Transgene-driven protein expression specific to the intermediate pituitary melanotrope cells of *Xenopus laevis*

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Abstract In the present study, we examined the amphibian *Xenopus laevis* as a model for stable transgenesis and in particular targeted transgene protein expression to the melanotrope cells in the intermediate pituitary. For this purpose, we have fused a *Xenopus* proopiomelanocortin (POMC) gene promoter fragment to the gene encoding the reporter green fluorescent protein (GFP). The transgene was integrated into the *Xenopus* genome as short concatemers at one to six different integration sites and at a total of one to ~20 copies. During early development the POMC gene promoter fragment gave rise to GFP expression in the total prosencephalon, whereas during further development expression became more restricted. In free-swimming stage 40 embryos, GFP was found to be primarily expressed in the melanotrope cells of the intermediate pituitary. Immunohistochemical analysis of cryosections of brains/pituitaries from juvenile transgenic frogs revealed the nearly exclusive expression of GFP in the intermediate pituitary. Metabolic labelling of intermediate and anterior pituitaries showed newly synthesized GFP protein to be indeed primarily expressed in the intermediate pituitary cells. Hence, stable *Xenopus* transgenesis with the POMC gene promoter is a powerful tool to study the physiological role of proteins in a well-defined neuroendocrine system and close to the in vivo situation.

Key words: Proopiomelanocortin; Pituitary; Gene expression; Transgenesis; *Xenopus laevis*

1. Introduction

Recently, a number of complete genome sequences have become available. Many of these sequences encode proteins with an unknown function, and analysis of the roles of these proteins will be the next scientific challenge. One approach for functional analysis concerns transfection of tumor cells in culture. However, this approach has several drawbacks, in particular when using cultured neuroendocrine tumor cells since such cells have lost many of their neuroendocrine characteristics [1]. A direct way to study protein function is by generating transgenic animals to specifically manipulate the expression of the protein of interest. A unique feature of transgenesis is namely the ability to target transgene expression in a restricted spatial and/or temporal manner by the use of specific promoter sequences. The most frequently used transgenic experimental animal is the mouse but this approach is time consuming and rather expensive, indicating the need for a system to rapidly screen for the functions of many unknown proteins. Recently, the stable introduction of a transgene in the South-African claw toed frog *Xenopus laevis* has been achieved [2,3]. This system can be used for the introduction of many transgene constructs in a relatively short time and in a cost-effective way.

Our interest lies in the molecular analysis of the functioning of neuroendocrine cells. To study these cells, we use the process of background adaptation of the amphibian *X. laevis* as a model system. The dispersion of black pigment in the melanophores of the skin of *Xenopus* is caused by the α-melanophore stimulating hormone (αMSH), which is secreted by the melanotrope cells of the intermediate pituitary [4]. The precursor protein giving rise to αMSH is the prohormone proopiomelanocortin (POMC) that is produced in high amounts in active *Xenopus* melanotrope cells, and POMC mRNA represents about 80% of the total mRNA content in these activated cells [5]. The first melanophores develop in stage 33/34 *Xenopus* tadpoles [6] and the first immunoreactivity for αMSH can be found in stage 35/36 embryos, just before the beginning of the differentiation of the adenohypophysis into the intermediate pituitary and anterior pituitary [7]. In situ hybridization analysis has shown that POMC mRNA can be detected in the pituitary primordium of stage 28 hatching embryos and in the forebrain of stage 29/30 embryos [8]. With the more sensitive reverse transcriptase (RT)-polymerase chain reaction (PCR) technique, POMC mRNA was detected already in the neural plate stage (stage 13) embryos [9]. In view of our research goal, we have used the new technique of stable *Xenopus* transgenesis and the reporter green fluorescent protein (GFP) under the control of an approximately 500-bp *X. laevis* POMC gene promoter fragment to target GFP to the melanotrope cells. This study reports for the first time the use of *Xenopus* transgenesis in the field of neurobiology and endocrinology, and demonstrates that this approach will be a helpful tool to establish the roles of proteins in neuroendocrine cells.

