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Molecular probing of the secretory pathway in peptide hormone-producing cells

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SUMMARY

The biosynthetic machinery in the melanotrope cells of the *Xenopus* intermediate pituitary is primarily dedicated to the generation of proopiomelanocortin (POMC)-derived, melanophore-stimulating peptides. Transfer of the animal to a black background stimulates the production of these peptides and causes a dramatic increase in POMC mRNA levels. To identify genes involved in the biosynthesis and regulated release of peptide hormones, we differentially screened an intermediate pituitary cDNA library of toads adapted to a black background with cDNA probes derived from intermediate pituitary mRNA of black- and white-adapted animals. Here we report the identification of twelve distinct genes whose expression levels in the melanotropes are regulated in coordination with that of POMC.

Four of these genes are novel while the others code for translocon-associated proteins, a luminal cysteine protease of the endoplasmic reticulum, prohormone-processing enzymes, members of the granin family and a transmembrane protein presumably involved in the assembly and/or specific functioning of vacuolar H⁺-ATPase from secretory granules. Our results indicate that a wide variety of both soluble and membrane-associated components of the secretory pathway is recruited in physiologically activated, peptide hormone-producing cells.

Key words: pituitary, secretory pathway, differential screening, *Xenopus laevis*

INTRODUCTION

During the last decade, considerable contributions have been made towards an understanding of the mechanisms by which proteins are secreted from eukaryotic cells. A number of the key molecules involved have been identified by genetic analysis of secretion-defective mutants in yeast (Pryer et al., 1992), whereas the utilization of mammalian cell-free systems led to a biochemical definition of vesicle-mediated protein transport (Rothman and Orci, 1992). Both lines of investigation primarily dealt with the constitutive route of protein secretion, which is common to all cell types. Specialized secretory cells, however, often contain additional pathways by which proteins are delivered to the cell surface (Burgess and Kelly, 1987). For instance, the production and release of peptide hormones by endocrine cells or peptidergic neurons is mediated through a regulated secretory pathway that, in various aspects, is clearly distinct from the constitutive route. Firstly, peptide hormones arise from inactive precursor molecules or prohormones, which acquire full biological activity by undergoing multiple post-translational modifications during their intracellular transport. Such modifications may include glycosylation (Loh and Gainer, 1982), sulfation (Huttner, 1988), endoproteolytic cleavage (Halban and Irminger, 1994), exoproteolytic cleavage (Fricker, 1988), amidation (Eipper and Mains, 1988) and acetylation (Glembotski, 1982). Several of the responsible processing enzymes have been identified and it appears that a number of these reside exclusively in the secretory pathway of peptidergic cells (Eipper and Mains, 1988; Halban and Irminger, 1994). Secondly, prohormones arriving in the trans-Golgi network (TGN) are segregated from proteins travelling via the constitutive route and packaged separately into secretory granules (Orci et al., 1987). The molecular systems controlling prohormone sorting and packaging are poorly understood but may involve specific targeting signals that interact with sorting receptors in the TGN lumen (Chidgey, 1993). Alternatively, these events may be the consequence of a selective aggregation of prohormones triggered by the low pH and calcium-rich milieu of the TGN lumen (Reaves and Dannies, 1991; Chanat and Huttner, 1991). Thirdly, whereas the contents of transport vesicles derived from the constitutive route of secretion is released continuously into the extracellular environment, the secretory granules containing mature peptides are stored in the cytosol and will be delivered to the cell surface only in response to a specific extracellular stimulus. This regulated form of protein export has been studied in various experimental settings and, thus far, only a limited number of the molecular components involved have been identified (Wollheim and Lang, 1994). Further insight into the mechanisms by which peptide hormones are produced and released is desirable, as their malfunctioning may constitute the basis of many neuroendocrine disorders.

