Alternative splicing of the proadrenomedullin gene results in differential expression of gene products

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ABSTRACT

The adrenomedullin (AM) gene codifies for two bioactive peptides, AM and proAM N-terminal 20 peptide (PAMP). We have found two forms of the AM mRNA. Form A is devoid of introns and results in a prohormone containing both peptides. Form B retains the third intron, which introduces a premature stop codon, producing a shorter prohormone with only PAMP. Tissues with a higher B/A ratio were more immunoreactive for PAMP than for AM. The form B message was found in the cytoplasmic compartment, thus excluding that the longer message was a result of contaminating nuclear mRNA. Form B was found in cells that express PAMP but not AM. mRNA expression in a variety of cell lines was investigated by ribonuclease protection assay and form B was found in significant amounts in two of them. Treatments that modify AM expression, such as exposure to hypoxia, were shown to change the B/A ratio and the relative secretion of AM and PAMP, indicating that the splicing mechanism for AM can be modulated and is physiologically relevant. Analysis of the sequence of the third intron and the fourth exon of the AM gene found motifs compatible with a highly regulated alternative splicing mechanism.

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INTRODUCTION

Adrenomedullin (AM) is a 52 amino acid peptide involved in many physiological actions as varied as hypotension (Nuki *et al*. 1993), bronchodilation (Kanazawa *et al*. 1994), renal function (Jougasaki *et al*. 1995), hormone regulation (Samson *et al*. 1995, Martínez *et al*. 1996), neurotransmission (Allen & Ferguson 1996) and growth (Miller *et al*. 1996, Whiters *et al*. 1996), among others.

The gene for human AM has been cloned and sequenced (Ishimitsu *et al*. 1994). It contains four exons and three introns which, through maturation of the message and cleavage of the introns, generates a 1·6 kb mRNA. After translation, a 181 amino acid long preprohormone is produced which, after post-translational modifications occur, originates two active α-amidated peptides: AM and proAM N-terminal 20 peptide (PAMP) (Fig. 1).

Given this scenario, equimolar amounts of AM and PAMP should be expected in the cells and tissues expressing the AM gene. On the contrary, several reports that quantified both molecules by RIA (Ichiki *et al*. 1994, Sakata *et al*. 1994, Washimine *et al*. 1994, Insatsu *et al*. 1996) found very variable PAMP/AM ratios in different organ extracts. For example, the ratios for human and rat lung (2·5 and 0·4% respectively) were very low compared with the ratio in the adrenals (29% human, 41% rat) or the heart atrium (45% in the rat). It has been reported that PAMP is rapidly cleaved by neutral endopeptidases (Nagatomo *et al*. 1996) but, although this may explain the low levels of PAMP when compared with AM, the variation in the ratios suggests that some differential regulation in the expression of AM and PAMP may occur.

Another intriguing series of observations that suggests an alternative splicing of the AM gene involves the localization of AM and PAMP immunoreactivities in different cell types of the same organ. This has been reported in the prostate, where AM is found homogeneously distributed

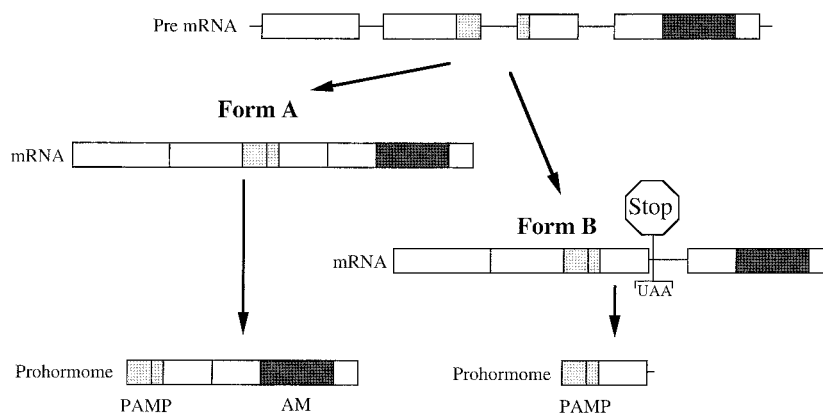Online version via http://www.endocrinology.org

FIGURE 1. Schematic drawing of the alternative splicing mechanism that results in the generation of two different prohormones. The freshly transcribed mRNA contains four exons and three introns. The removal of all the introns results in the form A of the mature mRNA, which in turn is translated into a long prohormone containing both PAMP and AM. If intron 3 is not cleaved off, a longer mRNA is produced (form B), but the presence of a stop codon in the intron results in a smaller prohormone that only contains PAMP.

throughout the epithelium whereas PAMP appears exclusively in cells of the diffuse endocrine system (Jiménez *et al.* 1999). Another striking example is found in the kidney. In this organ AM is expressed by the proximal convoluted tubules and the collecting ducts. PAMP, on the other hand, is localized only in the juxtaglomerular granular cells, co-stored with renin (López *et al.* 1999). A recent report has demonstrated that in the pituitary, AM has a very diffuse staining pattern throughout the anterior lobe, while PAMP has a more restricted expression in the gonadotropes (Montuenga *et al.* 2000).

In our previous studies (Martínez *et al.* 1995, 1997, Miller *et al.* 1996, Montuenga *et al.* 1997) we reported the presence of a double band after RT-PCR analysis of both human and rodent tissues and cell lines, and suggested that it may reflect the existence of alternatively spliced mRNA.

