

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; Chanut 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 gene is highly expressed and the level of POMC mRNA is up to 30-fold higher than in that of 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 cell 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 Hepes, 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 Hepes, pH 7.2, 140 mM NaCl, 1% Tween-20, 0.1% Triton X-100, 0.1% deoxycholate, 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 0.2× 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 [³²P]dATP (3000 Ci/mmol, Amersham), 35 units RNasin and 400 units Superscript reverse transcriptase. After 45 minutes at 42°C, the cellulose 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 [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: 0.5×10⁶ cpm; black probe, total activity: 4.0×10⁶ cpm). For a probe enriched in brain-specific sequences, labeled ss-cDNA fragments (total activity: 6×10⁶ cpm) were generated from 70 ng of cellulose-coupled brain cDNA, mixed with 140 ng cellulose-coupled liver cDNA in 200 µl 5× 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: 3.5×10⁶ cpm), and another fraction eluted from liver and heart cDNA containing mainly common sequences (common probe, total activity: 2.5×10⁶ cpm). Prehybridized filters were screened separately with black, white, brain and common probes overnight at 63°C

and washed to a final stringency of $0.2\times$ 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 selected by oligo(dT) chromatography and quantified by spectrophotometry. The recovery of RNA from NILs and ALs was aided by using yeast tRNA as a carrier.

RNase protection assay

Constructs to be used in the RNase protection assay were generated by subcloning appropriate restriction fragments from selected cDNA clones into pBluescript SK⁻ vector. After verification by sequencing, constructs were linearized by restriction digestion and antisense runoff transcripts were generated from the T3 or T7 RNA polymerase promoter. Transcripts labeled with [α -³²P]UTP (800 Ci/mmol, Amersham) were purified on 5% polyacrylamide/8 M urea gels. Transcript sizes generated from the constructs (with size of protected bands in parentheses) were: X0286, 402 nt (346); X1035, 359 nt (277); X1262, 216 nt (156); X1267, 347 nt (277); X1311, 362 nt (279); X6227, 319 nt (244); X8211, 429 nt (355); X8556, 385 nt (343); X8591, 301 nt (254); X8596, 297 nt (276); X8675, 410 nt (311); fascin, 406 nt (311); actin, 233 nt (210). About 1×10^5 cpm of each transcript was combined with total RNA samples in 25 μl hybridization mix (80% formamide, 400 mM NaCl, 40 mM Pipes, pH 6.4, and 1 mM EDTA). Samples were incubated at 80°C for 5 minutes before hybridization overnight at 50°C . Non-hybridized RNA was digested with RNase A and T₁ for 30 minutes at 37°C . Samples were treated with proteinase K, phenol/chloroform/isoamyl-alcohol extracted, supplemented with 10 μg yeast tRNA, ethanol precipitated and run on 5% polyacrylamide/8 M urea gels. Following autoradiography, quantification of protection signals was performed with a Ultrascan XL laser densitometer (LKB/Pharmacia).

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 $5\times$ SSPE, 50% formamide, $5\times$ Denhardt's, 0.5% SDS and 0.1 mg/ml denatured salmon sperm DNA, using 1×10^6 cpm/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 63°C to a final stringency of $0.1\times$ 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 $5\times$ SSC, 50% formamide, 50 mM sodium phosphate, pH 7.4, $2.5\times$ 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 $\mu\text{g}/\text{ml}$ of probe, sections were washed at 63°C to a final stringency of $0.1\times$ SSC and hybrids were detected by an enzyme-linked immunoassay (Boehringer Mannheim). Specificity of the hybridization signals obtained was assessed by using sense RNA probes as a control.

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.

