Volume 305, number 1, 45–50 FEBS 11187 June 1992

Structure and expression of *Xenopus* prohormone convertase PC2

Joanna A.M. Braks, Karin C.W. Guldemond, Maarten C.H.M. van Riel, Anton J.M. Coenen and Gerard J.M. Martens

Department of Animal Physiology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands

Received 24 April 1992

The multifunctional prohormone, proopiomelanocortin (POMC), is processed in the melanotrope cells of the pituitary pars intermedia at pairs of basic amino acid residues to give a number of peptides, including α-melanophore-stimulating hormone (α-MSH). This hormone causes skin darkening in amphibians during background adaptation. Here we report the complete structure of *Xenopus laevis* prohormone convertase PC2, the enzyme thought to be responsible for processing of POMC to α-MSH. A comparative structural analysis revealed an overall amino acid sequence identity of 85–87% between *Xenopus* PC2 and its mammalian counterparts, with the lowest degree of identity in the signal peptide sequence (28–36%) and the region amino-terminal to the catalytic domain (59–60%). The occurrence of a second, structurally different PC2 protein reflects the expression of two *Xenopus* PC2 genes. The expression pattern of PC2 in the *Xenopus* pituitary gland of black- and white-adapted animals was found to be similar to that of POMC, namely high expression in active melanotrope cells of black animals. This observation is in line with a physiological role for PC2 in processing POMC to α-MSH.

Prohormone convertase; Proopiomelanocortin; Background adaptation; cDNA sequence; *Xenopus* pituitary

1. INTRODUCTION

In the melanotrope cells of the pituitary pars intermedia of the amphibian *Xenopus laevis* the rate of production of the prohormone, proopiomelanocortin (POMC), is dependent on the color of the background on which the animal is placed. On a black background the melanotrope cells produce large amounts of POMC, while on a white background POMC gene expression is at a low level [1]. We use the *Xenopus* melanotrope cell as a model system to study regulatory mechanisms involved in the biosynthesis and secretion of biologically active peptides. In the melanotrope cells POMC is processed to a number of bioactive peptides, including α-melanophore-stimulating hormone (α-MSH) [2]. After secretion into the blood this hormone stimulates the dispersion of pigment granules in the melanophores of the skin, thus impairing a dark color to the animal. Processing of POMC, and of most other prohormones, occurs at pairs of basic amino acid residues which flank the bioactive peptides on both sides [3]. Until recently, the specific proteolytic enzymes responsible for such prohormone processing have remained elusive. Based on the homology with the endoprotease, KEX2, from yeast, three putative mammalian proprotein-cleaving enzymes, namely furin, and the prohormone convertases, PC1 (also referred to as PC3) and PC2, have been characterized by cloning and sequencing of their cDNAs [4–9]. Recently, two additional members of this family of subtilisin-related serine proteases have been cloned, and they were designated PC4 and PACE4 (PACE for Paired basic Amino acid residue Cleaving Enzyme) [10,11]. Furin appeared to be present in all mammalian cell types studied thus far, and it seems to be responsible for the proteolytic cleavage of proproteins for constitutively secreted proteins. PC1 and PC2, but not furin, are coordinately regulated with POMC, show tissue-specific expression, and are believed to be involved in the processing of prohormones at pairs of basic amino acid residues in the regulated secretory pathway of neurons and endocrine cells [12,13]. Recombinant vaccinia virus expression studies revealed that PC1 and PC2 are both capable of cleaving POMC. The specificity of the processing, however, appeared to be different for the two enzymes [14,15]. PC1 showed a preference for two of the five dibasic amino acid residues present within the structure of POMC, resulting in the production of adrenocorticotropin (ACTH) and β-lipotropin (β-LPH). In contrast, PC2 was found to recognize all five pairs generating α-MSH by further cleavage of ACTH, and β-endorphin from β-LPH. In view of the fact that α-MSH is of physiological importance in *Xenopus*, since it mediates the process of background adaptation, we were not only interested in the structure of *Xenopus* PC2, but also in obtaining physiological evidence for tissue-specific co-expression of PC2 with POMC in the pituitary of *Xenopus laevis*.

Correspondence address: G.J.M. Martens, Department of Animal Physiology, University of Nijmegen, Toernooiveld, 6525 ED Nijmegen, The Netherlands. Fax: (31) (80) 553 450.