The present study was designed to explore whether there exists alternative splicing in the maturation of the AM mRNA and, if so, what are the implications for the regulation of AM and PAMP expression.

MATERIALS AND METHODS

Cell lines and RNA extraction

Cell lines used in this study represent an array of the most widely distributed human cancer cell types as models. We used representatives of carcinomas (CA) or carcinoids of the lung (N417 (small cell CA), H1264 (adenoCA), H157 (squamous cell CA),

A549 (bronchioalveolar CA) and H720 (carcinoid)), breast (MCF7, ZR75 and H2380), colon (H716, H630 and SNUC-1), prostate (DU 145 and LNCap), ovary (SKOV3 and OVCAR3), liver (Hep 3B and Hep 62) and a chondrosarcoma (HTB-94). The cell lines HMC-1 (mast cell), KU 812 (basophil) and CRL-7922 (skin) were used as examples of non-tumoral cells. All cell lines were obtained from the National Cancer Institute, Navy Medical Oncology Branch, or purchased through the American Type Culture Collection (ATCC, Manassas, VI, USA). Cell lines were cultured in RPMI 1640 or DMEM media supplemented with 10% heat-inactivated fetal bovine serum (all tissue culture reagents purchased from Life Technologies, Gaithersburg, MD, USA). Cells were cultured at 37 °C in 20% O_2, 5% CO_2, 75% N_2 for normoxic conditions. The mRNA was extracted with the Micro-Fast Track kit (Invitrogen, San Diego, CA, USA) following the manufacturer's specifications, or by the guanidine isothiocyanate and cesium chloride method as reported (Garayoa *et al.* 2000). Human tissue mRNAs were purchased from Clontech (Palo Alto, CA, USA).

Cell treatments

The small cell lung CA cell line N417 was treated for 24 h with the following reagents: 10 ng/ml recombinant human tumor necrosis factor (TNF)-α (R&D Systems, Minneapolis, MN, USA), 50 ng/ml phorbol ester (PMA) (Sigma Chemical Co., St Louis, MO, USA), 0·1 µM N-(4-hydroxyphenyl)-retinamide (4 HPR) (Sigma), and 4 µg/ml dexamethasone (Elkins-Sinn, Cherry Hill, NJ, USA).

Oxygen deprivation has been shown to induce AM expression through the hypoxia-inducible factor-1 pathway (Garayoa *et al*. 2000) and was also applied to N417. Cells were subjected to an atmosphere of 1% O_2, 5% CO_2, 94% N_2 in a hypoxia chamber at 37 °C. After 24 h the cells were dissociated in guanidine isothiocyanate and the RNA isolated as above. The conditioned medium from these experiments was analyzed by RIA (see below). Exposure to TNF-α and hypoxia was done in the presence or absence of 10^{-5} M phosphoramidon (Sigma).

Cell fractionation

In selected cell lines, separation of cytoplasmic and nuclear fractions was performed by selective lysis with non-ionic detergents, following established protocols (Davis *et al*. 1994). The RNA from these fractions was purified as above.

RT-PCR and Southern blot

Reverse transcription was performed with the SuperScript Preamplification System (Life Technologies) following the manufacturer's instructions. The sequence of the primers used for PCR has been previously published (Martínez *et al*. 1996) and was based on the AM cDNA sequence (accession no. D14874). Here, the numbers for the nucleotides correspond to the AM human genomic DNA entry in GenBank (accession no. D43639). The sense primer (AM 2389–2413+2605–2619: 5′-AAG-AAG-TGG-AAT-AAG-TGG-GCT-3′) anneals into the beginning of the third exon, while the antisense primer (AM3122–3103: 5′-TGT-GAA-CTG-GTA-GAT-CTG-GT-3′) is located in the fourth exon of the AM gene, with an expected product of 293 bp if the third intron is cleaved and 524 bp if it is retained. All buffers, enzymes and nucleotides used for PCR were obtained from Applied Biosystems (Perkin Elmer/Cetus, Norwalk, CT, USA). After 1 min of denaturation at 94 °C, 35 cycles of 94 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min, were performed in a 9600 GeneAmp thermocycler (Perkin Elmer). PCR products were run in 1% agarose gels, transferred into nitrocellulose by capillary flow, and subjected to Southern analysis with an internal probe (AM3030–3010: 5′-TCT-GGC-GGT-AGC-GCT-TGA-CTC-3′) end-labeled with ^{32}P by standard methods.

Cloning and sequencing

The PCR products were cloned into a pCR II vector (Invitrogen) according to the manufacturer's instructions. Top 10 competent cells (Life Technologies) were transformed and plated into agar dishes containing X-gal and ampicillin. Plasmids from white colonies were purified by Insta-mini-preps (5 prime-3 prime, Inc., Boulder, CO, USA) and analyzed by restriction digestion. The proper clones were grown in larger volumes and plasmids purified by Qiagen maxiprep kit (Qiagen, Inc., Chatsworth, CA, USA). After a further cleaning with Wizard DNA Clean-up System (Promega, Madison, WI, USA), the plasmids were sent to Sequetech (Mountain View, CA, USA) to be sequenced.