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

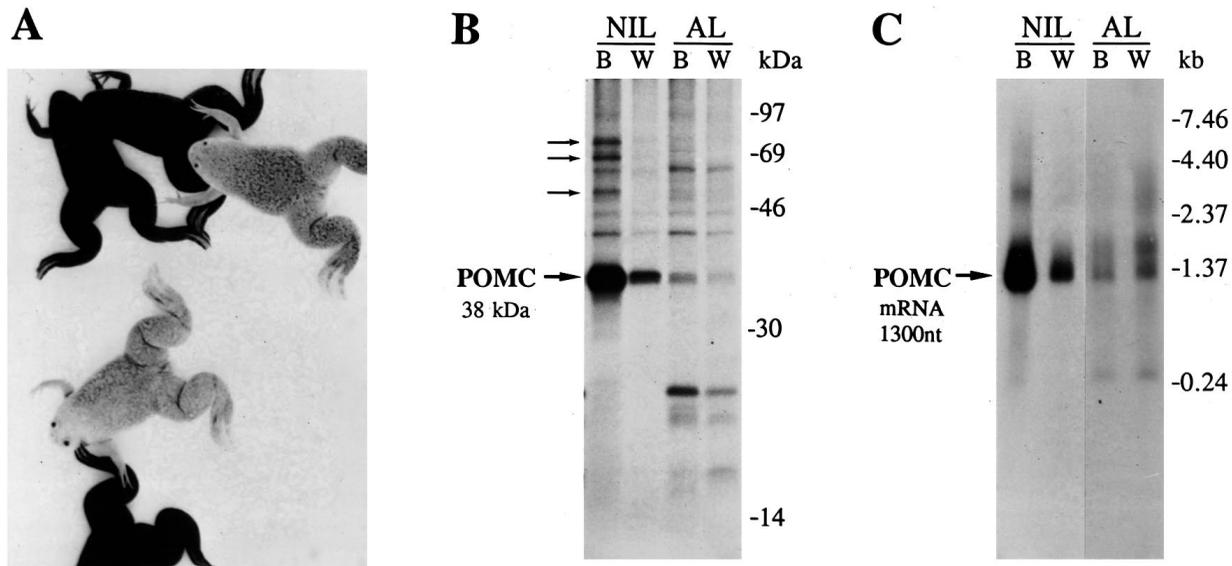


Fig. 1. Effect of background adaptation on levels of newly synthesized proteins and RNA expression in the pituitary gland of *Xenopus laevis*. (A) Animals adapted to black or white backgrounds for three weeks. (B) Radiolabeled proteins produced by pulse-incubated neurointermediate lobes (NIL) and anterior lobes (AL) of black (B) and white (W) animals. Arrows indicate POMC and three additional proteins whose production levels in the NIL are clearly enhanced in black animals with respect to white animals. Each lane contains protein extract equivalent to 1/25 part of a lobe. (C) Northern blot analysis of total RNA extracted from NILs and ALs of black (B) and white (W) animals using a cDNA probe derived from oligo(dT)-primed NIL RNA of black animals. The migration of POMC transcripts is indicated. Each lane contains RNA extracted from five pooled lobes.

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, calpactin 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