2. MATERIALS AND METHODS

Poly(A)$^+$ RNA was isolated with guanidineisothiocyanate and oligo(dT) cellulose from pituitary pars intermedia of *Xenopus laevis* adapted to a black background to increase the biosynthetic activity for production of POMC in the melanotrope cells. Using about 0.5 μg poly(A)$^+$ RNA as template a pars intermedia cDNA library was constructed in the vector λZAP-II (Stratagene). About 4,000 clones of this library were screened with a mixture of insert DNAs of human PC2 cDNA clone, pPC2, and mouse PC3 clone, pPC3, (kindly provided by Dr. D.F. Steiner, Chicago, IL, USA), labelled with ^{32}P by random priming according to standard procedures [16]. Screening was performed with replica nitrocellulose filters at 37°C in 6 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), 3 × Denhardt's solution, 0.1% SDS, 0.1% sodium pyrophosphate, 1 mM EDTA, 100 μg/ml salmon sperm DNA and 25% formamide. Hybridization-positive phage plaques were purified, and the recombinant pBluescript SK-phagemids were rescued from the bacteriophage (λZAP) clones by in vivo excision, according to the instructions of the manufacturer. Sequencing on both strands and with pBluescript subclones or specific primers was performed with single- and double-stranded DNA using T7 DNA polymerase and the dideoxy chain termination method [17]. For in situ hybridization, tissue was fixed en bloc in Bouin's fixative (70% picric acid, 25% formaldehyde, 5% acetic acid) for 16 h. Paraffin sections (5 μm) were pretreated with 0.1% pepsin in 0.2 N HCl at 37°C for 15 min, rinsed, post-fixed in 2% formaldehyde in PBS for 4 min, treated with 1% hydroxylammonium chloride in PBS for 15 min, rinsed with PBS and dehydrated. Hybridization (150 μl standard hybridization solution for each section) in the presence of 50% formamide was performed at 50°C for 16 h in a moist chamber. RNA probes were synthesized as run-off transcripts from linearized DNA of pBluescript *Xenopus* PC2 cDNA clone, XY2.1, with 350 μM digoxigenin-11-UTP and T7 RNA polymerase. Alkaline phosphatase-conjugated anti-digoxigenin antiserum was used for visualization of the hybridization signals. For PCR analysis, total RNA was extracted with Nonidet P40-containing buffer, and single-stranded cDNA was synthesized from total RNA using MLV reverse transcriptase (BRL). The cDNA template was amplified using 50 pmol each of primer 1 (5'-GGAGCTATGGTTAAAATGGC-3') and primer 2 (5'-AGCTCTTCTTTCTTGGACAT-3') for 30 cycles (93°C, 1 min; 60°C, 1.5 min; 70°C, 1 min; Perkin Elmer-Cetus Thermal Cycler) with Ampli-Taq DNA polymerase (Perkin Elmer-Cetus). Primer 1 corresponds to nucleotides 1,351–1,370, and primer 2 to nucleotides 1,340–1,859 of *Xenopus* PC2 cDNA clone, XY2.1 (Fig. 1). PCR products were run on a 1.5% agarose gel, the gel was blotted, and the nitrocellulose filter was hybridized under standard hybridization conditions [16] with *Xenopus* pars intermedia PC2 cDNA clone, XY2.1, as a probe. Quantification of the PCR products was performed by scanning of the autoradiograms with a laser densitometer (LKB-Pharmacia).

3. RESULTS

3.1. *Isolation and characterization of pars intermedia cDNA encoding* Xenopus *PC2*

Low-stringency screening of 4,000 clones of the *Xenopus* pituitary pars intermedia cDNA library with a mixture of human PC2 and mouse PC3 cDNA clones as probes resulted in the isolation of seven hybridization-positive clones. Fig. 1 shows the nucleotide sequence of the hybridization-positive cDNA clone with the longest insert (clone XY2.1 with an insert size of 2.2 kb) coding for a 639 amino acid protein (with a calculated M_r of 70,532 and isoelectric point of 5.8). From the high degree of identity between the amino acid sequence of the XY2.1 protein, and human, rat and mouse PC2 structures (Fig. 2) we conclude that XY2.1 cDNA encodes a *Xenopus* PC2 protein. Further analysis of the hybridization-positive cDNA clones revealed four clones (XU2.2, XS2.1, XX2.1 and XU2.1) corresponding to XY2.1, and two cDNA clones (XT2.1 and XT2.2) encoding a PC2 protein structurally different from the XY2.1 protein (95% nucleotide sequence identity; 98% amino acid sequence identity). None of the positive clones analysed was found to correspond to PC1/PC3.