To explore the pathway of peptide hormone secretion at the
molecular level we use, as a model system, the neurointermediate lobe (NIL) from the pituitary gland of *Xenopus laevis*. This tissue consists of a nearly homogeneous population of melanotrope cells with a well-defined neuroendocrine function: namely, the production and release of α-melanophore-stimulating hormone (α-MSH) during adaptation of the animal to a dark background (Jenks et al., 1977). α-MSH is cleaved from the prohormone proopiomelanocortin (POMC) and stimulates the dispersion of black pigment in dermal melanophores, thus imparting a dark colour to the toad. Consequently, in the NIL of black background-adapted animals the POMC mRNA is highly expressed and the level of POMC is up to 30-fold higher than that in white-adapted animals (Martens et al., 1987). Moreover, the melanotropes of black-adapted animals display the ultrastructural features of cells with high biosynthetic and secretory activities, including an enlarged cell nucleus, extensively elaborated endoplasmic reticulum and Golgi complex, and a low amount of storage granules (Hopkins, 1970; de Rijk et al., 1990). Conversely, the melanotropes of white-adapted animals resemble inactive storage cells with a poorly developed synthetic apparatus and a high content of storage granules. Hence, the biosynthetic and secretory activity of *Xenopus* melanotropes can be manipulated at the physiological level simply by changing the background colour of the animal. As an approach to identify genes associated with the specialized secretory function of peptide hormone-producing cells, we differentially screened a NIL cDNA library of black-adapted *Xenopus* with cDNA probes derived from NIL mRNA of black- and white-adapted animals. Here we report the identification of twelve distinct genes whose transcript levels in the melanotropes are regulated in coordination with that of POMC during background adaptation.

**MATERIALS AND METHODS**

**Animals**

South-African clawed toads, *Xenopus laevis* (40-60 g), were adapted to black or white backgrounds under constant illumination for three weeks at 22°C.

**Metabolic intermediate lobes labeling studies**

Neurointermediate lobes (NILs) and anterior lobes (ALs) from black and white *Xenopus* were dissected and preincubated in incubation medium (IM: 112 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 15 mM Heps, pH 7.4, 0.3 mg/ml BSA, 2 mg/ml glucose) at 22°C for 15 minutes. Pulse labeling of newly synthesized proteins was performed by incubating lobes in IM containing 1.7 mCi/ml [³⁵S]methionine (Tran³⁵S-label, ICN Radiochemicals) for 10 minutes at 22°C. Lobes were homogenized on ice in lysis buffer containing 50 mM Heps, pH 7.2, 140 mM NaCl, 1% Tween-20, 0.1% Triton X-100, 0.1% deoxycytate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1% mg/ml soybean trypsin inhibitor. Homogenates were cleared by centrifugation, before addition of 0.1 volume of 10% SDS. Newly synthesized proteins were resolved by SDS-PAGE and visualized by fluorography.

**cDNA library construction and prescreening**

Cytoplasmic RNA was isolated from NILs of 100 black-adapted *Xenopus* toads using the NP40 method and subjected to oligo(dT) chromatography according to Sambrook et al. (1989). cDNA appropriate for directional cloning was synthesized using a commercial cDNA synthesis kit (Stratagene), size-fractionated on CL4B Sepharose and ligated into lambda uni-ZAP XR (Stratagene). About 50,000 primary plaques were hybridized on duplicate filters at a density of 10 plaques per cm² using standard procedures (Sambrook et al., 1989). Hybridization was with a random prime-labeled POMC cDNA (poly(A) tail removed) and with a single-stranded (ss) cDNA probe synthesized from oligo(dT)-primed *Xenopus* liver RNA using Superscript reverse transcriptase (Gibco-BRL). Filters were washed at 63°C to a final stringency of 0.2x SSC and exposed to X-ray film at -70°C with two intensifying screens.