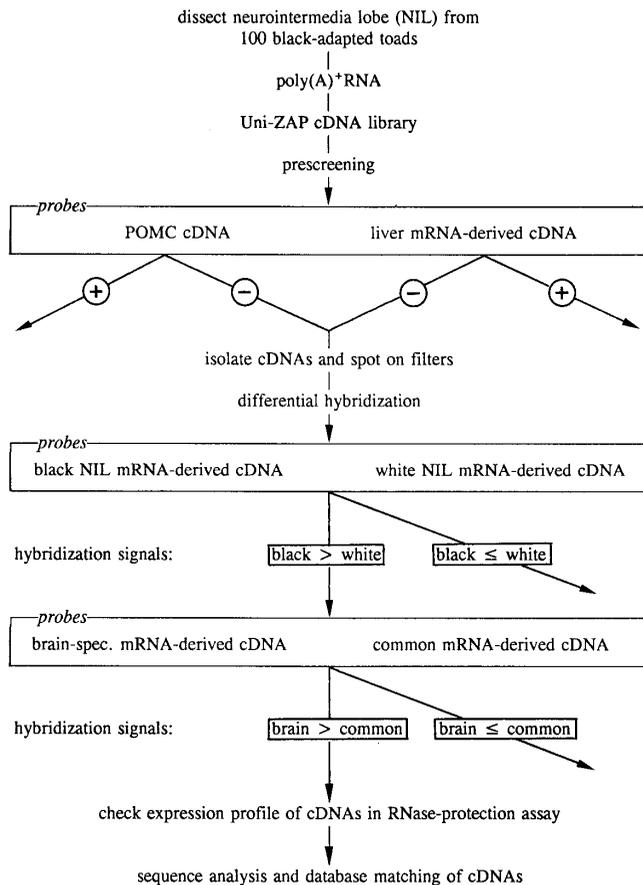


Fig. 2. Strategy for the identification of differentially expressed genes. See text for details.

(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/SWISS databases.

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 con-

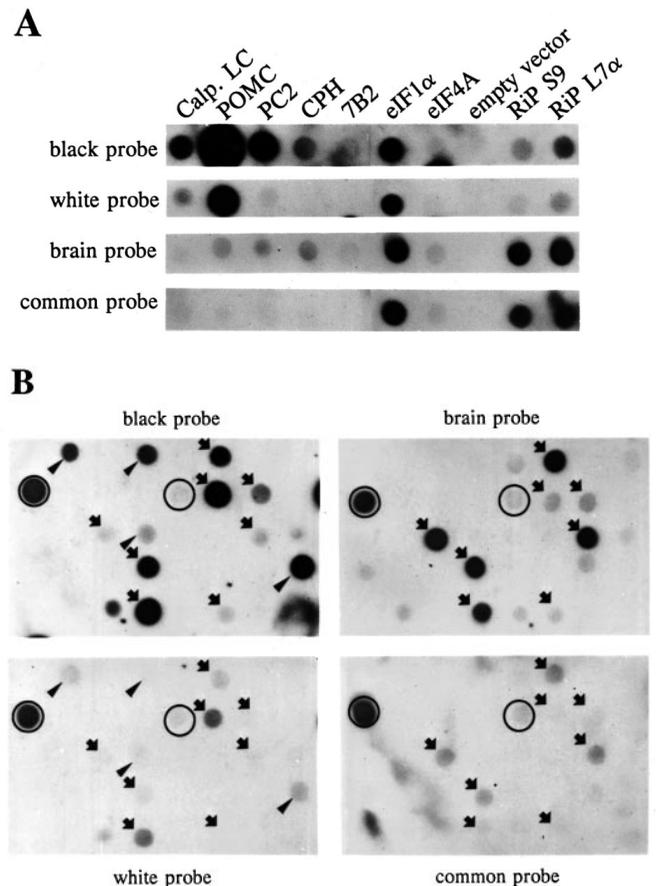


Fig. 3. Differential hybridization of NIL cDNA clones. Filters containing spotted DNA from isolated NIL cDNA clones were hybridized with cDNA probes derived from NIL mRNA of black and white animals (black and white probes), or cDNA probes enriched in brain-specific (brain probe) or commonly expressed sequences (common probe). (A) Previously identified NIL cDNA clones serving as a reference for the differential screening procedure. Calp. LC, calpactin light chain; POMC, proopiomelanocortin; PC2, prohormone convertase 2; CPH, carboxypeptidase H; 7B2, neuroendocrine protein 7B2; eIF1 α , translation initiation factor 1 α ; eIF4A, translation initiation factor 4A; empty vector, pBluescript SK⁻; RiP S9, ribosomal protein S9; RiP L7 α , ribosomal protein L7 α . (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.