3.2. *Comparison between* Xenopus *and mammalian PC2 structures*

The overall degree of amino acid sequence identity between the *Xenopus*, and human, rat and mouse PC2 structures is 87, 85 and 85%, respectively (Fig. 2), while on the nucleotide sequence level in the protein-coding regions in all three cases the identity is 75%. The amino acid sequence identity between the signal peptide of the *Xenopus* PC2 protein and the mammalian PC2 signal peptides is 28–36%. The catalytic domains (residues 122–413) and the regions carboxy-terminal to the catalytic domain (residues 414–639) show 95–97 and 88–90% identity, respectively, between *Xenopus* and mammalian PC2. In contrast, the portions of the PC2 proteins amino-terminal to the catalytic domain (residues 26–121) display only 59–60% sequence identity (Fig. 2).

3.3. *Expression of* Xenopus *PC2 mRNA*

To study the dynamics of PC2 expression in the *Xenopus* pituitary gland during background adaptation of the animal, in situ hybridization and PCR analysis was performed on pituitaries of black- and white-adapted animals. Using antisense digoxigenin-labelled *Xenopus* PC2 cDNA clone, XY2.1, as probe in the in situ hybridization experiments strong signals were found in the melanotrope cells of the pituitary pars intermedia of black-adapted *Xenopus*, while only weak signals could be detected in these cells of white animals (Fig. 3). The difference in size between the pars intermedia of black and white animals reflects the difference in biosynthetic and secretory activity of the melanotrope cells between the two physiological conditions. In both white- and black-adapted animals no hybridization-positive areas could be detected in the brain or pituitary pars distalis. PCR analysis revealed that the level of PC2 mRNA is about 15-fold-higher in pars intermedia of black animals than in white animals, whereas for the two conditions PC2 mRNA levels were approximately the same in the pituitary pars distalis, with these levels comparable to that in pars intermedia of white animals. Furthermore, Southern blot analysis of PCR products derived from pars intermedia RNA of black-adapted animals, and digested by restriction enzymes with specific cleavage sites in the two structurally different PCR products, showed similar amounts of products derived from the two *Xenopus* PC2 gene transcripts (data not shown).

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                                                                5'----GAATTCCTGAAAAG   -1
METArgGluGlyValValThrValTrpMetLeuAlaAlaLeuValLeuHisLeuAlaSerLeuSerValSerAlaGlyArgProValLeu   30
ATGAGGGAAGGTGTTGTGACAGTGTGGATGCTCGCGGCTCTTGTTCTGCATCTTGCCAGTCTTTCCGTCTCCGCTGGAAGACCTGTCCTC   90

ThrAspHisPheLeuValAspLeuArgGluGlyGlyGluAlaGluAlaGluGlnLeuAlaAlaGluTyrGlyPheSerGlyThrArgLys   60
ACCGATCATTTTTTGGTTCACTTACGTGAAGGAGGAGAAGCCGAAGCCGAGCAACTTGCGGCGGAATATGGCTTCAGTGGGACAAGAAAG  180

LeuProPheSerGlnSerLeuTyrHisPheTyrGlyAsnGlyIleThrThrSerArgSerArgArgSerValAsnLysLysLysHisLeu   90
CTACCTTTTTCCCAAAGTTTATACCATTTTTACGGAAATGGAATCACCACATCCCGAAGTCGACGCAGTGTCAATAAAAAGAAACATTTA  270

AlaMetAspProLysValAsnLysValGluGlnGlnGluGlyPheHisArgLysLysArgGlyTyrArgAspIleAsnAspIleGluIle  120
GCCATGGATCCCAAGGTAAATAAAGTGGAGCAACAAGAAGGTTTCCACAGGAAAAAACGAGGATACAGAGACATCAATGATATTGAAATT  360

AsnMetAsnAspProLeuPheThrLysGlnTrpTyrLeuIleAsnThrGlyGlnAlaAspGlyThrProGlyLeuAspLeuAsnValAla  150
AACATGAACGATCCATTATTTACAAAACAGTGGTACTTGATCAATACAGGCCAAGCTGATGGGACACCTGGACTTGATCTTAATGTCGCG  450