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Abbreviations: ACTH, adrenocorticotropic hormone; AL, anterior lobe; GFP, green fluorescent protein; α-MSH, α-melanophore stimulating hormone; NIL, neurointermediate lobe; POMC, proopiomelanocortin; VH, ventral hypothalamic nucleus

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2. Material and methods

2.1. Generation of DNA constructs

A 529-bp DNA fragment containing nucleotides −487 to +41 of the *Xenopus* POMC gene A promoter (with +1 being the transcription initiation site) was amplified by PCR using *Xenopus* genomic clone λXPA5 [10] as a template. The primers used were: pPOMC5′: 5′-ACGCGTCGACGGTACCCCGTGTAAATGTCCCTCTCC-3′ and pPOMC3′: 5′-TAAGAAGCTTCACTAGTCCCAAGCTGTGC-3′. The pPOMCGFP construct was generated by replacing the CMV promoter in the pCSGFP2 construct (kindly provided by Dr. E. Amaya, Wellcome, Cambridge, UK) by the *Xenopus* POMC promoter (*Sal*I/*Hin*dIII fragment). The pPOMCGFP construct was checked by cycle sequencing using the Big Dye Ready Reaction system (Perkin Elmer), and the pPOMC5′ primer. Fig. 1 presents a schematic overview of construct pPOMCGFP with the *Xenopus* POMC gene promoter fragment fused to GFP cDNA.

2.2. Genomic DNA analysis

Genomic DNA was isolated either from liver of transgenic frogs or from whole tadpoles, as described previously [11]. 5 μg DNA, or the total amount isolated from one tadpole, was digested with *Hin*dIII in a volume of 100 μl, precipitated and separated on an 0.7% agarose gel. The gel was depurinated with 0.25 M HCl and the DNA was transferred to Nytran Super Charge nylon transfer membrane (Schleicher and Schuell) in 40 mM NaOH. The blot was then prehybridized in Church (0.5 M Na-P, 7% sodium dodecyl sulfate (SDS), 10 mM EDTA) for 30 min, at 68°C. 25 ng *Bam*HI/*Xba*I fragment of the pPOMCGFP construct (corresponding to the open reading frame (ORF) of GFP) was labelled with ^{32}P using the Random Primers Labeling system (Gibco-BRL) and [^{32}P]dCTP (Amersham-Pharmacia Biotech), and used for hybridization at 68°C in Church. The blot was subsequently washed until 0.5×SSC/0.1% SDS at 22°C and exposed to Kodak X-Omat X-ray film at −70°C.

2.3. Preparation of Xenopus unfertilized eggs

Mature female *Xenopus* (Xenopus Express, Cape Town, South Africa), were injected in their dorsal lymphatic cavities with 375 IU human gonadotropic hormone (Pregnyl, Organon, The Netherlands) 18 h before the removal of eggs. Eggs were squeezed from the females onto the bottom of a petri dish, dejellied with 2% cysteine (pH 8.2) and immediately used for transgenesis. All animal experiments were carried out in accordance with the European Communities Council Directive 86/609/EEC for animal welfare, and permit TRC 99/15072 to generate and house transgenic *X. laevis*.

2.4. Generation of transgenic Xenopus embryos

A 1557-bp *Sal*I/*Not*I fragment containing the pPOMCGFP construct and the SV40 pA signal (Fig. 1) was purified using a Qiaex II Gel Extraction Kit (Qiagen). This fragment (100 ng/2 μl) was mixed with sperm nuclei (2.5×10^5 in 2.5 μl). The mixture was diluted to 500 μl and ∼10 nl was injected per egg. As described previously [3,12], a Percoll gradient was used to purify the sperm nuclei, digitonin was used for permeabilization and the restriction enzyme mediated integration reaction was omitted. Normally cleaving embryos were selected at the four-cell stage and cultured in 0.1×MMR/6% Ficoll-400 with 50 μg/ml gentamicin at 18°C until gastrulation (stage 12) was reached. At that time point, embryo culturing was continued in 0.1×MMR with 50 μg/μl gentamicin and the temperature was raised to 22°C. From stage 45 onwards, tadpoles were raised in tap water at 22°C. The presence of GFP fluorescence was examined in living embryos anesthetized with 0.25 mg/ml MS222 (3-aminobenzoic acid ethyl ester; Sigma) using a Leica MZ FLIII fluorescent stereomicroscope and photographs were taken with a Leica DC200 color camera using the Leica DCviewer software.