servation 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

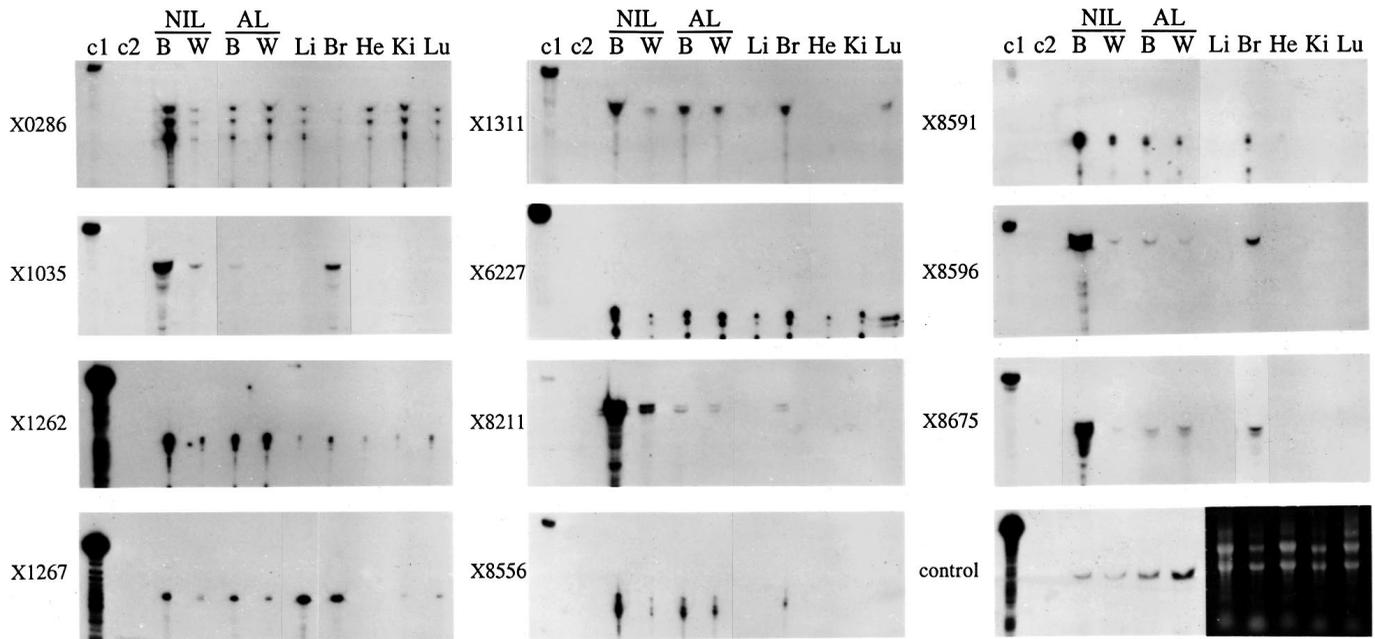


Fig. 4. RNase-protection analysis of differentially expressed genes. Radiolabeled anti-sense RNA was transcribed from selected NIL cDNA clones and hybridized to total RNA extracted from the NIL and AL of black (B) and white (W) animals, or to 5 µg total RNA from liver (Li), brain (Br), heart (He), kidney (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 demon-

strated that in rat intermediate pituitary these genes are coregulated 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

Clone	Freq*	Black/white Ratio†	mRNA (kb)‡	Protein (kDa)§	Database match	Entry	Species¶	Matched length (aa)	% Identity
X1035	10	25	2.2	71	Prohormone convertase PC2	S23118	X	614	100
X6243	4	25	4.4	71	Prohormone convertase PC2	S23118	X	183	100
X8211	4	20	1.2	23	Neuroendocr. polypeptide 7B2	S03938	X	209	100
X8675	4	35	4.4	–	Carboxypeptidase H	A40469	R	65	79
X1151	3	35	2.0	–	Carboxypeptidase H	A40469	R	132	89
X8556	2	15	n.d.	68	Secretogranin II	S02180	R	574	49
X8596	2	30	2.1	57	Secretogranin III	A37180	R	435	61
X0286	1	20	0.8	18	Translocon-associated protein δ	S33295	R	150	81
X1262	1	20	1.2	20	Glycoprotein 25L precursor	G25lcanfa	D	183	32
X1267	1	6	2.5	–	ERV1 protein	ERV1yeast	Y	72	27
X1311	1	10	2.3	48	Vacuolar H ⁺ -ATPase subunit	BT10039	B	430	60
X6227	1	10	n.d.	–	ER60 protease precursor	ER60mouse	M	165	72
X8290	1	8	8.0	–	no match	–	–	–	–
X8591	1	10	n.d.	–	no match	–	–	–	–

*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.

||cDNAs corresponding to larger transcripts generated by the use of alternative polyadenylation sites.

n.d., not determined.

