seen. The size corresponded to a site 311 bp upstream from the translation start site. This major initiation site (designated from now on as +1) was 22 bp downstream from a putative TATA box (Figs. 1 and 2). A CCAAT box-like sequence (CCAAAAT) was present 68 nucleotides upstream of the −22 TATA box. A comparison of the rat and mouse sequences from this area of the APA gene (Fig. 2) indicated that the transcription initiation site for the rat gene is 58 nucleotides upstream of the corresponding start site for the mouse gene (42). The primer extension product was also longer than either the previously characterized cDNA or 5′-RACE products (40).

We then proceeded to sequence the remainder of the 5′-flanking region sequence contained within the g1–7 clone (Fig. 1). Of note, in addition to the TATA box sequences at position −22 and +30, there were 14 TATA box sequences distributed throughout the 5′-flanking region sequence (Fig. 1). Consistent with the APA gene having a functional TATA box, the GC content of the entire 5′-flanking region was only 44%. In general, genes that do not use a TATA box tend to have high (>60%) GC content and have multiple consensus sites for the Sp1 transcription factor (9), of which there is only one in the entire 5′-flanking region of the APA gene (see below). Interestingly, a dinucleotide GT repeat sequence was seen from −1503 to −1420 (Fig. 1). Close inspection of the GT repeat indicated a further pattern of a stretch of 18 GT repeats followed by a repetitive CT(GT)2-4 sequence [namely 3 CT(GT)2 repeats followed by CT(GT)2 and CT(GT)3].

To identify the core promoter region of the rat APA gene, we tested a series of constructs containing progressively truncated regions of the 5′-flanking region placed upstream of the promoterless CAT reporter plasmid (pCAT-Basic) for CAT activity in transiently transfected mammalian cells (Fig. 4). When transfected into LLC-PK1 cells, a porcine renal epithelial cell line that expresses APA (40), a construct that extended from −2425 to +248 (pCAT-2425) had high activity. When placed in the antisense orientation (pCAT+248/−2425), the construct had no activity, indicating that the APA promoter was contained within this portion of DNA and that promoter activity was orientation dependent. A series of constructs with successively truncated 5′-flanking regions were then made based on identified restriction sites and transfected into LLC-PK1 cells. The highest activity was seen with a construct containing only 110 bp of 5′-flanking sequence (pCAT-110). These results suggest that the core promoter is contained within this region. Interestingly, whereas the differences in CAT activity between the pCAT-2425, pCAT-1404, pCAT-348, and pCAT-110 constructs were only two- to threefold, the pCAT-1063 construct was totally devoid of activity, suggesting that there was a strong negative element between −348 and −1063. As this area is contained within the pCAT-1404 and pCAT-2425 constructs, it is likely that additional elements between −1063 and −2425 are able to offset the suppressor activity of the downstream sequence.