Staging of *Xenopus* embryos was carried out according to Nieuwkoop and Faber [13].

2.5. Cryosectioning and immunohistochemistry

Brain-pituitary preparations were dissected from juvenile transgenic frogs and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS). After cryoprotection in 10% sucrose–PBS, sagittal 20 μm cryosections were mounted on poly-L-lysine coated slides and dried for 2 h at 45°C. To study GFP expression, sections were directly viewed under the fluorescent microscope.

For immunohistochemistry, sections were rinsed for 30 min in 50 μM Tris-buffered saline (pH 7.6), containing 150 μM NaCl and 0.1% Triton X-100 (TBS-TX). To prevent non-specific binding, blocking was performed with 0.5% bovine serum albumin (BSA) in TBS-TX. An anti-serum directed against the POMC-derived adrenocorticotropic hormone (ACTH) [14] was used as primary antibody in a dilution of 1:2000 for 16 h at 37°C in TBS-TX containing 0.5% BSA. After rinsing the slides with TBS-TX, a second antibody, goat anti-rabbit Texas Red (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at a dilution of 1:50, was applied and sections were incubated for 1 h at 37°C. Following an additional washing step, the sections were mounted in Citifluor (Agar Scientific, Stansted, Essex, UK) and coverslipped. Immunofluorescence was viewed under a Leica DM RA fluorescent microscope and photographs were taken with a Cohu High Performance CCD Camera using the Leica Q Fluoro software.

2.6. Metabolic cell labelling and immunoprecipitation analysis

Neurointermediate lobes (NILs) and anterior lobes (ALs) of pituitaries from transgenic and wildtype *Xenopus* were dissected and starved in methionine- and cysteine-free XL-15 medium (67% L-15 medium; Gibco-BRL, Gaithersburg, MD, USA) for 30 min at 22°C. The lobes were subsequently pulsed in methionine-and cysteine-free medium containing 5 mCi/ml ^{35}S- Promix (Amersham/Pharmacia Biotech) for 3 h at 22°C. The lobes were then homogenized on ice in lysis buffer (50 mM HEPES pH 7.2, 140 mM NaCl, 1% Tween 20, 0.1% Triton X-100, 0.1% deoxycholic acid, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg/ml soyabean trypsin inhibitor). The lysates were incubated on ice for 15 min, cleared by centrifugation (7 min, 13 000×*g*, 4°C) and supplemented with 0.1% SDS. 10% of the lysate was directly separated on SDS–PAGE (total lysates), while the remainder was used for immunoprecipitation, employing a 1:500 dilution of an anti-GFP anti-serum (kindly provided by Dr. B. Wieringa, Department of Cell biology, University of Nijmegen, The Netherlands). Immune complexes were precipitated with protein-A Sepharose (Amersham-Pharmacia Biotech) and resolved by SDS–PAGE, and radiolabelled proteins were visualized by fluorography.

3. Results

3.1. Generation of Xenopus embryos transgenic for the Xenopus POMC gene promoter linked to GFP cDNA

For our transgenesis studies, we cloned the promoter of *Xenopus* POMC gene A (nucleotides −487 to +41, with +1 representing the transcription initiation site [10]) by performing a PCR reaction with specific POMC promoter primers on *Xenopus* POMC genomic clone λXPA5 [15]. This amplification resulted in a 529-bp DNA fragment (pPOMC) that contains several transcriptional regulatory elements, such as a TATA box, a serum responsive element, activator protein-1 and -2 binding sites and a negative glucocorticoid responsive element [15]. Also, the binding site for the pituitary-specific factor PitX1 [16], the NurRE for Nur77 [17] and elements for Neuro1D/BETA2 binding [18] but not the binding site of the recently identified PitX1 binding Tbox factor (Tpit [19]) are present in this *Xenopus* POMC gene promoter fragment. The fragment was subsequently used to replace the CMV promoter in pCSGFP2, giving the construct pPOMCGFP (Fig. 1). To generate *Xenopus* transgenic for the pPOMCGFP construct, the recently described [2] and subsequently modified [3,12] method for stable *Xenopus* transgenesis was used. To establish that the construct had integrated into the genome, we isolated genomic DNA from transgenic tadpoles and from livers of transgenic frogs. *Hin*dIII-digested genomic DNA was analyzed on Southern blots using a probe corresponding to the ORF of GFP. The transgene was found to be integrated into the genome of the tadpoles and frogs in multiple concatemeric copies, and the number of integration sites varied