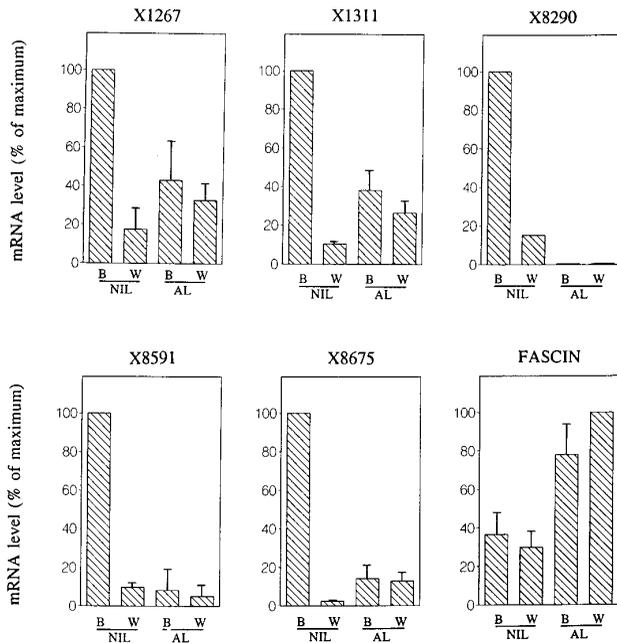


Fig. 5. mRNA levels of differentially expressed genes in the NIL and AL of black (B) and white (W) animals. mRNA levels were determined by RNase-protection analysis using radiolabeled anti-sense RNA transcribed from selected NIL cDNA clones (X1267, X1311, X8290, X8591 and X8675) and aliquots of total 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 \pm s.e.m. of three independent experiments.

1989). The protein sequences deduced from clones X8556 and X8596 display 49% and 61% identity with rat secretogranin (SG)II (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 and 30 for SGIII. All three proteins belong to a class of acidic secretory granule-associated proteins, also known as the granin 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, 1995) 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 TRAP δ (clone X0286). Both ER60 and TRAP δ 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 TRAP δ 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. TRAP δ 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 TRAP δ 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-

ocrine tissues and regulated in coordination with the biosynthetic activity of peptide hormone-producing cells.

Novel gene products

Four of the regulated transcripts isolated in this study are derived from previously unknown genes. Two of these, clones X8290 and X8591, gave no match with sequences in the database. Northern blot experiments revealed a single brain- and pituitary-specific transcript of 8 kb for X8290 (Fig. 6A,B), yet failed to produce a specific hybridization signal for X8591. RNase protection analysis showed that the X8591 transcript is predominantly, but not exclusively, expressed in brain and pituitary (Fig. 4). Levels of X8290 and X8591 transcripts in NILs of black animals are 8- and 10-fold higher, respectively, than in those of white animals (Fig. 5). The X8290 and X8591 cDNAs lack open-reading frames of significant length. Rescreening of a *Xenopus* hypothalamus cDNA library yielded clones with insert sizes up to 3.5 kb. None of these, however,

contained open-reading frames and they all seem to represent 3'-untranslated regions of long transcripts. Work is in progress to obtain 5'-extended cDNAs.