**Preparation of cellulose-coupled cDNAs**

As the first step towards the generation of the probes used in the differential hybridization, cellulose-coupled cDNAs were prepared from RNA from various *Xenopus* tissues using a modified version of the method from Rodriguez and Chader (1993). A 100 μg sample of total RNA from brain, liver or heart was annealed to 5 mg oligo(dT)-cellulose (Pharmacia) in 100 μl of 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 1 M NaCl and 0.25% SDS by preheating the mixture to 70°C and rotation for 20 minutes at room temperature. Excess RNA was removed by washing the cellulose three times in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, pH 8.0, 100 mM NaCl and two times in RT buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl and 3 mM MgCl₂). For cDNA synthesis, the cellulose was suspended in 50 μl RT buffer supplemented with 10 mM DTT, 500 μM dNTPs, 5 μCi [α-³²P]dATP (3000 Ci/mmol, Amersham), 35 units RNAsin and 400 units Superscript reverse transcriptase. After 45 minutes at 42°C, the cDNA was washed three times at room temperature and two times at 94°C with TE, pH 7.5. cDNA synthesized was quantified by measuring the amount of incorporated ³²P. In a similar way, cDNA from total NIL RNA of six black and six white animals was synthesized on 2 mg oligo(dT)-cellulose.

**Differential hybridization**

Recombinant pBluescript SK⁺ phagemids were excised in vivo from selected lambda ZAP clones and rescued as ss-antisense DNA according to the instructions of the manufacturer. A 5 ng sample of ss-antisense DNA derived from 204 isolated cDNA clones was spotted on four separate nitrocellulose filters. Each of these was then hybridized with different ss-sense cDNA probes prepared by random prime-labeling of cellulose-coupled antisense cDNAs. For this, the cellulose-coupled cDNAs were suspended in 15 μl water, heated for 2 minutes at 95°C, and cooled on ice before addition of 35 μl buffer containing random primers (Sambrook et al., 1989), 1 mg/ml BSA, 60 μCi [α-³²P]dATP, and 8 units Klenow fragment (Pharmacia). Following 30 minutes incubation at 37°C and 60 minutes rotation at room temperature, unincorporated label was removed by washing the cellulose three times in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA and 100 mM NaCl. Labeled ss-cDNA fragments were recovered by incubating the cellulose twice at 94°C for 2 minutes in 75 μl water containing 2 μg yeast tRNA and collected by centrifugation. Probes were generated from cellulose-coupled NIL cDNA of white and black animals (white probe, total activity: 0.5x10⁶ cpm; black probe, total activity: 4.0x10⁶ cpm). For a probe enriched in brain-specific sequences, labeled ss-cDNA fragments (total activity: 6x10⁶ cpm) were generated from 70 ng of cellulose-coupled brain cDNA, mixed with 140 ng cellulose-coupled liver cDNA in 200 μl 5x SSPE, denatured at 94°C for 2 minutes and allowed to anneal at 55°C for 1 hour. Non-hybridizing ss-cDNA fragments were annealed to 220 ng cellulose-coupled heart cDNA. Consequently, two fractions of the original probe were isolated: one that fails to anneal to liver and heart cDNA, thus enriched in brain-specific sequences (brain probe, total activity: 3.5x10⁶ cpm), and another fraction eluted from liver and heart cDNA containing mainly common sequences (common probe, total activity: 2.5x10⁶ cpm). Prehybridized filters were screened separately with black, white and common probes overnight at 63°C.
and washed to a final stringency of 0.2x SSC at 63°C. Exposure to X-ray film was for three days at -70°C with two intensifying screens.

**DNA sequence analysis and database matching**

Sequencing of selected cDNA clones on both strands and with pBlue-script subclones or specific primers was performed with single- and double-stranded DNA using T7 DNA polymerase (Pharmacia) and the dideoxy chain termination method (Sanger et al., 1977). Nucleotide sequences and deduced protein sequences were compared with those present in the EMBL/GenBank and Swissprot/PIR databases using computer facilities of the CAOS/CAMM center at the University of Nijmegen.

**RNA isolation**

For expression studies, total RNA was prepared according to the method of Chomczynski and Sacchi (1987), using acid-guanidine isothiocyanate-phenol-chloroform extraction. After recovery by ethanol precipitation, the RNA was quantified by spectrophotometry and its integrity checked by running samples on denaturing agarose gels followed by ethidium bromide staining. Poly(A)+ RNA was separated by electrophoresis on 2.2 M formaldehyde-containing 5% polyacrylamide gels. Following autoradiography, quantification of protection signals was performed with a LKB/Pharmacia Ultrascan XL laser densitometer (LKB/Pharmacia).