Immunohistochemistry

Paraffin blocks containing normal human tissues were obtained from the Cooperative Human Tissue Network (Philadelphia, PA, USA). Tissue procurement was approved by the National Institutes of Health's Office of Human Subjects Research. Sections were subjected to the avidin–biotin complex immunohistochemical method as previously described (Montuenga *et al*. 1997). Briefly, after an overnight incubation with the primary antibody at optimal concentration (AM 1:1000; PAMP 1:900), the sections were incubated with biotinylated goat anti-rabbit immunoglobulin (1:200) (Vectastain, Burlingame, CA, USA) and then with avidin–biotin peroxidase complex (1:100) (Vectastain). The bound antibodies were visualized using diaminobenzidine (Sigma) and H_2O_2. Sections were lightly counterstained with hematoxylin. Pre-incubation of the antisera with 10 nmol/ml of the proper antigen was used as a negative control.

In situ hybridization

A probe for the third intron was generated from H720 genomic DNA by PCR using as primers: sense (AM2755–2775 5′-TAA-CTA-CGC-CCT-GTG-CTG-TCC-3′) and antisense (AM2986–2966 5′-CTG-CGG-GCG-GGG-GGA-GGG-GGA-3′). The 233 bp product was cloned into pCR-Blunt II-TOPO (Invitrogen) and sequenced to confirm amplification fidelity. The plasmid was linearized with either EcoRV or BamHI and transcribed with SP6 or T7 RNA polymerases in the presence of digoxigenin-11-dUTP (Boehringer-Mannheim, Gaithersburg, MD, USA) to generate labeled sense and antisense probes. The probes were hybridized to paraffin-embedded human prostate sections following previously published protocols (Jiménez *et al*. 1999). Alternatively, the digoxigenin-labeled probe was detected with a specific monoclonal antibody, followed by a fluorescein-donkey anti-mouse conjugate (Jackson

ImmunoResearch, West Grove, PA, USA). After hybridization, the antibody against PAMP or AM was applied and its presence detected with Texas Red-goat anti-rabbit (Jackson ImmunoResearch). The localization of the signals was accomplished with a confocal microscope (Zeiss LSM 510, Thornwood, NY, USA).

RNase protection assay

A probe bridging parts of intron 3 and exon 4 was prepared by PCR with the following primers: sense (AM2891–2910 5′-TCT-GGC-TCT-AGA-ATG-GCT-CC-3′) and antisense (AM3253–3233 5′-CGT-GTG-CTT-GTG-GCT-TAG-AA-3′). The PCR product obtained from the cell line H720 genomic DNA was cloned into pCR-Blunt II-TOPO and sequenced to confirm sequence fidelity. The plasmid was linearized with BamHI and an antisense probe was generated with T7 RNA polymerase using MAXIscript (Ambion, Austin, TX, USA). The RNase protection assay was performed with the RPA II kit from Ambion, following the manufacturer's instructions. A probe for the 18S rRNA subunit (Ambion) was also transcribed and added together with the AM probe to standardize loading efficiency. The protected products were separated by size on 6% denaturing polyacrylamide gels. Gels were dried under vacuum at 80 °C for 2 h and placed into phosphor cassettes and exposed for 16–72 h. The images were visualized using PhosphorImager SI analysis and evaluated with ImageQuaNT (Molecular Dynamics, Sunnyvale, CA, USA). The expected sizes for the AM protected fragments are 362 nt if the intron is retained and 266 nt if it is not. The protected fragment for the 18S rRNA is 80 nt long.

RIAs

The AM and PAMP contents of N417 supernatants were evaluated by RIA. Our protocol for the AM RIA has been extensively published (Martínez *et al.* 1997, 1999, Garayoa *et al.* 2000). The PAMP RIA was performed using a kit from Phoenix (Mountain View, CA, USA), following the manufacturer's instructions.

RESULTS

RNA samples from different normal human tissues were reverse transcribed and amplified by PCR with the AM primers, followed by Southern analysis with an internal probe (Fig. 2). As previously reported (Martínez *et al.* 1995, 1997, Miller *et al.*
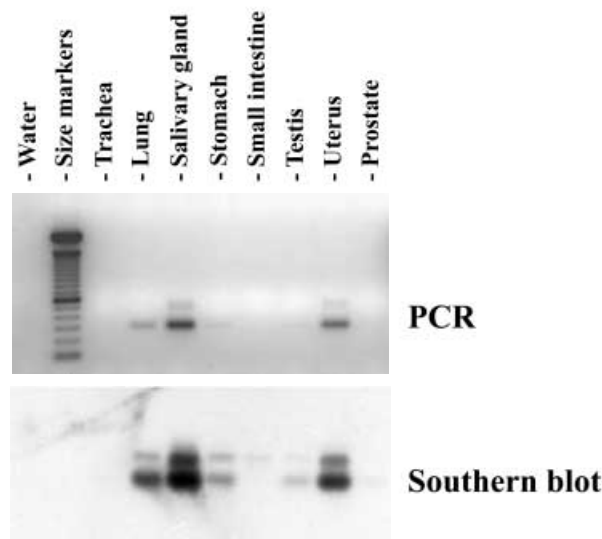

FIGURE 2. RT-PCR amplification for AM in RNA extracts from several human organs. The double bands can be seen in the ethidium bromide stained gel (upper panel), but are much more prominent after Southern blotting with an internal probe (lower panel).