GluAlaTrpGluLeuGlyTyrThrGlyArgGlyValThrIleAlaIleMetAspAspGlyIleAspTyrLeuHisProAspLeuAlaSer  180
GAAGCATGGGAACTTGGATACACAGGAAGAGGGGTTACCATAGCAATTATGGATGATGGAATTGATTATCTGCACCCAGATCTTGCCTCC  540

AsnTyrAsnAlaGluAlaSerTyrAspPheSerSerAsnAspProTyrProTyrProArgTyrThrAspAspTrpPheAsnSerHisGly  210
AATTACAACGCAGAGGCAAGCTATGACTTCAGCAGCAATGATCCCTACCCGTATCCTCGATATACAGACGACTGGTTCAACAGCCATGGG  630

ThrArgCysAlaGlyGluValSerAlaSerAlaAsnAsnAsnIleCysGlyValGlyValAlaTyrAsnSerLysValAlaGlyIleArg  240
ACCCGATGTGCAGGAGAAGTGTCAGCATCTGCCAACAATAATATATGTGGAGTTGGAGTGGCTTATAATTCCAAAGTGGCAGGCATTCGA  720

MetLeuAspGlnProPheMetThrAspIleIleGluAlaSerSerIleSerHisMetProGlnValIleAspIleTyrSerAlaSerTrp  270
ATGCTGGACCAGCCCTTCATGACTGATATAATAGAAGCTTCATCCATCAGTCACATGCCCCAGGTTATTGACATATACAGTGCAAGCTGG  810

GlyProThrAspAspGlyLysThrValAspGlyProArgGluLeuThrLeuGlnAlaMetAlaAspGlyValAsnLysGlyArgGlyGly  300
GGTCCTACTGATGATGGCAAGACGGTTGATGGACCAAGAGAACTAACTTTACAGGCAATGGCAGACGGTGTGAATAAGGGTCGTGGTGGC  900

LysGlySerIleTyrValTrpAlaSerGlyAspGlyGlySerTyrAspAspCysAsnCysAspGlyTyrAlaSerSerMetTrpThrIle  330
AAGGGAAGCATCTATGTCTGGGCATCTGGAGATGGAGGAAGTTATGATGACTGCAACTGTGACGGCTATGCATCAAGCATGTGGACTATT  990

SerIleAsnSerAlaIleAsnAspGlyArgThrAlaLeuTyrAspGluSerCysSerSerThrLeuAlaSerThrPheSerAsnGlyArg  360
TCCATAAACTCTGCTATTAATGATGGACGCACTGCCCTGTATGATGAGAGTTGCTCCTCAACTCTGGCCTCCACTTTTAGCAATGGTAGA 1080

LysArgAsnProGluAlaGlyValAlaThrThrAspLeuTyrGlyAsnCysThrLeuArgHisSerGlyThrSerAlaAlaAlaProGlu  390
AAAAGAAATCCAGAGGCTGGCGTGGCTACAACAGACTTGTATGGAAACTGCACTTTGCGTCATTCAGGAACATCTGCAGCCGCACCAGAA 1170

AlaAlaGlyValPheAlaLeuAlaLeuGluAlaAsnProGlyLeuThrTrpArgAspLeuGlnHisLeuSerValLeuThrSerLysArg  420
GCAGCTGGAGTATTTGCATTGGCCCTGGAGGCTAACCCAGGTCTGACATGGAGGGACTTGCAGCATCTCTCAGTGCTAACATCGAAAAGG 1260

AsnGlnLeuHisAspGluValHisLysTrpArgArgAsnGlyValGlyLeuGluPheAsnHisLeuPheGlyTyrGlyValLeuAspAla  450
AATCAGCTTCACGATGAAGTGCACAAATGGCGTAGAAACGGAGTCGGTTTGGAGTTCAATCATTTGTTCGGCTATGGCGTACTTGATGCT 1350

GlyAlaMetValLysMetAlaAsnGluTrpLysThrValProGluArgPheHisCysIleGlyGlyAlaIleGlnGluProArgLysIle  480
GGAGCTATGGTTAAAATGGCCAACGAGTGGAAAACTGTTCCGGAAAGGTTTCATTGCATTGGAGGAGCCATACAAGAGCCAAGGAAAATA 1440

ProSerAspGlyLysLeuIleLeuThrLeuSerThrAspAlaCysGluGlyLysGluAsnPheValArgTyrLeuGluHisValGlnAla  510
CCTTCTGATGGGAAGCTGATCCTTACTCTTTCAACTGATGCCTGTGAAGGAAAAGAAAACTTTGTTCGCTACCTGGAGCACGTTCAAGCA 1530