**RESULTS AND DISCUSSION**

**Xenopus melanotropes as a model system for identifying genes involved in peptide hormone production and release**

The biosynthetic activity of the melanotrope cells in the NIL of *Xenopus* can be manipulated in vivo by changing the background colour of the animal (Fig. 1). Metabolic cell labeling experiments revealed that in the melanotropes of black-adapted toads the production levels of a subset of the newly synthesized proteins are dramatically increased compared to those in melanotropes of white-adapted animals (Fig. 1B). Within this group, POMC clearly represents the major newly synthesized protein as it constitutes over 80% of all radiolabeled products. These findings are consistent with northern blot experiments in which NIL RNA from black and white animals was hybridized with a cDNA probe prepared from NIL RNA of black animals. As shown in Fig. 1C, POMC transcripts represent by far the most abundant mRNA species in the NIL. Moreover, as reported previously (Martens et al., 1987), the levels of POMC mRNA are 20- to 30-fold higher in the NILs of black animals compared to that in white animals. These physiologically induced changes in protein biosynthesis and RNA expression are confined to the melanotrope cells of the NIL and do not occur in cells of the anterior lobe (AL) of the pituitary (Fig. 1B, C).

The above results led us to use *Xenopus* melanotropes in a strategy aimed at the identification of genes involved in peptide hormone production and release. Our strategy is based on the assumption that, following physiological manipulation of these cells, at least part of the genes implicated in the translocation, sorting, processing and exocytotic release of peptide hormones will be coordinately expressed with the POMC gene.

**Northern blot analysis**

RNA was separated by electrophoresis on 2.2 M formaldehyde-containing 1.2% agarose gels in MOPS buffer and blotted onto nitrocellulose filters as described by Ausubel et al. (1989). Hybridization was overnight at 45°C in 5x SSPE, 50% formamide, 5x Denhardt’s, 0.5% SDS and 0.1 mg/ml denatured salmon sperm DNA, using 1x10⁶ cpmp/ml of probe. A cDNA probe prepared from oligo(dT)-primed NIL RNA of black animals was used to hybridize a blot containing RNA of NILs and ALs from black and white animals. Other tissue RNA blots were hybridized with random prime-labeled inserts from selected cDNA clones. Blots were washed at 65°C to a final stringency of 0.1x SSPE and autoradiographed at -70°C using two intensifying screens.

**In situ hybridization**

Black and white animals were anesthetized in water containing 1 mg/ml 3-aminobenzoic acid ethyl ester (Sigma). Brains with pituitaries still attached were perfused with XPBS (101 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄ and 1.4 mM KH₂PO₄) and subsequently with 4% paraformaldehyde in XPBS (PFA/XPBS), dissected and incubated in 4% PFA/XPBS for 4 hours at 4°C. Following overnight incubation in 30% sucrose/XPBS at 4°C, tissues were frozen and cut into 15 μm sections that were collected on binding silane-treated glass slides. Each slide contained pituitary sections of both black and white animals to be processed under identical conditions. Sections were incubated with 0.2% pepsin in 0.1 M HCl for 10 minutes, fixed in 2% PFA/XPBS for 10 minutes, acetylated with acetic anhydride and dehydrated in a graded series of ethanol at room temperature. Hybridization was overnight at 55°C in 5x SSC, 50% formamide, 50 mM sodium phosphate, pH 7.4, 2.5x Denhardt’s, 10% dextran sulfate, 0.1% SDS, 0.2 mg/ml denatured proteinase K-treated salmon sperm DNA and 0.2 mg/ml yeast tRNA. Digoxigenin-labeled antisense RNA probes were prepared according to the instructions of the manufacturer (Boehringer Mannheim). Linearized plasmid constructs used in the RNase protection assay served as templates. After hybridization with 0.2 μg/ml of probe, sections were washed at 63°C to a final stringency of 0.1x SSC and hybrids were detected by an enzyme-linked immunosassay (Boehringer Mannheim). Specificity of the hybridization signals obtained was assessed by using sense RNA probes as a control.