Clone X1267 represents a transcript of 2.5 kb, which was detected by northern blot analysis in *Xenopus* liver (Fig. 6B). RNase protection assays revealed lower amounts of the transcript in a variety of other tissues (Fig. 4). NILs of black animals contain sixfold higher levels of this mRNA than those of white animals (Fig. 5). X1267 cDNA contains an open-reading frame that shares 27% identity over 72 matched amino acids with the yeast *ERV1* gene product (Lisowsky, 1992). A zinc finger-like motif (HXF/YXCXXCAXXFXR/K, corresponding to *Erv1p* residues 54-67) was found within a well-conserved region. The *ERV1* gene is essential for yeast viability and experimental data suggest that it encodes an important component for the expression or stability of nuclear and mitochondrial transcripts (Lisowsky, 1992). The structural resemblance between the X1267 protein and the *ERV1* gene

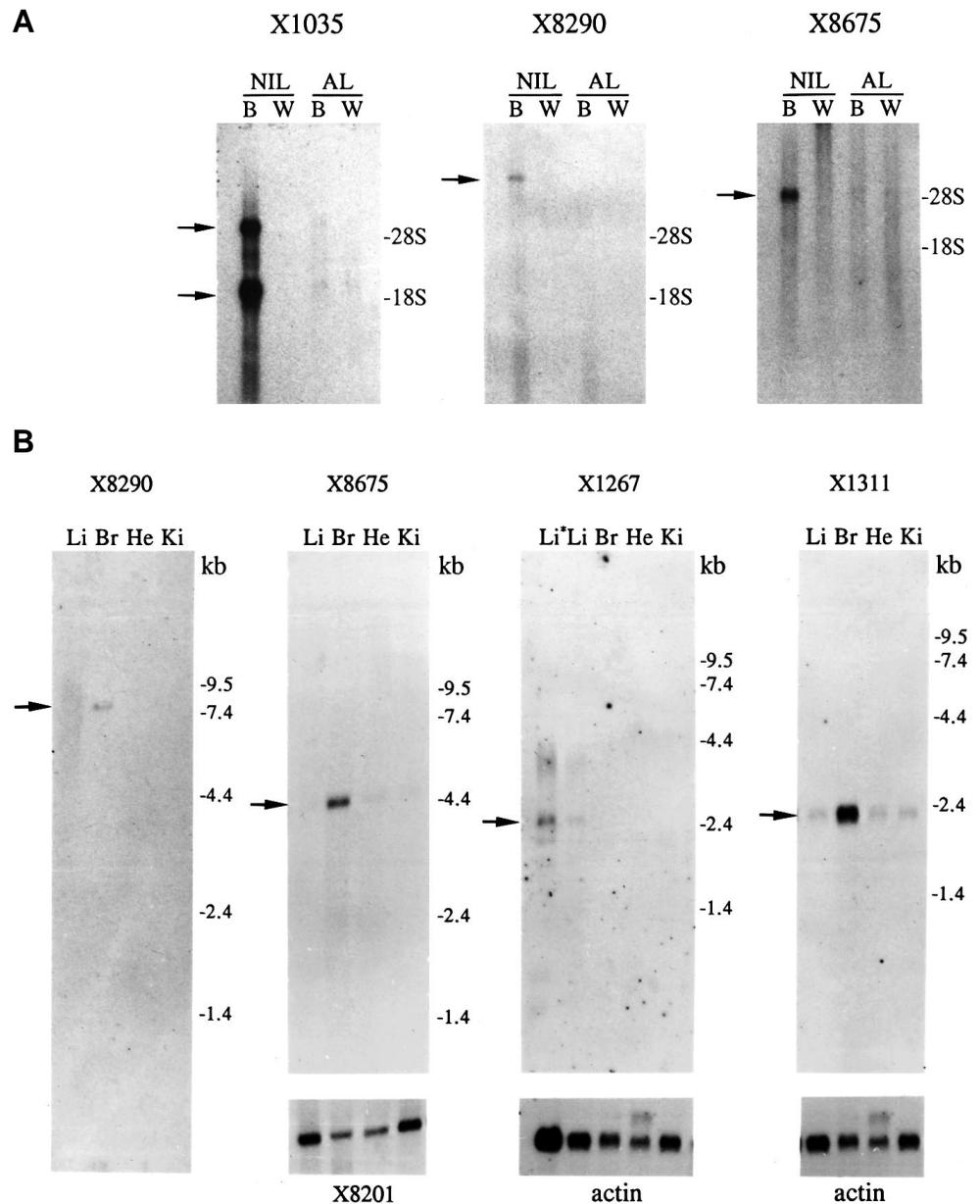


Fig. 6. Northern blot analysis of differentially expressed genes. (A) Random prime-labeled inserts from NIL cDNA clones X1035, X8290 and X8675 were hybridized to total RNA extracted from NILs and ALs of black (B) and white (W) animals. Each lane contains RNA extracted from five pooled lobes, except those hybridized with the X8290 probe (ten pooled lobes). The mobility of 18 S and 28 S ribosomal RNA is indicated. (B) Hybridization of random prime-labeled inserts from NIL cDNA clones X8290, X8675, X1267 and X1311 to 6 µg poly(A)⁺ RNA from liver (Li), brain (Br), heart (He) and kidney (Ki) or 20 µg liver poly(A)⁺ RNA (Li*). As a control for loading, blots were stripped and rehybridized with radiolabeled inserts from cDNA clones representing ubiquitously expressed transcripts (X8211 or actin). The mobility of size markers is indicated.

product together with the finding that its transcript levels in *Xenopus* melanotropes are regulated in parallel with those of POMC make X1267 an attractive candidate for a regulator of gene expression in these cells.