ValIleThrValAsnSerThrArgArgGlyAspLeuAsnIleAsnMetThrSerProMetGlyThrLysSerIleLeuLeuSerArgArg  540
GTTATAACCGTCAATTCCACTCGGCGAGGGGACTTGAACATCAACATGACATCACCAATGGGAACTAAGTCCATTTTATTAAGTCGTCGT 1620

ProArgAspAspAspSerLysValGlyPheAspLysTrpProPheMetThrThrHisThrTrpGlyGluAspProArgGlyThrTrpVal  570
CCTAGGGATGACGACTCAAAAGTTGGTTTTGATAAATGGCCATTTATGACAACACACACATGGGGGGAAGATCCAAGAGGGACTTGGGTT 1710

LeuGluValGlyPheValGlySerIleProGluLysGlyValLeuLysGluTrpThrLeuMetLeuHisGlyThrGlnSerAlaProTyr  600
CTAGAAGTTGGGTTTGTTGGGAGCATACCAGAGAAAGGTGTTTTAAAAGAATGGACACTGATGTTACACGGCACTCAAAGTGCCCCCTAT 1800

IleAspGlnIleValArgAspTyrGlnSerLysLeuAlaMetSerLysLysGluGluLeuGluGluGluLeuAspGluAlaValGluArg  630
ATAGACCAAATAGTTAGAGATTATCAGTCTAAGTTAGCAATGTCCAACAAAGAAGAGCTGGAGGAGGAACTAGATGAAGCAGTAGAGAGA 1890

SerLeuLysSerLeuLeuSerLysAsn***                                                              639
AGTCTCAAGAGCCTTTTGAGCAAGAACTAGCACTATTCTGCATGTCTGTATTATGTTTATATTCTCCATTATTTTCTGTTAATCTTTTCA 1980

ACACCTTTACCAAGTATAATTTCCTCGCTGTGTTACAGTAATGGAATCTTTCTAGATATTTTCTTTTGTGTAGAAAAAAATAAATATATA 2070

AATATAAAAAAAAAAAAAAAAA---3'                                                                 2093
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Fig. 1. Nucleotide sequence and deduced amino acid sequence of pituitary pars intermedia cDNA clone, XY2.1, encoding *Xenopus* PC2. Amino acid sequence numbering starts with the presumptive 25-amino acid signal peptide sequence. The subtilisin-related catalytic domain consists of amino acid residues 122–413. The arrow indicates the presumptive signal peptide cleavage site, arrow heads indicate the active sites, and dots indicate potential *N*-linked glycosylation sites.