**Differential screening strategy**

As outlined in Fig. 2, the first step in our strategy concerned the construction of a cDNA library from NIL mRNA of fully black-adapted *Xenopus*. Screening of the library with a POMC...
cDNA probe revealed that 75% of the 50,000 primary clones harbour POMC-encoding sequences. This proportion of POMC cDNAs is in line with the high rate of POMC biosynthesis and the abundant presence of POMC transcripts in NILs of black animals (Fig. 1). The library was subsequently hybridized with a liver mRNA-derived cDNA probe to eliminate clones encoding ribosomal proteins, mitochondrial enzymes and other highly expressed 'housekeeping' proteins. Following screening with the POMC and liver probes, about 7,500 non-hybridizing clones remained for further analysis. From this group, clones representing genes that are coordinately expressed with the POMC gene were selected by differential hybridization with cDNA probes derived from NIL RNA of black and white animals (black and white probes, respectively). To determine which of the differentially hybridizing clones represent neuroendocrine-specific genes, an additional screening was performed with cDNA probes enriched in brain-specific or commonly expressed sequences (brain and common probes, respectively).

For testing the specificity of our differential hybridization procedure, we first screened a number of NIL cDNAs that had been isolated and identified following a random pick approach. Differential hybridization signals in the black/white screening were obtained for cDNAs encoding POMC, prohormone convertase PC2, carboxypeptidase H (CPH), the neuroendocrine polypeptide 7B2, calpain light chain, calreticulin and binding protein BiP (Fig. 3A and data not shown). Non-differentially hybridizing cDNAs or cDNAs showing only a minor (below threefold) difference in signals included those for ribosomal proteins, translation initiation factors, ferritin-H, mitochondrial NADH-oxidoreductase chain 4, actin and the actin-bundling protein fascin (Fig. 3A and data not shown). Only cDNAs encoding neuroendocrine-specific proteins (POMC, PC2, CPH and 7B2) were positive with the brain probe, not with the common probe (Fig. 3A).

Selection of differentially expressed genes
Having established the feasibility of the various probes for differential screening, we then randomly picked 204 clones from the group of 7,500 non-POMC and non-liver clones, and single-stranded DNA corresponding to these was spotted onto nitrocellulose filters. Screening of the filters with the black and white probes yielded 58 differentially hybridizing cDNAs (Fig. 3B). Seven of these were removed, since they displayed strong hybridization signals with the common probe. Of the remaining 51 cDNAs, 36 were brain-specific, whereas no hybridization signals above background were observed for the other 15 cDNAs. Partial sequence analysis and cross-hybridization experiments with the cDNA inserts as probes revealed that the 51 cDNAs were derived from 27 distinct genes (data not shown). Fifteen genes were excluded from subsequent studies, since their presumed differential expression, based on the dot blot hybridization procedure, could not be confirmed in RNase protection assays (data not shown). The RNA levels of the remaining twelve genes were at least sixfold higher in the NILs of black animals than in those of white animals (Fig. 4, Table 1). These physiologically induced changes in gene expression are tissue specific because they were not observed in the AL (Fig. 4). Eight of the differentially regulated genes are selectively or preferentially expressed in Xenopus brain and pituitary (clones X1035, X1311, X8211, X8290, X8556, X8591, X8596 and X8675) whereas four represent ubiquitously expressed genes (clones X0286, X1262, X1267 and X6227) (Fig. 4). The above results were highly reproducible.
Characterization of differentially expressed genes

As summarized in Table 1, the database search revealed that eight of the twelve differentially expressed genes correspond to previously identified genes. These genes can be classified according to the functions and/or subcellular distributions of the proteins they encode.