Inspection of the DNA sequence of the Hind III-Pst I fragment revealed that there were six TATA boxes and three CCAAT boxes within this region. At least one TATA box (−833) had a surrounding sequence that was very similar to the functional downstream TATA box at −22 (compare −834TTATATA−828 to −23TTATATT−18). Also, because other peptidase genes have been shown to use multiple promoters (36), we considered that the region between −1063 and −348 may contain an alternate upstream promoter whose activity is somehow adversely affected in the context of the pCAT-1063 reporter construct. To test whether this region functioned as an independent promoter, we placed the Hind III-Pst I fragment upstream of the promoterless pCAT-Basic construct and measured CAT activity after transfection into LLC-PK1 cells (Fig. 4). Interestingly, this construct (pCAT-1063/−348) had no activity, suggesting that it could not function as an independent alternate promoter in LLC-PK1 cells. To determine whether the −1063/−348 region did impart some suppressor effect on the larger constructs, we deleted this region from the pCAT-1404 construct and measured CAT activity in LLC-PK1 cells. Deletion of this region (pCAT-1404−1063/−348) increased activity twofold over the pCAT-1404 construct to reach the level of activity of the pCAT-348 construct (Fig. 4). Thus, possibly through binding of trans-acting factors to cis-elements contained within, the −1063/−348 region imparts suppressor activity on the APA gene when expressed in renal epithelial cells. Finally, because the −1063/−348 re-
The organization of the rat APA gene is not consistent with it being a housekeeping gene. Housekeeping genes generally are TATA box-less genes, have high GC content in the 5'-flanking region, have multiple sites for the Sp1 transcription factor, and initiate transcription from multiple sites (9). In contrast, the major APA gene transcript initiates 22 nucleotides downstream from a TATA box and 311 bp upstream of the translation start site, has low GC content (44%), and a single Sp1 consensus site at position +6. Moreover, the ~3 kb of 5'-flanking sequence contained multiple TATA box sequences. In this regard, the APA gene is more similar to the closely related aminopeptidase-N (APN) gene, which also contains a TATA box and initiates transcription in the kidney from a single major site (36), than it is to dipeptidyl peptidase IV, which is a TATA-less gene with high GC content and initiates transcription from more than six sites (4). The APA gene expressed in rat kidney apparently uses the TATA box at position +22. There is a CCAAT-like box (CCAAAAT) 68 bp upstream from this site. ATATA box at position +30 is apparently not used. This is interesting because the mouse APA gene as expressed in pre-B cells, i.e., the BP-1/6C3 antigen, uses a TATA box corresponding to this 3' site. Close inspection indicates that the surrounding sequence in the rat gene is TATA_C, whereas the mouse sequence is TATA_T. The upstream sequence at ~22 in
the rat gene is TATAT, suggesting that the TATAT sequence might be preferred for binding of TFIID and the transcriptional machinery for initiation of transcription of the APA gene in rodents. One can only speculate as to whether any of the remaining 14 upstream TATA box sequences in the rat 5′-flanking region are functional. This point is relevant because the closely related APN gene has been reported to use two alternative promoters depending on the cell type (11, 29, 36). In our hands, in most tissues there appears to be a single major transcript of ~4.1 kb in size (39, 40), a size that is identical to the BP-1/6C3 transcript seen in mouse pre-B cells (44). The existence of multiple upstream TATA boxes in the region from −1063 to −348 led us to think that the reduced level of expression of the pCAT-1063 construct may be related to this region functioning as an independent promoter. To test this, we placed the −1063 to −348 region upstream of the promoterless CAT reporter gene, but it still failed to have any activity. Thus, whereas this region does not appear to contain an independent promoter that is functional in renal epithelial cells, this possibility cannot be ruled out for other cell types.

APA is highly expressed in the kidney proximal tubule cells (39). To identify the minimal promoter for APA gene expression in proximal tubule cells, we characterized the transcriptional activity of a series of successively truncated 5′-flanking regions placed upstream of a CAT reporter gene and transfected into the porcine proximal tubule-like established cell line LLC-PK₁ (18). The largest construct (pCAT-2425, PvuII-PvuII) contained the transcription start site and 22425 bp of 5′-flanking sequence and 248 bp of the 5′-UTR and was very active when transfected into LLC-PK₁ cells. Placement of this fragment in the reverse orientation completely abolished transcriptional activity, indicating that promoter activity was orientation specific. With the exception of the pCAT-1063 construct, all the other constructs (pCAT-1404, pCAT-348, and pCAT-110) were also active, with pCAT-110 being the most active. Thus in LLC-PK₁ cells the minimal APA core promoter is contained within the first 110 bp of 5′-flanking sequence.