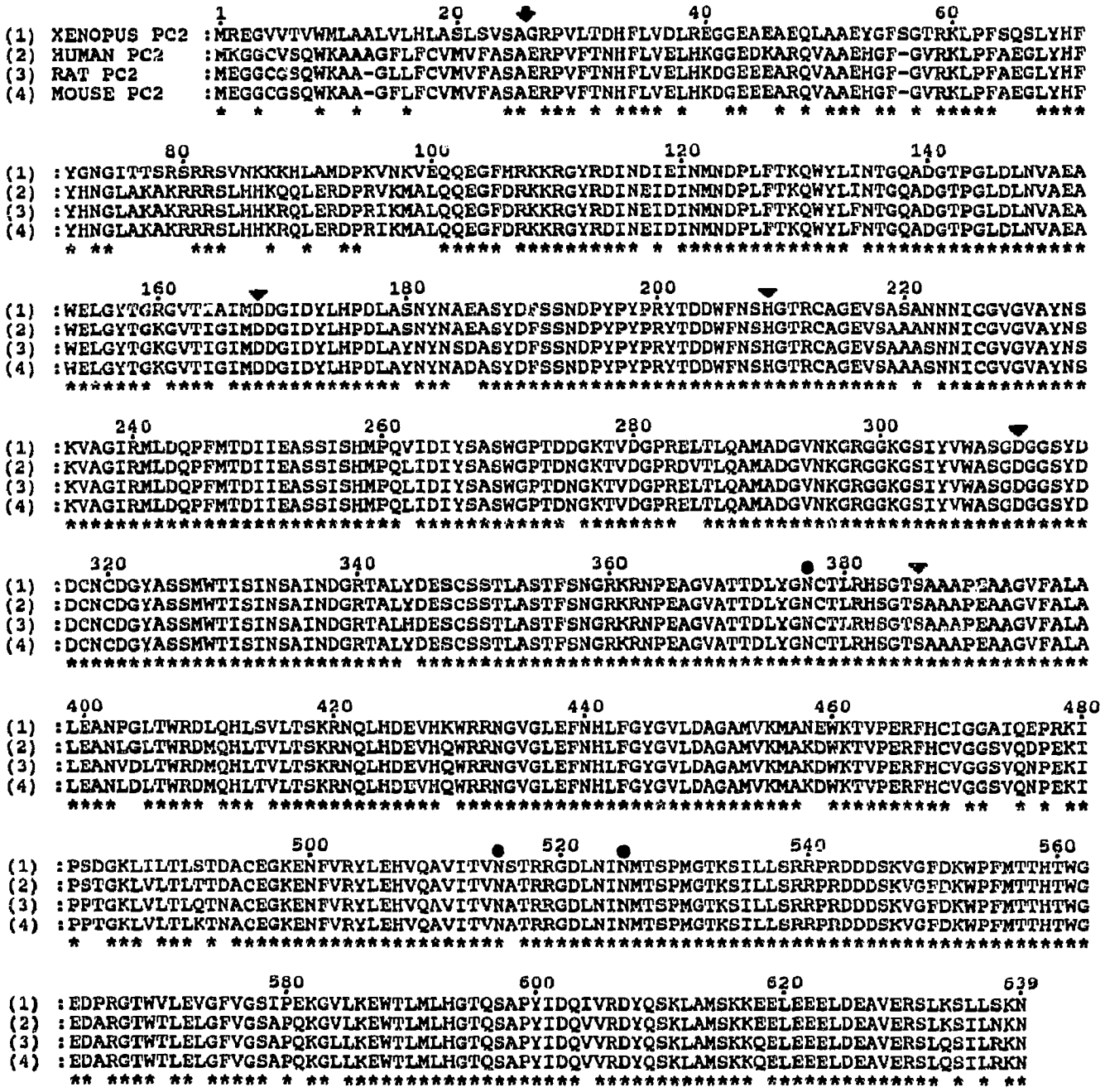

Fig. 2. Alignment of the amino acid sequences of *Xenopus*, human, rat and mouse PC2. The one-letter amino acid notation is used. Gaps introduced in the mammalian sequences to optimize the alignment are represented by dashes. Residues identical among the PC2 structures of the four species are indicated by asterisks. The subtilisin-related catalytic domain consists of amino acid residues 122–413. The arrow indicates the presumptive signal peptide cleavage site, arrow heads indicate the active sites, and dots indicate potential *N*-linked glycosylation sites. The human, rat and mouse PC2 sequences have been taken from [6], [25] and [7], respectively.

4. DISCUSSION

The occurrence of two gene transcripts coding for structurally different PC2 proteins reflects the expression of two PC2 genes in *Xenopus laevis*. The nucleotide sequence divergence between the two PC2 transcripts (5%) is similar to that reported for the protein-coding regions of *Xenopus laevis* gene pairs encoding globins [18], albumins [19], vitellogenins [20], proenkephalins [21] and POMCs [22] (4–8%). These gene pairs are believed to represent duplicated genes resulting from a duplication of the entire genome in the genus *Xenopus* [23]. The two PC2 genes were found to be expressed to approximately the same level in the pituitary pars inter-