Prohormone processing enzymes

Clone X1035 encodes the prohormone convertase PC2, a neuroendocrine-specific endoprotease that cleaves its substrate at pairs of basic amino acids (Benjannet et al., 1991; Thomas et al., 1991). Identification of this gene is reassuring as its transcript levels in Xenopus melanotropes were previously found to be regulated in parallel with POMC mRNA (Braks et al., 1992). Clone X8675 codes for the Xenopus homolog of rat carboxypeptidase H (CPH), an enzyme removing the basic amino acid residues from C-terminal ends of prohormone-derived peptides (Fricker, 1988). An amino acid sequence identity of 84% over 197 matched residues reflects a high degree of conservation for this enzyme between mammals and amphibians. Both the PC2 gene and CPH gene produce two differentially regulated transcripts (Fig. 6A, Table 1) that differ in size due to the use of alternative polyadenylation sites (data not shown). The frequency by which cDNAs for these transcripts were selected in our study indicates that they represent the most abundant of all regulated non-POMC mRNAs in the NIL (Table 1). RNase protection assays revealed 35-fold higher levels of CPH mRNA in the NILs of black animals than in those of white animals (Fig. 5). This black/white ratio in transcript levels is 25 for PC2. In situ hybridization experiments

![](image)

**Fig. 2.** Strategy for the identification of differentially expressed genes. See text for details.

(Fig. 5) and consistent with northern blot analysis (Fig. 6A,B) and in situ hybridization experiments (Fig. 7 and data not shown). The cDNAs corresponding to the twelve differentially expressed genes were therefore sequenced, and their nucleotide and deduced protein sequences compared with those present in the EMBL/GenBank and NBRF/GENSWISS databases.

**Fig. 3.** Differential hybridization of NIL cDNA clones. Filters containing spotted DNA from isolated NIL cDNA clones were hybridized with cDNA probes derived from NIL mRNA of black and white animals (black and white probes), or cDNA probes enriched in brain-specific (brain probe) or commonly expressed sequences (common probe). (A) Previously identified NIL cDNA clones serving as a reference for the differential screening procedure. Calp. LC, calpain light chain; POMC, proopiomelanocortin; PC2, prohormone convertase 2; CPH, carboxypeptidase H; 7B2, neuroendocrine protein 7B2; eIF1α, translation initiation factor 1α; eIF4A, translation initiation factor 4A; empty vector, pBluescript SK-; Rip S9, ribosomal protein S9; Rip L7α, ribosomal protein L7α. (B) Differential hybridization of newly isolated NIL cDNA clones. Arrows indicate clones displaying differential hybridization signals in both the black/white and brain/common screenings. Clones giving differential hybridization signals in the black/white screening only are marked with an arrowhead. Non-differentially hybridizing clones are encircled.
Fig. 4. RNase-protection analysis of differentially expressed genes. Radiolabeled anti-sense RNA was transcribed from selected NIL cDNA clones and hybridized to total RNA extracted from the NIL and AL of black (B) and white (W) animals, or to 5 μg total RNA from liver (Li), brain (Br), heart (He), kidney (Ki) and lung (Lu). In each assay, total RNA from 2 NILs or ALs was used (except for clone X8211, 1/5 part of a NIL, one AL; clones X1035 and X8675, 1/25 part of a NIL and one AL each). RNase-treated samples were loaded onto denaturing polyacrylamide gels and autoradiographed. Undigested RNA probes (c1) or probes hybridized to 20 μg yeast tRNA prior to digestion (c2) served as controls for specificity of protection signals. Quality of RNA was checked by hybridizing samples to antisense RNA probe against the non-regulated transcript for fascin (Holthuis et al., 1994) or by running samples on ethidium bromide-stained gels (see control).

demonstrate that these dynamics in gene expression occur in the melanotrope cells of the intermediate pituitary (Fig. 7; Braks et al., 1992). Since both enzymes are indispensable for the conversion of prohormones into bioactive peptides, the vigorous regulation of their mRNAs in Xenopus melanotropes is not surprising. Our findings concerning PC2 and CPH are in line with those of other investigators who previously demonstrated that in rat intermediate pituitary these genes are co-regulated with POMC by dopaminergic agents (Bloomquist et al., 1991, 1994).