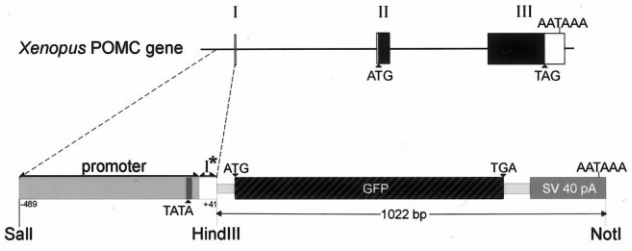

Fig. 1. Schematic representation of the structural organization of the *Xenopus* POMC gene and the pPOMCGFP construct used for *Xenopus* transgenesis. The protein-coding parts of exons I, II and III of the *Xenopus* POMC gene are indicated with black boxes. The pPOMCGFP transgene construct contains the 529-bp *Xenopus* POMC gene promoter fragment including part of exon I (I*) and a cDNA encoding the marker GFP. Indicated are restriction sites used for isolation of the fragment for transgenesis experiments (*Sal*I, *Not*I) and for analysis of genomic DNA (*Hin*dIII).

between one and six per genome. The total number of copies integrated varied between one and ~20 per genome, as determined by densitometric analysis of the Southern blot. The prominent presence of the 2044-bp hybridizing fragment, which is the result of tail to tail concatemerization, indicates that most of the fragments are indeed integrated as concatemers (Fig. 2).

3.2. GFP expression during early development of pPOMCGFP transgenic Xenopus

Injected, healthy looking *Xenopus* embryos were selected at the four-cell cleavage stage, cultured and screened for fluorescence during early development. The first indication of GFP expression in the transgenic embryos was observed around stage 25 (i.e. before hatching). A diffuse fluorescence was detected in a broad area around the presumptive eye, indicating that the promoter fragment is active in the total prosencephalon and especially in the region differentiating into the eye vesicle and future diencephalon (ST25, Fig. 3). During further development the localization of the fluorescent signal became more restricted to the forebrain, although some fluorescent signal was also detected in the olfactory placode (ST31, Fig. 3). At stage 37/38, most of the fluorescence was observed in the lower part of the forebrain, whereas the signal in the olfactory placode still remained detectable (ST37/38, Fig. 3). At stage 40, a clear bright signal was visible near the midbrain in the region where the pituitary differentiates. In about 50% of the transgenic tadpoles, GFP fluorescence was also observed in the anterior border region of the diencephalon (ST40, Fig. 3). The fluorescent pituitary signal could be detected until stage 50; in subsequent stages, the tissue surrounding the pituitary prevented the excitation of pituitary GFP. To examine transgene GFP expression in >stage 50 tadpoles, we dissected the brain with the pituitary attached of a stage 56 transgenic tadpole. The most intense fluorescent signal was located at the ventral side of the brain, in the region of the intermediate pituitary. In whole-mount preparations, no fluorescence was observed in other brain regions (Fig. 4).