Surprisingly, the pCAT-1063 construct was totally inactive when expressed in LLC-PK₁ cells. It was also inactive in nonrenal cells, including HepG2, SV40Mes13, and NIH3T3 cells. Because this region contains numerous TATA boxes and there is some evidence of larger APA transcripts being present in kidney RNA (40) and because there is evidence that the APN gene uses alternative promoters in different tissues (11, 29, 36), we placed the HindIII-PstI fragment (−1063 to −348) upstream of the promoterless pCAT-Basic construct and determined if it had activity in both renal and nonrenal cells. However, this construct (pCAT-1063/-348) was again totally inactive in all cells tested, suggesting that this region did not contain an alternative promoter that could be revealed when expressed in different cells. Because both larger (pCAT-1404) and smaller (pCAT-348) constructs had high activity, it seemed that the low activity of the pCAT-1063 construct was due principally to the presence of a negative element within the region of −1063 to −348. To test this possibility we placed this region upstream of a heterologous promoter (TK) reporter construct and found that it suppressed reporter activity by 75% when expressed in LLC-PK₁ cells. This indicates that a negative or suppressor element(s) is contained within this region and that it has suppressor activity independent of the APA gene. The finding that the USA region of the APA gene inhibited transcription in both the APA and TK promoters suggests that the mechanism might involve a direct interaction with the basal transcription...
machinery [so-called active repression (15)] rather than a mechanism that involves steric hindrance or interaction with activator proteins binding to flanking DNA sequences in the APA gene. The fact that the next larger pCAT-1404 construct had high activity indicated that the suppressor activity of the USA region could be overcome by distal elements located between −1404 and −1063. Whereas this region may contain enhancer elements, the fact that deletion of the −1063 to −348 region from the pCAT-1404 construct (i.e., pCAT-1063/−348) resulted in only a modest increase in activity over pCAT-1404 indicated that, by itself, the region between −1404 and −1063 does not function as a strong enhancer for the core promoter. On the other hand, an interaction between positive upstream promoter elements with downstream negative elements may play an important role in cell-specific expression of the APA gene, where the proximal promoter negative elements predominate in absence of specific trans-acting factors binding to the upstream elements. The regulation of the rat APA gene is somewhat similar to that of the mouse renin Ren-1c gene, where an upstream enhancer interacts with the proximal promoter to override the effects of an intervening negative regulatory region (31). However, the regulation is not identical, because the renin gene proximal promoter has very low activity in the absence of the upstream elements, whereas the rat APA core promoter does not require upstream elements for high activity. Finally, as se-

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**Fig. 4.** Transcriptional activity of rat APA gene expressed in renal epithelial LLC-PK1 cells. A: schematic representation of chloramphenicol acetyltransferase (CAT) reporter gene constructs with increasing deletions of APA gene 5'-flanking region. Orientation and position of start codon at position +1 is shown by arrow for each construct. B: transcriptional activity of each construct after transient transfection into LLC-PK1 cells. Transcriptional activity is expressed relative to promoterless pCAT-Basic construct. Construct labeled pCAT-1404-delta is identical to pCAT-1063–1404/−348 described in METHODS. Values represent mean ± SE of 4 separate experiments, each point determined in triplicate. E, EcoRI; P, PvuII; S, SphI; H, HindIII; P, PstI; X, XhoI.

**Fig. 5.** Transcriptional activity of a heterologous promoter/reporter construct with upstream suppressor of APA (USA) region of APA gene positioned upstream. A: schematic representation of heterologous thymidine kinase (TK) promoter/CAT construct (pTK-CAT) and APA gene sequence from −1063 to −348 placed upstream in sense orientation and tested for enhancer or suppressor activity (USA-pTK-CAT). B: transcriptional activity of each construct after transient transfection into LLC-PK1 cells, with activity expressed as percent pTK-CAT activity. Values represent means ± SE of 4 separate experiments, each point determined in triplicate.

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**Fig. 6.** Cell specificity of APA gene transcriptional activity using rat APA gene reporter constructs. APA gene reporter constructs were transiently transfected into LLC-PK1 cells, SV40-transformed mesangial cells SV40Mes13, hepatic cells HepG2, and fibroblast cells NIH3T3, and CAT activity was measured. Transcriptional activity is expressed relative to promoterless pCAT-Basic construct. Values represent mean ± SE of 3 separate experiments, each point determined in triplicate.
sequence analysis indicated that there are numerous cis-acting elements contained within this region, further experiments are required to ascertain whether this suppressor activity can be attributed to binding of a known trans-acting factor or whether this activity represents binding of a novel suppressor protein(s).