1996, Montuenga *et al.* 1997), the PCR reaction gave two bands of 293 and 526 bp respectively. Both bands from the salivary gland PCR reaction were cloned and sequenced. The smaller band had the expected size, and sequence analysis demonstrated that it contained the exact sequence reported by Kitamura *et al.* (1993) for human AM cDNA. Sequence analysis of the upper band revealed that it contained the third intron of the gene (233 bp), which apparently is not cleaved off in a proportion of the molecules of AM mRNA, resulting in a longer form of the message. The implications of this insertion for the resulting protein are very relevant. When the longer mRNA species is translated into protein, the first 82 amino acids remain unchanged, but the serine in position 83 is substituted by an arginine and immediately after that, a nonsense codon stops the translation of the mRNA. This means that the longer mRNA (form B) produces a shorter preprohormone which only contains the sequence for PAMP but not for AM (Fig. 1). The upper band was not the result of genomic contamination since PCR performed in the same samples without reverse transcription failed to produce any band (results not shown).

Analysis of the intensity ratio of both bands in different tissues shows a great degree of variation (Fig. 2), suggesting an organ-dependent regulation of the proportion of either spliced form. In most tissues, form A (shorter mRNA codifying for both
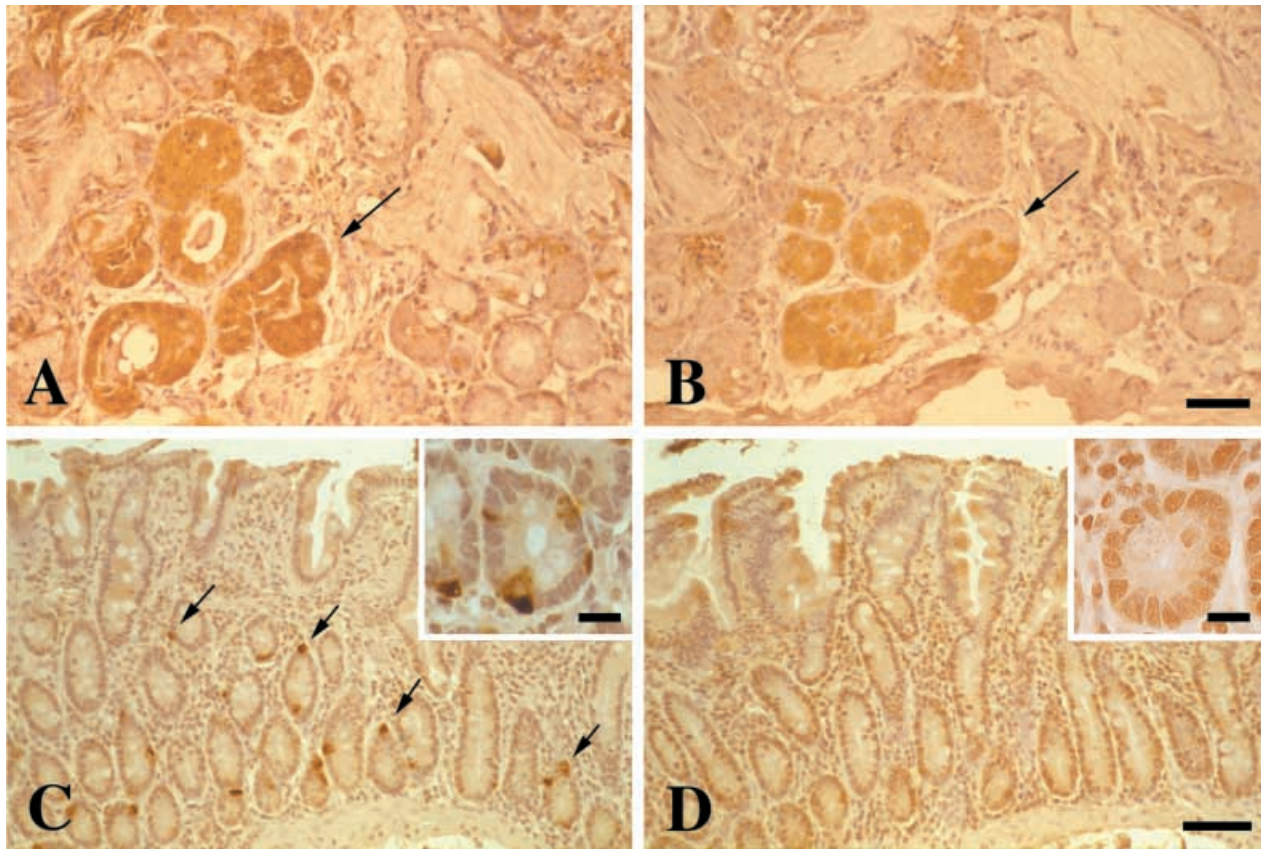
FIGURE 3.   Immunohistochemical staining for PAMP (A, C) and AM (B, D) in serial sections of human salivary gland (A, B) and duodenum (C, D). Insets in (C) and (D) are higher magnifications of comparable areas. A PAMP-positive endocrine cell can be easily recognized in (C). Both peptides are highly expressed by the salivary gland secretory units (arrows) whereas only PAMP (short arrows) is found in the intestine. (A, B) bar=30 μm; (C, D) bar=50 μm; insets, bar=10 μm.

peptides) is more abundant than form B, but there are examples as in the case of the small intestine in which this relationship is reversed. In the case of the prostate, only form A is apparent in Fig. 2, although overexposure of the Southern blot showed a light band for form B as well (results not shown).

If the previous hypothesis is true, there must be a correlation between the band pattern and the protein expression in these tissues. To demonstrate this, immunohistochemical staining with specific antibodies against AM and PAMP was performed in serial sections of paraffin-embedded human tissues. The salivary glands, which express both forms of the message, were immunoreactive for both peptides (Fig. 3A and B). On the other hand, the small intestine, which is especially rich in form B, had a strong positivity for PAMP in the cells of the epithelial diffuse endocrine system (Fig. 3C), but was negative after application of the anti-AM antibody (Fig. 3D).