3.3. GFP expression is localized to the intermediate pituitary cells of pPOMCGFP transgenic Xenopus embryos

In order to investigate the exact site of GFP expression, we dissected the brain, with the pituitary attached, of a juvenile

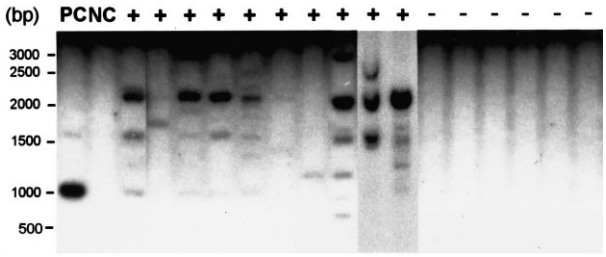

Fig. 2. Integration of the pPOMCGFP transgene into the genome of *X. laevis*. Genomic DNA was isolated from tadpoles and frogs displaying fluorescence in the pituitary or from animals with no fluorescence (controls). 5 μg stage 45 embryonic genomic DNA or genomic liver DNA was digested with *Hin*dIII and analyzed by Southern blot using the ORF of GFP as a probe. Note the prominent presence of the 2044-bp fragment that results from tail to tail ligation, indicating that most of the fragments are integrated as tail to tail concatemers. PC, 10 ng *Sal*I/*Not*I plasmid control digested in the presence of 5 μg genomic DNA isolated from a control animal. NC, negative control genomic DNA isolated from a control animal. +, fluorescence observed in pituitary; −, no fluorescence observed in pituitary.

pPOMCGFP transgenic frog and prepared sagittal cryosections. We observed a bright fluorescent signal in the intermediate lobe of the pituitary, the tissue consisting of POMC-producing melanotrope cells. No clear GFP signal was detected in sections containing the anterior pituitary, in which the POMC-producing corticotrope cells are located (Fig. 5b). To study whether the GFP protein produced by the transgene colocalizes with endogenously expressed POMC and its cleavage products, the sections were incubated

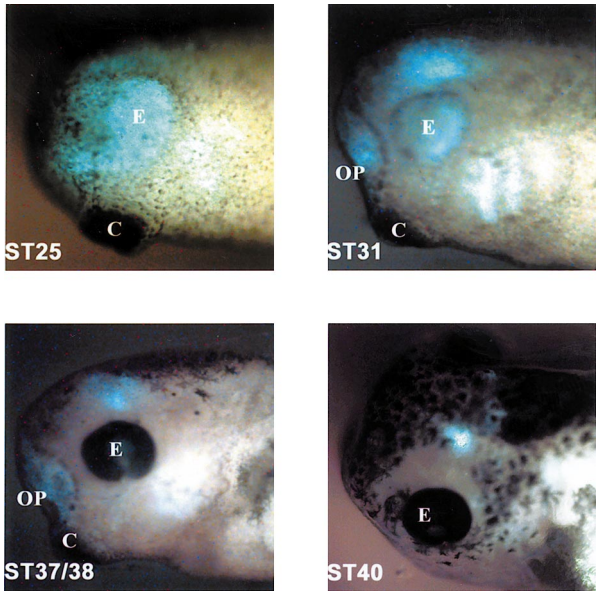

Fig. 3. GFP expression driven by the 529-bp POMC promoter fragment during early *Xenopus* development. Expression of GFP was detected from stage 25 onwards. At this stage (ST25) GFP is expressed in the total prosencephalon and especially in the region differentiating into the eye vesicle and the diencephalon. At stages 31 and 37/38 (ST31 and ST37/38), expression of GFP becomes restricted to the forebrain and the olfactory placode (OP). Stage 40 tadpoles (ST40) show high GFP expression in the region where the pituitary is located. In a number of animals, GFP expression is also found in the anterior part of the diencephalon. Note that the GFP signal is blue, due to the correction for yolk autofluorescence. E, (presumptive) eye; C, cement gland; OP, olfactory placode.