Wang et al. (42) reported that a 2.1-kb fragment of the mouse BP-1 gene promoter was not sufficient for complete cell-specific and stage-specific expression in pre-B cells. Whereas the 2.1-kb promoter fragment placed upstream from a reporter gene was inactive in fibroblast cells, the construct was active both in pre-B cells (1H6A) and in a BP-1-negative plasma cell line (Ag8.653), indicating that additional regulatory elements outside of the 2.1-kb fragment were required for cell-specific and stage-specific expression. In contrast, as little as 110 bp of the rat APA gene was sufficient for expression in porcine renal epithelial LLC-PK1 cells. To determine the minimal promoter fragment necessary for cell-specific expression in nonlymphoid cells, we also transfected the APA promoter constructs into APA-positive and APA-negative cells and measured reporter gene activity. Positive cells included a mouse mesangial cell line that had been transformed with SV40 (SV40Mes13) and HepG2 cells, a human hepatic cell line. APA-negative cells were the NIH3T3 mouse fibroblast cell line. Interestingly, the APA-positive cell lines (SV40Mes13 and HepG2) had transcriptional activity similar in magnitudes to LLC-PK1 cells, whereas the APA-negative cell line NIH3T3 was virtually devoid of activity. Likewise, the pCAT-1063 and pCAT-1063/−348 constructs were equally inactive in all cells tested. Thus the minimal promoter contained within the first 110 bp of 5′-flanking region sequence is sufficient for cell-specific expression in vitro.

In general, cell-specific gene expression does not involve cell-specific trans-acting factors, but rather the unique combination of core promoter and upstream and downstream activator (or positive) and suppressor (or negative) trans-acting factors. Whereas the presence of consensus cis-acting elements within gene sequences does not necessarily indicate the involvement of the cognate trans-acting factors, to begin to understand how the rat APA gene might be regulated, we scanned the 5′-flanking region sequence for transcription factor binding sites using the TFSEARCH program (by Y. Akiyama) and the TRANSFAC database (17). A number of putative cis-acting responsive elements were revealed, but the functional significance remains to be determined. However, because the APA gene is expressed in a cell-specific manner, consensus sites for several tissue-selective transcription factors deserve comment. Hepatic nuclear factor-5 (HNF-5) is a transcription factor expressed in liver that recognizes the sequence TTTTG (12). There are six HNF-5 sites throughout the APA 5′-flanking region, with two being on opposite strands in an inverted repeat pattern separated by 3 bp (TTTGTGTTTAAACAA, −2281 to −2283) and further downstream (−1211 to −1098) in a tandem repeat (TTTTGT GTTTGT). YY-1 was shown to be a negative regulator of skeletal 3-actin gene transcription in cardiomyocytes (30). There are three YY-1 sites within the APA gene, two of which (−2234 and −2206) are identical and separated by 19 bp. A single myosin light chain inducible element (MLC) (37) is located on the negative DNA strand at position −343. These muscle-selective elements are of interest, because we showed previously that APA is expressed in mesangial cells and pericytes, both of which are vascular myoepithelial cells associated with glomerular capillaries and microvessels from various tissues, respectively (40, 41). Finally, although hematopoietic cells were not tested here, there are multiple consensus sites for trans-acting factors known to be involved in gene expression in lymphoid tissue, including Oct 1 and GATA 1 (17).