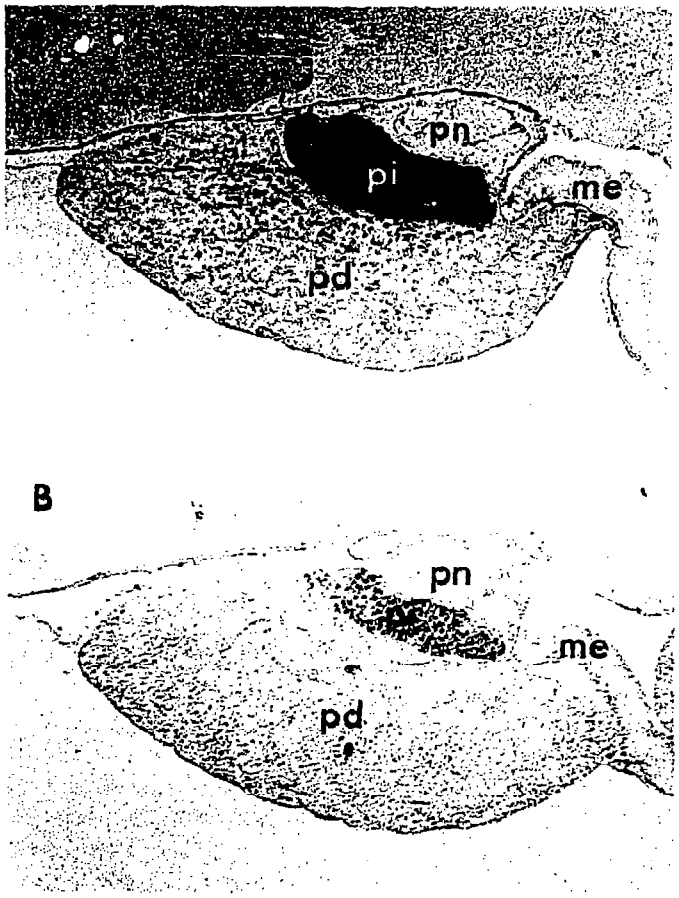

Fig. 3. Non-radioactive in situ hybridization showing the pattern of PC2 expression in the pituitary gland of *Xenopus laevis* adapted to a black background (A) or a white background (B). pn, pars nervosa; pi, pars intermedia; pd, pars distalis; me, median eminence; magnification × 120.

media, similar to the expression of the two *Xenopus* POMC genes [22].

Elucidation of the structure of PC2 in an amphibian species, and comparative analysis of the *Xenopus* and mammalian PC2 structures, revealed that this prohormone-converting enzyme has been highly conserved over 350 million years of vertebrate evolution. As expected, the regions around the active sites (Asp-168, His-209, Asp-311 and Ser-385; Fig. 2) are especially well conserved. It is noteworthy that the carboxy-terminal region of PC2 is remarkably well conserved, indicating that this region is functionally important. As is the case with other secretory proteins, the amino-terminal hydrophobic stretch of amino acid residues, comprising the putative signal peptide sequence of PC2, is the least-conserved region of the protein.

In corticotropes of the pituitary pars distalis POMC is processed to ACTH, and in the melanotrope cells to α-MSH [2]. The enzymes responsible for this tissue-specific processing of POMC have recently been suggested to be PC1 and PC2 [13–15]. Our screening of the pituitary pars intermedia cDNA library with PC1/PC3 and PC2 cDNA probes resulted in the isolation of only PC2 cDNA clones. Moreover, we recently applied a differential screening approach to identify genes co-expressed with POMC, and whose products may be involved in POMC biosynthesis and release of POMC-derived peptides. This approach involved screening of the pars intermedia cDNA library with single-stranded cDNA probes derived from pars intermedia RNA of either white or black animals, and it led to the identification of only PC2 clones (our unpublished results). These findings indicate that PC2, and not PC1, is the specific protease responsible for in vivo POMC processing to α-MSH in the melanotrope cells. In line with these results are two recent observations concerning PC1 and PC2 expression in mammalian cells. First, PC2 is much more abundant in rat pars intermedia cells than in the ACTH-producing corticotropes of the anterior lobe, while PC1 displayed the reverse expression pattern [8,12,24]. Second, with gene transfer experiments it has been shown that PC1 cleaves POMC to ACTH while PC2 processes POMC to yield α-MSH [14,15].

The results of the in situ hybridization experiments and PCR analysis provide evidence for tissue-specific regulation of the *Xenopus* PC2 gene. We detected high levels of PC2 mRNA in melanotrope cells of black-adapted animals, low levels in cells of white animals and low levels in the pars distalis of either white or black animals. The observed dynamics of PC2 gene expression during background adaptation of *Xenopus* is similar to that previously reported for the POMC gene [1]. We conclude that the POMC and PC2 genes are co-expressed, and that the PC2 protein is a crucial component of the biosynthetic machinery which ultimately leads to the production of α-MSH in *Xenopus* pituitary melanotropes. The expression pattern of the *Xenopus* PC2 gene during the physiological process of background adaptation of the animal is in line with the notion that genes encoding prohormone convertases are active in cells where specific cleavages of prohormones at pairs of basic amino acid residues are known to occur.

Acknowledgements: This work was supported by a PIONIER grant from the Netherlands Organization for Scientific Research (NWO). We thank Mr. R.J.C. Engels for animal care.

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