To ensure that the presence of longer species of proAM mRNA was not a result of contamination from immature nuclear RNA, cell fractionation was performed in the cell lines, H510 and H720. After RT-PCR, the two PCR bands were observed in both the nuclear and the cytoplasmic fractions with the cytoplasmic band being more intense, indicating that the longer form is translocated into the cytoplasm (Fig. 4). To further demonstrate the presence of the form B mRNA in the cytoplasm, we prepared a riboprobe containing exclusively the third intron of the AM gene and performed *in situ* hybridization in prostate tissue sections. The antisense intron probe detected a few endocrine cells scattered throughout the epithelium of the central glands and the utriculus (Fig. 5A), while the sense probe gave a negative signal, therefore confirming the specificity of the reaction (Fig. 5B). In addition, consecutive sections stained with anti-PAMP antibody showed a similar pattern to
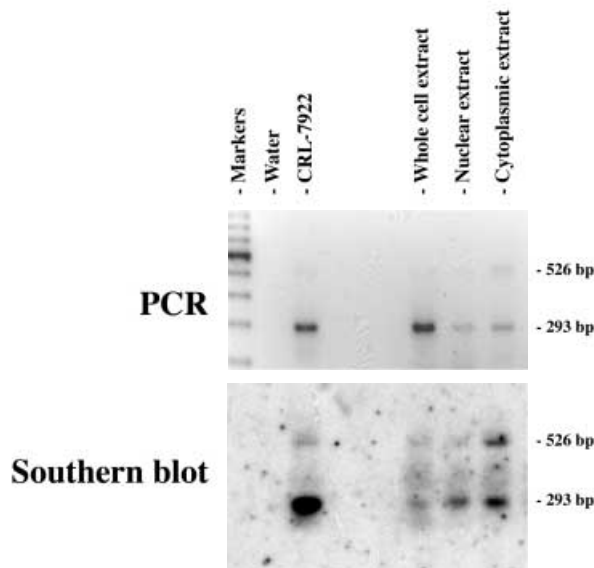
FIGURE 4.   The cell line H510 was fractionated and RNA from the nuclear and cytoplasmic fractions was extracted and subjected to RT-PCR with AM primers. The skin cell line, CRL-7922 was included as a positive control. The 526 bp band is visible in the cytoplasmic extract both by ethidium bromide staining (upper panel) and after hybridization with an internal probe (lower panel). To determine the size of the PCR products a 100 bp DNA ladder was used.

the intron-containing cells although, given the small size of the endocrine cells, it was difficult to confirm the co-localization of both signals (Fig. 5C). AM staining was negligible in this area (Fig. 5D), although it was clearly found in other areas of the prostate, including the peripheral glands, the urethra, ejaculatory ducts and squamous glands (for a complete description of the anatomical distribution of AM and PAMP in prostate, see Jiménez et al. 1999). To ensure that the cells showing PAMP immunoreactivity are the same as those that contain transcripts for the third intron in their cytoplasm, double immunofluorescence followed by confocal microscopy was performed. The digoxigenin-labeled riboprobe was demonstrated in green (Fig. 5F) whereas the antibody against PAMP (Fig. 5E and H) was labeled in red. Superposition of both signals (Fig. 5G) demonstrates the co-localization of both labels.

When RNAs from different cell lines were analyzed by RNase protection assay, the existence of the alternatively spliced RNA was confirmed. In particular cell lines (CRL-7922 and N417), these assays showed a protected band of 362 nt, consistent

with the retention of the third intron, in addition to the lower 266 nt band, corresponding to the intronless species. Comparison of different cell lines shows also that the basal levels of proAM gene expression are variable (Fig. 6).

The differential expression of both bands in the various cell lines suggests that there may be a regulatory process by which cells are able to modulate their ratio of secreted AM/PAMP. To investigate this, we subjected the cell line N417 to a variety of stimuli that have been previously shown to affect the expression levels of proAM mRNA (Cuttitta et al. 1998, Garayoa et al. 2000). Treatments with 4 HPR and hypoxia resulted in a marked elevation of AM mRNA expression, this elevation being more prominent for the second treatment. On the other hand, exposure to PMA reduced the expression of proAM mRNA, whereas dexamethasone did not greatly modify the overall expression levels (Fig. 7). More interestingly, variations were observed in the ratios between both forms of the message. Cells treated with TNF-α showed a clear diminution in the upper band while the lower band remained with a similar intensity to the control. Conversely, in cells exposed to 1% O_2 for 24 h the upper band's increase in intensity was higher than the increase in the intensity of the lower band. After quantifying the bands, the B/A ratios are 0·68 for the TNF-α treatment and 1·85 after hypoxia. These ratios, compared with 1·33 for the control, clearly point to the existence of a cellular mechanism that can regulate the balance between both messages. To investigate whether this variation at the mRNA level has a real impact in the secretion of AM and PAMP from the cell, supernatants from the cell line N417 were analyzed by RIA after being treated with TNF-α and hypoxia for 24 h. The results of this evaluation are presented in Table 1. The experiment was done in the presence or absence of 10^{-5} M phosphoramidon, an inhibitor of neutral endopeptidases (Nagatomo et al. 1996). No apparent change in morphology or behavior due to the addition of the inhibitor was observed in this cell line at 24 h. In the presence of the inhibitor, the PAMP/AM ratios are consistent with the B/A ratios described above for the mRNA forms, although there are not enough data for a statistical comparison, given that only one RNase protection assay was performed. Nevertheless, both ratios tend to be lower than the control after treatment with TNF-α and both get higher after hypoxia. On the other hand, the PAMP/AM ratios in the absence of phosphoramidon were confounded by the activity of the degrading enzymes, with AM being more stable and PAMP rather labile.