The mouse BP-1/6C3 gene has been shown to be regulated by type 1 interferons (INF) and interleukin (IL)-7 (42). Whereas there is some evidence that APA expression in renal cells can be regulated by a variety of cytokines (21), whether expression of APA in the kidney is directly influenced by cytokines has not been reported. However, the sequence of the rat APA 5′-flanking region contains a very high number of consensus sites for cytokine-dependent transcription factors, suggesting a possible role for cytokines in regulating APA gene transcription. For example, there are 13 γ-INF regulatory elements (IRE) (CTKKNNY), 21 α,β-IRE sites (CWWKNNNY), 12 α-INF sites (AARKGA), and 13 γ-INF inducible elements (GAS; TTNCNNA) (6, 10, 46). There are eight consensus sites for IL-1 and IL-6 class I gene response elements (TKNNNNAK) and four class II gene response elements (CTGGGA) (47). A similar site (CTGGAA) has been shown to be the IL-6 response element in the human fibrinogen gene expressed in liver (27), and there are four such sites in the APA gene. The Smad proteins are transcription factors that are activated by the transforming growth factor-β (TGF-β)-superfamily and translocate to the nucleus to influence gene transcription (19). There are 14 Smad binding elements (CAGACA) within the 5′-flanking region of the rat APA gene. Because TGF-β has been implicated in the development of glomerulosclerosis (1, 20, 22) and because APA expression has been shown to be upregulated within glomeruli of hypertensive rats (16, 38), the presence of multiple consensus sites for cytokine-dependent transcription factors suggests that cytokines might play a role in regulation of APA expression in glomeruli.

A unique feature of the APA gene was the presence of a GT (or TG) dinucleotide repeat region, where there were 18 GT repeats followed by 4 repeats of CTGGTGTG, a single CTGTGG, and a single CTGTGTTGTG. Dinucleotide repeats such as GT/CA are common in eukaryotes and are more frequently found within 5′-flanking regions, 5′-UTR, or introns than within coding regions and have been termed microsatellites (7, 35). Variations in the length of the dinucleotide repeats create substantial polymorphism and are used to generate genetic markers for complex genetic traits such as...
hypertension (2). Repetitive dinucleotide tracts such as CA/GT also have been shown to influence recombination activity and increase genomic rearrangements (8). The repeated stretches of DNA alter the structure of the DNA, producing so-called Z-DNA, where alternating purine/pyrimidine expenses produce negative supercoiling (33). Poly GT has been reported to modulate promoter activity from a distance (3, 14) and to have enhancer activity in lymphoid and nonlymphoid cells (26). The APA gene GT repeat also bears resemblance to several consensus sites. For example, there are five Smad binding elements on the negative strand (CAGACA) (19). Also, four overlapping repeats of a sequence similar to Ig3 site (25) are found on the negative strand (CACAGACAC) within this region. This is interesting, because multiple tandem repeats of Ig3 binding sites confer TGF-β inducibility in lymphoid cells (25).

Perspectives

The generally held principle is that peptidases are housekeeping enzymes. Early studies where plasma APA levels were unchanged in renal hypertensives seemingly supported this principle (28). However, more and more evidence now suggests that APA is regulated. First of all, we recently showed that conditions that result in changes in the systemic levels of ANG II, one of the principal biological substrates for APA, result in alterations in APA levels in the kidney (16, 38). These studies have further shown that plasma levels of APA do not accurately reflect the level of APA expression in the tissues and may explain why early studies concluded APA levels were static. Second, APA (i.e., BP-1/6C3) has been well characterized as being developmentally regulated in lymphocytes (32). Finally, here we have shown that the organization of the APA gene is unlike that of a housekeeping gene. Whereas APA is clearly regulated at the transcriptional level in B cells, it has not yet been determined whether the APA gene in other tissues is regulated principally at the level of transcription. Cell-specific expression of the APA gene probably involves the strong USA region within the 5′-flanking region. The transfer of this suppressor activity to a heterologous promoter and the fact that constructs containing larger portions of the 5′-flanking region are able to overcome the suppression together suggest that an interaction between multiple cis-acting elements and their cognate trans-acting factors plays a critical role in regulating expression of the APA gene.

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