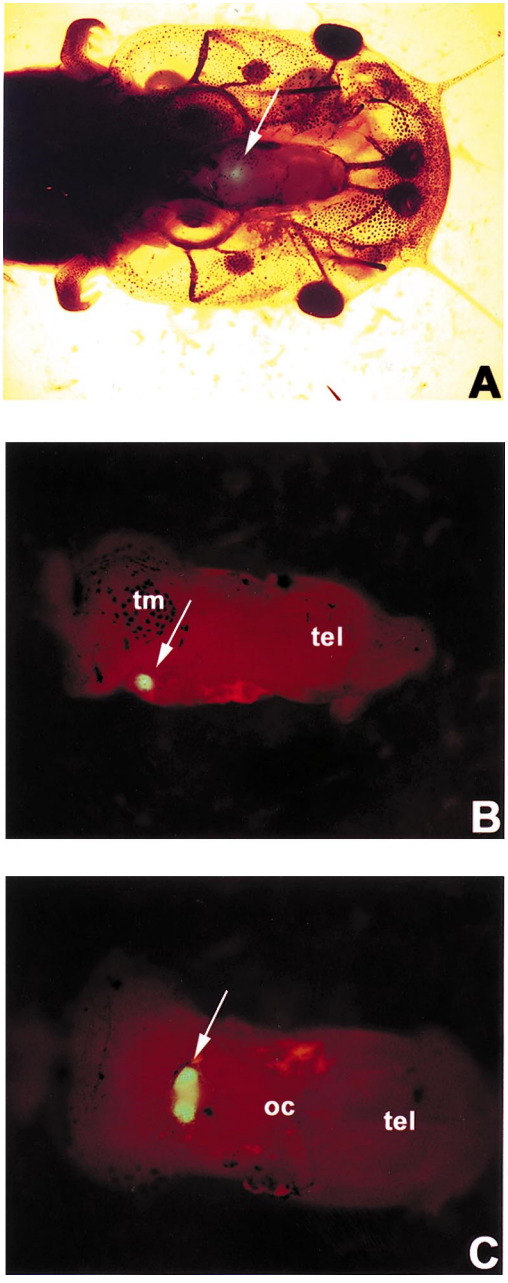

Fig. 4. Whole-mount analysis of GFP expression in a stage 56 pPOMCGFP transgenic *Xenopus* tadpole. Dorsal view on the tadpoles brain where GFP fluorescence can be observed in the pituitary region only after exposing the brain (A). Lateral (B) and ventral (C) views on the transgenic brain show bright fluorescence in the pituitary. tm, tectum mesencephali; tel, telencephalon; OC, optic chiasma.

with an anti-ACTH antibody recognizing ACTH as well as POMC itself ([14] and our unpublished observations). Immunoreactivity was found in the intermediate pituitary, colocalizing with the site of GFP expression (Fig. 5a,c). In addition, the POMC-producing cells in the anterior pituitary and several brain regions, for instance the ventral hypothalamic nucleus (VH), were immunopositive for ACTH/POMC. We used higher magnifications to study the sections at the cellular level and to compare endogenous POMC expression with the transgene-driven GFP expression in more detail. The melanotrope cells in the intermediate pituitary were indeed found to express both POMC and GFP. The subcellular localization of the two proteins was however different. POMC and POMC-derived peptides were restricted to the Golgi and secretory granules, while GFP was found both cytoplasmic and in the nuclei of these cells (Fig. 6a–c). POMC immunoreactivity was