Granins
Clone X8211 corresponds to a previously isolated Xenopus gene encoding the neuroendocrine protein 7B2 (Martens et al.,

Table 1. Identification of differentially expressed genes

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<th>Clone</th>
<th>Freq*</th>
<th>Black/white Ratio†</th>
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<th>Protein (kDa)§</th>
<th>Database match</th>
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<th>Species¶</th>
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*Frequency by which independent cDNAs representing one transcript were isolated.
†Ratio of transcript levels in the intermediate pituitary between black- and white-adapted animals.
‡Transcript size as determined by northern blot analysis.
§Calculated size of protein without signal peptide as deduced from cDNA sequence.
¶B, bovine; D, dog; M, mouse; R, rat; X, Xenopus; Y, yeast.
IlcDNAs corresponding to larger transcripts generated by the use of alternative polyadenylation sites.

n.d., not determined.
Fig. 5. mRNA levels of differentially expressed genes in the NIL and AL of black (B) and white (W) animals. mRNA levels were determined by RNase-protection analysis using radiolabeled antisense RNA transcribed from selected NIL cDNA clones (X1267, X1311, X8290, X8591 and X8675) and aliquots of total RNA extracted from pooled lobes. Aliquots of RNA from each pool were used to determine the level of the non-regulated transcript for fascin. mRNA levels were quantified by densitometric scanning of autoradiographs. Data shown are the mean ± s.e.m. of three independent experiments.

The protein sequences deduced from clones X8556 and X8596 display 49% and 61% identity with rat secretogranin (SGII) (Gerdes et al., 1989) and SGIII (Ottiger et al., 1990), respectively. These clones therefore likely represent the Xenopus homologs of SGII and SGIII. Transcripts for 7B2, SGII and SGIII are selectively present in neuroendocrine tissues (brain and pituitary; Fig. 4) and are fairly abundant among the regulated messengers in the NIL (Table 1). The black/white ratio in transcript levels is 20 for 7B2, 15 for SGII family (Huttner et al., 1991). Several functions for these proteins have been proposed. They may represent precursors of biologically active peptides (Saria et al., 1993), assist in sorting and secretory granule biogenesis (Chanat and Huttner, 1991; Huttner and Natori, 1993) or serve as molecular chaperones for prohormone-processing enzymes (Braks and Martens, 1994). The differential regulation of three distinct granins in Xenopus melanotropes during background adaptation reinforces the physiological relevance of this class of proteins in neuroendocrine cells.

ER resident proteins

Two differentially regulated genes represent Xenopus homologs of known mammalian genes for proteins residing in the endoplasmic reticulum (ER): namely, the cysteine protease precursor ER60 (clone X6227) and the translocon-associated protein TRAP8 (clone X0286). Both ER60 and TRAP8 are encoded by ubiquitously expressed genes (Fig. 4) and exhibit a high degree of conservation during vertebrate evolution (Table 1). ER60 transcript levels in the NIL show a black/white ratio of 10 whereas this ratio is 20 for TRAP8 mRNA. ER60 belongs to the class of reticuloplasmins, a group of soluble and abundant proteins: that is retained in the ER lumen by the C-terminal retention signal KDEL. ER60 protease shows cysteine proteolytic activity and catalyzes proteolytic cleavage of itself as well as of other reticuloplasmins (Urade et al., 1992; Urade and Kito, 1992). Therefore, ER60 protease could well be part of the proteolytic system that clears the ER of abnormal or incorrectly folded secretory proteins. TRAP8 is a subunit of a tetrameric complex of transmembrane proteins that is located in proximity to the site where secretory proteins are translocated across the ER membrane (Hartmann et al., 1992). The TRAP complex is not essential for the translocation process (Migliaccio et al., 1992) and its function is unknown. It may have a role in the proper release of newly synthesized proteins from the translocon or participate in the folding or quality control systems of the ER. The enhanced transcript levels observed for ER60 protease and TRAP8 in the melanotropes of black-adapted Xenopus probably relate to an expanding capacity of the ER to accommodate the high quantity of newly synthesized prohormone.