The 1.3 kb transcript corresponding to clone X1262 was detected in all tissues examined, both by RNase protection assay (Fig. 4) and northern blot analysis (data not shown). About 20-fold more X1262 mRNA was measured in NILs of black animals compared to those of white animals. The protein encoded by X1262 has a low but significant degree of identity (32% over 183 matched residues) with ER-associated glycoprotein gp25L (Table 1). The proteins are similar in size and both carry an N-terminal signal sequence, a C-terminal transmembrane domain and a double lysine motif that may function as a retention signal for ER transmembrane proteins. gp25L has been isolated from dog pancreatic microsomes as a complex together with two members of the TRAP tetramer (TRAP α and TRAP β) and the Ca²⁺ binding protein calnexin (Wada et al., 1991). The role of gp25L and the significance of its association with calnexin and members of the TRAP complex remain to be established. We noticed that gp25L and the X1262 protein share an overall sequence identity of 27% with protein P24B from yeast. P24B is a component of ER-derived transport vesicles with an essential role in the delivery of a subset of secretory proteins to the Golgi complex (Schimmöller et al., 1995).

X1262 is clearly not the *Xenopus* homolog of gp25L or P24B. An amino acid sequence deduced from two overlapping human expressed sequence tags (EMBLnew accession numbers hs23814 and t10797) displays 85% sequence identity over 100 matched residues with the X1262 protein. Hence, the X1262 protein is highly conserved during evolution and belongs to a novel family of ER transmembrane proteins whose members occur throughout the animal kingdom.

The nature of the genes described above demonstrates that our differential screening method can be successfully applied as a strategy for the isolation of components from the secretory pathway in peptide hormone-producing cells. We identified a number of regulated secretory proteins (PC2, CPH, 7B2, SGII and SGIII) whose recruitment in the melanotropes of black-adapted *Xenopus* is not an unexpected finding, given that such proteins are generally stored and released together with peptide hormones. We also tracked down one soluble and several transmembrane proteins representing permanent residents of the secretory pathway. Three of these (ER60, TRAP δ and the gp25L-like protein) are retained in the ER, whereas a fourth protein (Ac45) belongs to the membrane moiety of V-ATPase from secretory granules. The impressive (10- to 20-fold) rise in transcript levels for these non-secreted proteins in stimulated melanotropes most likely reflects, at the molecular level, the