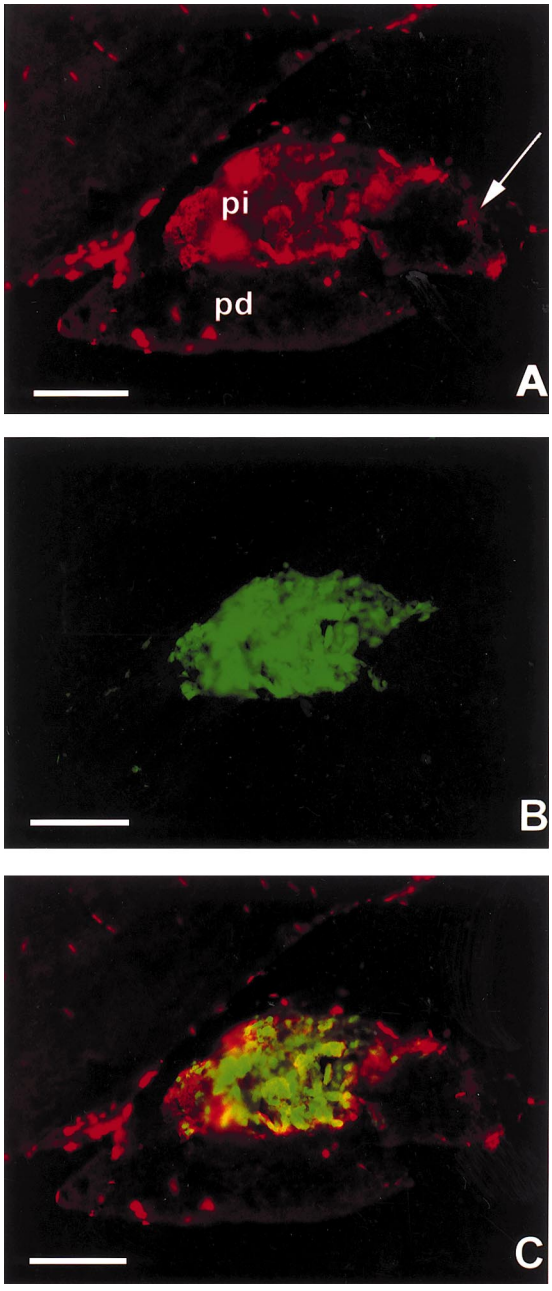

Fig. 5. GFP is primarily expressed in the intermediate pituitary of pPOMCGFP transgenic juvenile *Xenopus*. Sagittal brain-pituitary cryosections of a pPOMCGFP transgenic frog were analyzed. A: Endogenous POMC expression. Sections were stained for POMC using an anti-ACTH antibody and a Texas Red conjugated second antibody. High levels of POMC expression were found in the intermediate pituitary (pi), whereas lower levels of expression were detected in a subregion of the anterior pituitary (pd; indicated by an arrow). Other fluorescent signals were the result of autofluorescence. B: GFP fluorescence found in the intermediate part of the *Xenopus* pituitary. C: Merge of direct GFP fluorescent signal and endogenous POMC signal, showing colocalization of GFP and POMC in the intermediate pituitary. Scale bar, 100 μM.