DISCUSSION

The present study demonstrates that the AM gene produces two different mRNAs by alternative splicing, which in turn code for two different preprohormones; one containing both AM and PAMP and the other with only PAMP (Fig. 1). We propose naming the originally reported mRNA form A and the longer message form B. In addition, there is a good correlation between the pattern of expression of both messages and the protein expression pattern observed by immunohistochemical and RIA methods.

We are beginning to realize that alternative splicing appears to be a common mechanism for regulating gene expression. In fact, a recent study indicates that up to 38% of human mRNAs contain possible alternative splice forms (Bretta *et al*. 2000). The usual splicing mechanism consists in exon shuffling and several regulatory peptides, including calcitonin gene-related peptide, gastrin-releasing peptide, and the pro-opiomelanocortin family, are produced in this fashion (Sausville *et al*. 1986, Garrett *et al*. 1989, Lou & Gagel 1998). Intron retention is not the most common mechanism for alternative splicing, but many references can be found in the literature describing this process (Lupetti *et al*. 1998, Unsworth *et al*. 1999, Moutsaki *et al*. 2000, Weiss *et al*. 2000). In the AM gene, the retention of the third intron creates a truncated preprohormone because it introduces a premature in-frame termination codon. This phenomenon has also been reported for a number of genes (Pollard *et al*. 1998, Van Heumen *et al*. 1999, Whittock *et al*. 1999, Wistow *et al*. 2000, Xie *et al*. 2000).

In other systems, it has been shown that expression modulators can induce differently the various spliced forms. For instance, in liver cells, addition of PMA induces production of the soluble interleukin-1 receptor accessory protein mRNA over the membrane-bound alternative (Jensen *et al*. 2000). We studied the impact of different treatments known to influence AM expression. Hypoxia was shown to elevate the B/A and the PAMP/AM ratios, whereas TNF-α seemed to reduce both the B/A ratio and the ratio of PAMP/AM secretion, although this failed to reach statistical significance. These facts reveal the existence of a selective mechanism that controls the removal of the third intron in the AM gene.

In general, the splicing process is regulated by a number of soluble proteins called splicing factors (Manley & Tacke 1996, ten Dam *et al*. 2000) that recognize consensus sequences in the gene and which are cell type specific. We have also seen a clear variation between cell types in our model that

may indicate an involvement of the soluble splicing factors in the regulation of proAM mRNA processing.

A critical step in the splicing process appears to be the initial recognition of the intron's 5′- and 3′-splice sites by the splicing apparatus (Shapiro & Senapathy 1987). The 5′-consensus sequence is GURAGU, whereas the 3′-consensus contains a branchpoint sequence (YNRAY) and a polypyrimidine tract, followed by YnNCAG (Seong *et al*. 1999). The analysis of the third intron of the AM gene reveals the presence of a mismatch on the 5′-splice site (GUAAcU) and another one on the branchpoint sequence (UCAAg). These features may explain the lower efficiency of the splicing apparatus on cleaving intron 3 in the AM gene. The other introns of the gene contain sequences that are perfectly compatible with the consensus sequences, with the only exception of intron 1, that contains a single mismatch in the 5′-splice site (GUAgGU).

Another feature that influences splicing performance is the presence of purine-rich sequences, known as exonic splicing enhancers. These regions lie within exons and facilitate cleavage of the upstream intron (Tanaka *et al*. 1994, Seong *et al*. 1999). Although there are some short purine-rich regions in the second and third exons of the AM gene, the longest one is situated in the fourth exon (ACAGAUAAGGACAAGGACAACG), 133 bp downstream of the intron/exon boundary. Enhancers are usually found associated with introns that are considered to be weak (poor matches to the consensus) and that are frequently subject to alternative splicing (Manley & Tacke 1996). This fact further supports the notion of the third AM intron being a likely candidate for splicing regulation.

To see whether this is a conserved feature in different species, we also studied the structure of intron 3 in the mouse AM gene (GenBank accession no. D78349). We found a mismatch in the 5′-consensus sequence (GUAAcU instead of GUAAGU) that may be also responsible for a partial splicing of the intron. An exonic splicing enhancer was also found in the fourth exon of the mouse gene. As with the human gene, if the third intron of the mouse AM gene is retained, an arginine and a stop codon are introduced before the AM sequence and only PAMP is produced.