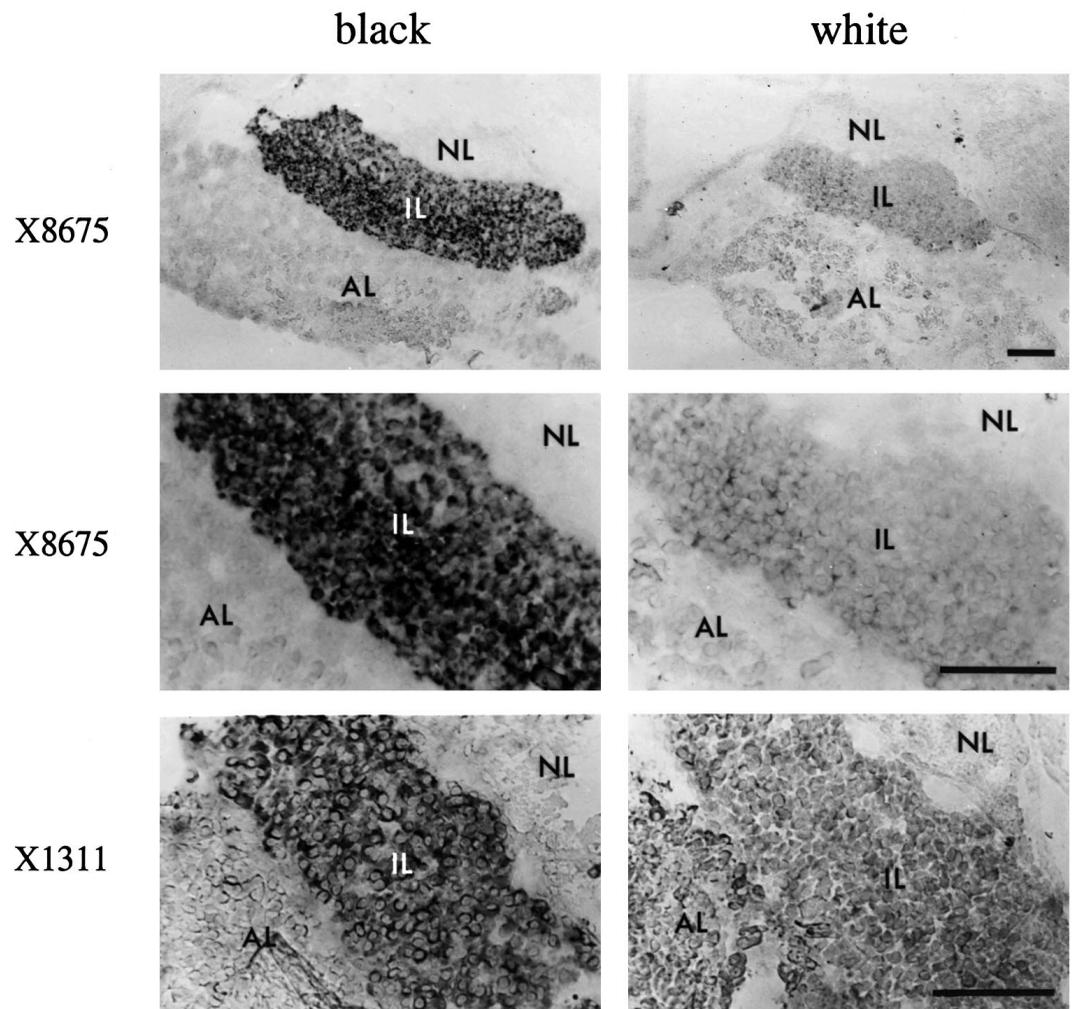


Fig. 7. In situ hybridization analysis of X8675 and X1311 transcripts in pituitary glands of black- and white-adapted *Xenopus*. Cryosections of pituitaries from black and white animals were hybridized under identical conditions with anti-sense digoxigenin-labeled RNA probes. Both probes give strong hybridization signals in the melanotropes of the intermediate lobe (IL) of black animals, whereas only weak signals are obtained in melanotropes of white animals. Note that cells of the anterior lobe (AL) are moderately stained without any difference in intensity between black and white animals. No staining is present in the neural lobe (NL). Control experiments using sense digoxigenin-labeled RNA probes failed to produce any signal above background (not shown). Bar, 250 μ m.

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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