An accessory subunit for vacuolar H⁺-ATPase

Clone X1311 corresponds to a single mRNA of 2.3 kb, which is expressed predominantly in pituitary and brain (Figs 4, 6B). NILs of black animals contain ten times more of this transcript than those of white animals (Fig. 5). In situ hybridization experiments indicate that these changes in gene expression originate from the melanotrope cells (Fig. 7). The 48 kDa protein encoded by X1311 carries an N-terminal signal sequence and contains seven potential N-linked glycosylation sites as well as a membrane-spanning segment close to its C-terminal end. A database search revealed 60% amino acid sequence identity with Ac45 (Table 1), a novel accessory subunit of the vacuolar H⁺-ATPase (V-ATPase) from bovine adrenal medulla chromaffin granules (Supek et al., 1994). X1311 therefore probably represents the Xenopus homolog of Ac45. The function of Ac45 is not known. V-ATPases are ancient multimeric enzymes responsible for the generation of the acidic gradients within the central vacuolar system of eukaryotic cells (Forgac, 1989). In neuroendocrine cells, the perturbation of these gradients by weak bases or with specific V-ATPase inhibitors leads to missorting and impaired proteolytic processing of regulated secretory proteins (Moore et al., 1983; Henomatsu et al., 1994). How V-ATPases are directed to, and achieve their specialized functions in such diverse organelles as endosomes, lysosomes and secretory granules is unclear. Studies with yeast mutants lacking genes for specific V-ATPase subunits have indicated that the assembly of the enzyme starts with its membrane sector (Manolson et al., 1992; Kane et al., 1992). Consequently, specific accessory membrane proteins are likely to be required for correct assembly and specific functioning of V-ATPases in the various organelles. Hence, an attractive possibility is that Ac45 is a membrane-associated modulator of V-ATPase activity in secretory granules. Such a role would be consistent with our finding that transcripts for this protein are primarily expressed in neuroen-
Figure 6. Northern blot analysis of...
X131

X8675

X8675

White

Black

The nerve growth factor receptor, a transmembrane protein required for neuronal survival, is a receptor for the EGF-like and TGF-α-like growth factors. The receptor is composed of two subunits, α and β, which associate to form a heterodimer. The α subunit binds to the extracellular domain of the receptor, while the β subunit contains the transmembrane and intracellular domains. The signal transduction pathway initiated by the binding of nerve growth factor to the receptor leads to the activation of several intracellular signaling pathways, including the Ras-MAPK pathway and the PI3K-AKT pathway.

The diagram illustrates the interactions between the nerve growth factor receptor and its ligands, as well as the downstream signaling pathways activated by receptor activation. The green arrows represent the activation of signaling pathways, while the red arrows indicate inhibitory effects.

The nerve growth factor receptor plays a critical role in the regulation of neuronal survival, growth, and differentiation. Mutations in the receptor or its ligands can lead to a variety of neurological disorders, including neuropathies and cancers.
extensive elaboration of the synthetic apparatus observed within these cells (Hopkins, 1970; de Rijk et al., 1990). Thus far, our collection of differentially expressed genes does not include genes for cytosolic components involved in vesicular traffic. Such genes may have simply not been encountered in this study due to their relatively low levels of expression.

Clearly the most abundant species among the regulated transcripts in Xenopus melanotropes is that for POMC, which constitutes approximately 75% of all mRNAs. Our data indicate that transcripts for PC2 represent the second most abundant type (~1% of total mRNA), followed by those for CPH, 7B2, SGII and SGIII (~0.5%, ~0.3%, ~0.2% and ~0.2% of total mRNA, respectively). In agreement with our metabolic cell labeling studies, these data indicate that the biosynthetic machinery in Xenopus melanotropes is primarily dedicated to the production of POMC and does not produce any other protein in stoichiometric amounts with this prohormone. Interestingly, we recently showed that 7B2 is a molecular chaperone in the secretory pathway of Xenopus melanotropes, which is produced in comparable amounts with its physiological target PC2 (Braks and Martens, 1994). Our screening results suggest that a similar neuroendocrine secretory protein with chaperone-like activity towards prohormones does not exist.

Undoubtedly, many more components from the biosynthetic and secretory machinery in neuroendocrine cells remain to be identified. The in vivo model system of Xenopus melanotropes can be put forward as a valuable means to trace some of these and to gain further insight into the molecular mechanisms responsible for the proper generation and release of bioactive peptides.

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