The existence of two forms of AM mRNA helps in understanding the differential expression of AM and PAMP reported in many tissues (Ichiki *et al*. 1994, Sakata *et al*. 1994, Washimine *et al*. 1994, Insatsu *et al*. 1996, Jiménez *et al*. 1999, López *et al*. 1999, Montuenga *et al*. 2000). Cells expressing a higher proportion of the longer message have a
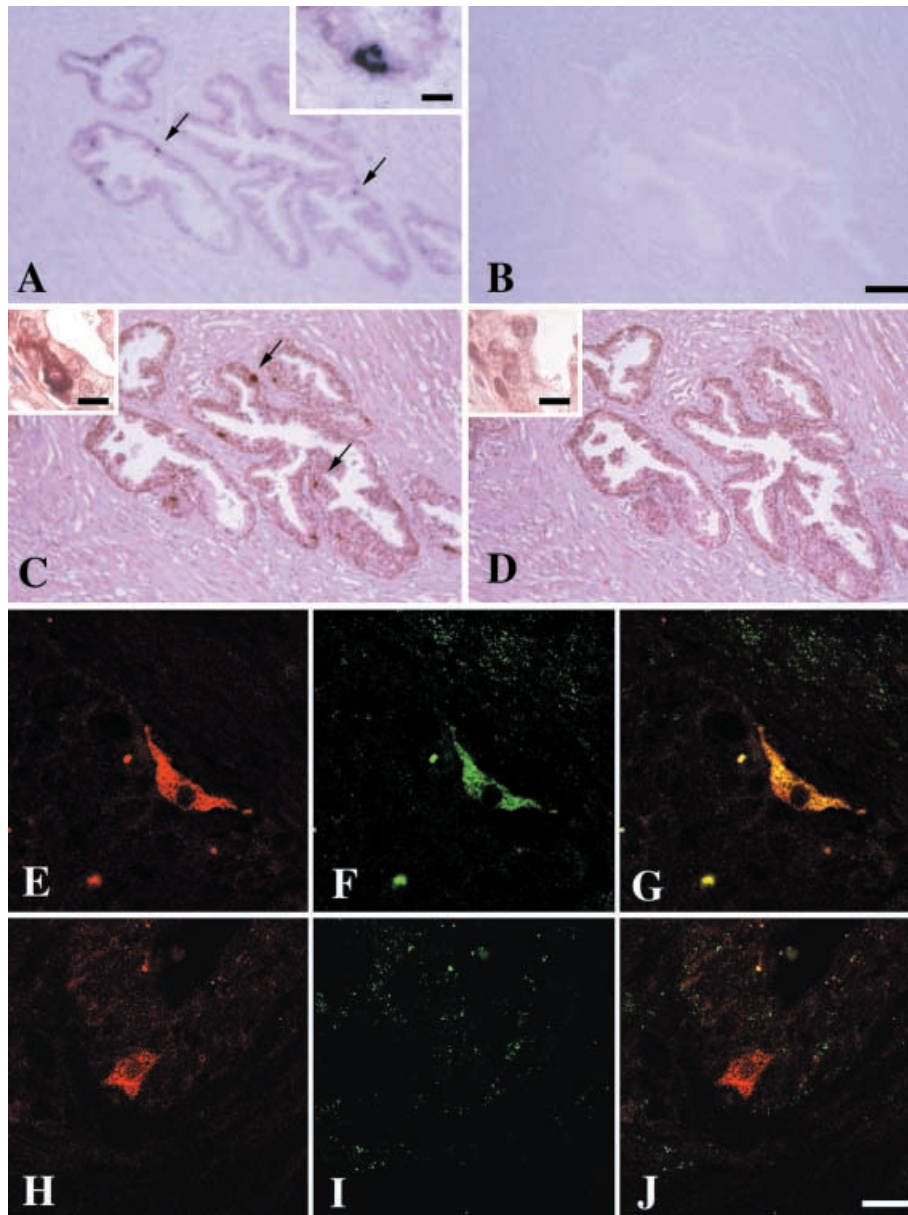
FIGURE 5.   Serial sections of human prostate stained by *in situ* hybridization with the antisense probe for the third intron (A), the sense probe as a negative control (B), and by immunohistochemistry with antibodies against PAMP (C) and AM (D). The inset in (A) is a higher magnification of one of the positive cells (arrows) to appreciate the cytoplasmic staining. Insets in (C) and (D) are also higher magnifications of the glandular epithelium. A PAMP-positive endocrine cell can be observed in (C). (E–J) Confocal microscopy of the same organ. PAMP immunoreactivity is detected in red (E, H), and the digoxigenin-labeled riboprobe in green (F). The sense probe (I) did not produce any significant staining. The last column (G, J) represents a composite of the previous ones. Combination of red and green results in yellow. (A–D) bar=100 μm, insets bar=10 μm; (E–J) bar=10 μm.

higher PAMP/AM secretion ratio. This idea was corroborated by the histochemical staining, the localization by *in situ* hybridization of the intron transcript in the cytoplasm of cells known to contain PAMP but not AM, and by analyzing the contents of PAMP and AM in the supernatant of cells with artificially modified B/A levels. Interestingly, when AM and PAMP levels are monitored in tissue extracts or culture supernatants, the AM contents are always higher (Ichiki *et al*. 1994, Sakata *et al*.
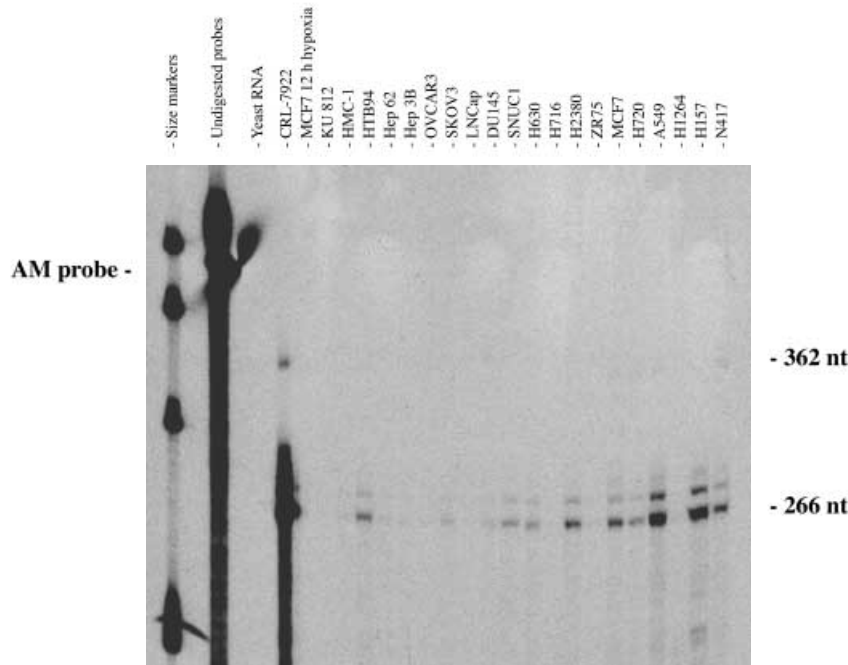
FIGURE 6.   Ribonuclease protection assay in a variety of human cancer cell lines. The protected fragment of 266 nt corresponds to the form A of the message (intronless), whereas the 362 nt can only be obtained when the third intron remains.

1994, Washimine *et al*. 1994, Insatsu *et al*. 1996). This observation may seem in conflict with our findings that would predict higher PAMP levels, but in fact it reflects the existence of additional levels of regulation. One of the strategies leading to higher circulating AM levels is its resistance to degradation by neutral endopeptidases in contrast with the high susceptibility of PAMP (Nagatomo *et al*. 1996). Another factor contributing to this difference may be the existence of an AM-binding protein in the serum that may protect AM from degradation and/or clearance (Elsasser *et al*. 1999). This serum binding protein has been recently identified as complement factor H (Pío *et al*. 2001).

The study of the alternative splicing of the AM gene in particular organs may be obscured by their complex anatomy. A good example is the prostate. In this organ, AM is widely expressed throughout the epithelial surfaces whereas PAMP is expressed by only a few scattered cells in particular regions of the gland (Jiménez *et al*. 1999). This morphological pattern can only be appreciated by careful immuno-histochemical or *in situ* hybridization analysis. When the prostate is homogenized and studied by molecular means (Fig. 2), a dilution effect takes

TABLE 1. RIA values for AM and PAMP in the supernatant of the cell line N417 exposed to different treatments for 24 h in the presence or absence of the neutral endopeptidase inhibitor, phosphoramidon. Values are the means ± S.D. of three determinations

	Without inhibitors				**Phosphoramidon** (10^{-5} M)			
	AM (fmol/ml)	PAMP (fmol/ml)	PAMP/AM ratio	*P* value[a]	AM (fmol/ml)	PAMP (fmol/ml)	PAMP/AM ratio	*P* value[a]
Control	2·5 ± 0·23	0·85 ± 0·12	0·34 ± 0·04	—	2·7 ± 0·15	1·10 ± 0·24	0·41 ± 0·05	—
TNF-α	3·0 ± 0·13	1·22 ± 0·08	0·41 ± 0·02	0·074	2·2 ± 0·26	0·65 ± 0·16	0·30 ± 0·05	0·052
Hypoxia	23·0 ± 1·42	6·79 ± 0·47	0·30 ± 0·01	0·068	21·0 ± 0·95	11·87 ± 1·30	0·57 ± 0·04	<0·001

[a]Compared with control. *P*<0·05 was considered statistically significant.
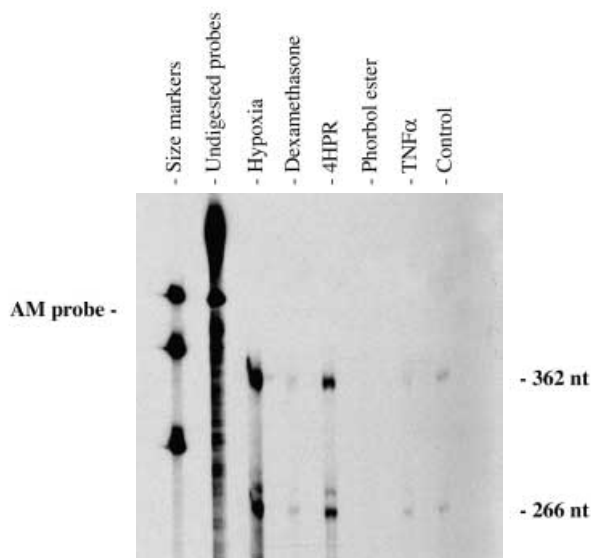
FIGURE 7. Ribonuclease protection assay in RNA obtained from the cell line N417 after exposure for 24 h to the indicated treatments. Hypoxia and 4 HPR clearly elevated overall expression whereas PMA reduced it.

place and the form A of the message, which is more abundant in the prostate as a whole, tends to obliterate the presence of form B that is expressed only by a few cells.

Although there are many more studies on AM physiology than on PAMP functions, in most cases both peptides seem to act in parallel. They elevate blood pressure when injected into the brain and reduce it when provided intravenously. They are both bronchodilators, and reduce release of several hormones including adrenocorticotropin, aldosterone and epinephrine (Samson 1999). On the other hand, they elicit these activities through different signal transduction pathways, possibly as a fail-safe redundant mechanism. AM acts through a cholera toxin-sensitive G-protein whereas PAMP signals are mediated by a pertussis toxin-sensitive mechanism (Samson 1999). Modulation of the levels of PAMP/AM secreted by a particular cell type would therefore influence the kind of signal received by the target cells.

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