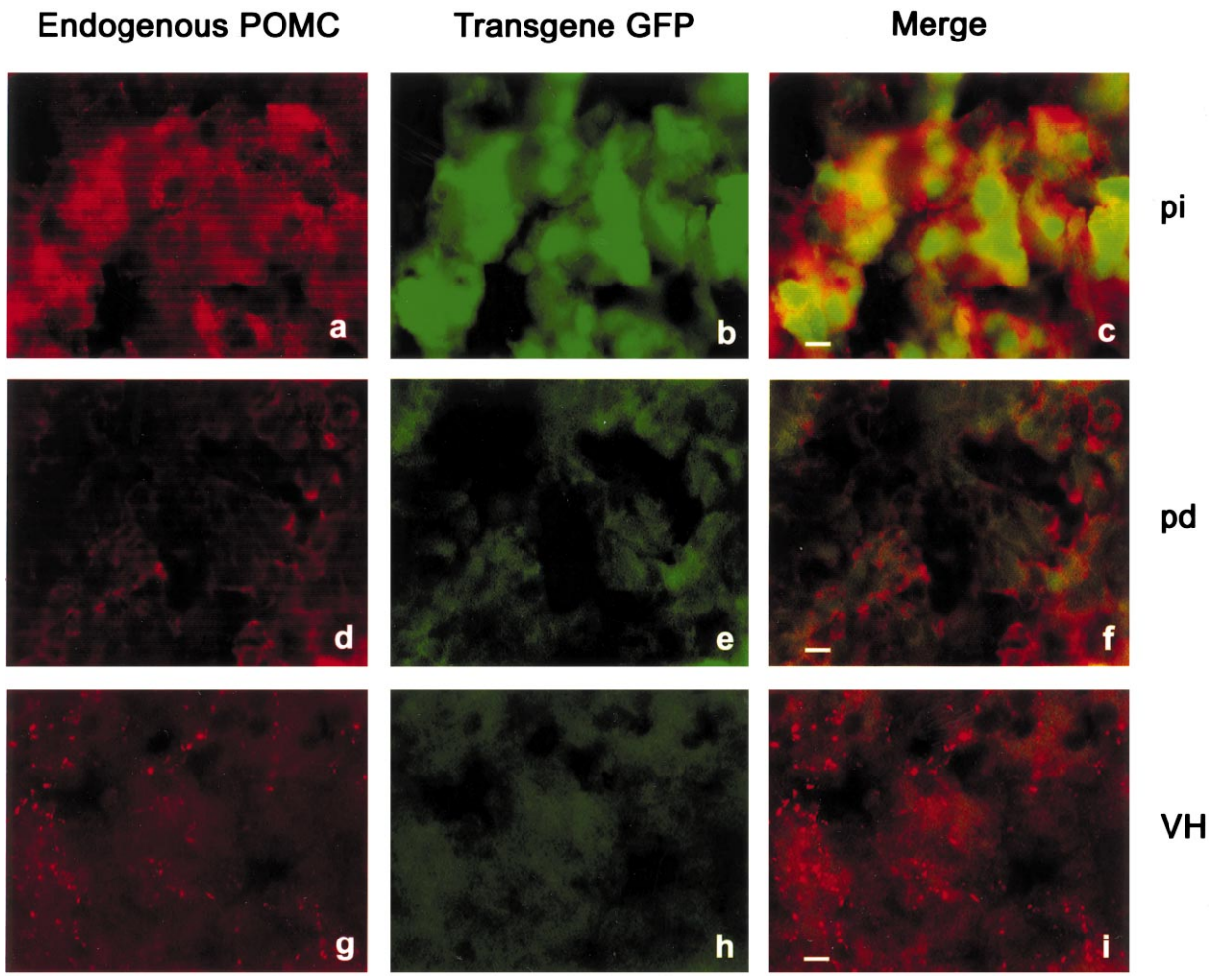

Fig. 6. Analysis at the cellular level of brain-pituitary cryosections of pPOMCGFP transgenic juvenile *Xenopus*. Using an anti-ACTH antibody, endogenous POMC was detected in the melanotrope cells of the intermediate pituitary (A), in the corticotrope cells of the anterior pituitary (D) and in brain cells, e.g. in the VH (G). GFP expression was observed only in the melanotrope cells of the intermediate pituitary (B), and not in the corticotrope cells or brain cells (E, H). Merging of the signals revealed co-expression of GFP and endogenous POMC in all melanotrope cells (C) and not in the corticotrope cells or brain cells (F, I). pi = intermediate pituitary, pd = anterior pituitary, VH = ventral hypothalamic nucleus. Scale bar, 10 μM.

also found in the corticotrope cells, whereas no signal above background was detected for GFP, indicating that the POMC promoter fragment used for transgenesis is not or only at low levels active in these cells (Fig. 6d–f). Similarly, the POMC-producing cells in the VH of the brain showed immunoreactivity with the anti-ACTH antibody but no GFP could be detected in these cells (Fig. 6g–i). Taken together, these results indicate that the transgene POMC promoter fragment is active only in the melanotrope cells and not in other cells producing POMC through the endogenous POMC gene promoter (anterior pituitary, brain VH).

3.4. *Biosynthesis of GFP in pPOMCGFP transgenic Xenopus intermediate pituitary cells*

To examine the amount of transgenic protein produced relative to endogenous protein expression, we metabolically labelled NILs and ALs of control and pPOMCGFP transgenic juvenile frogs, and compared transgene-driven GFP protein biosynthesis with endogenous POMC synthesis. Similar amounts of newly synthesized POMC were produced in the control and transgenic NILs, indicating that the POMC transgene promoter fragment did not interfere with endogenous POMC expression. Also, the 37 kDa POMC precursor protein was similarly converted to 14–18 kDa POMC, showing that the presence of the transgene product did not influence the transport and cleavage of the endogenous POMC protein. Analysis of total lysates of the pulsed tissues showed the 37-kDa POMC precursor protein to be the major newly synthesized protein produced in the transgenic and the wild type NIL, whereas no prominent band of newly synthesized GFP was observed in the transgenic tissue (Fig. 7, lanes 1,3). The level of POMC biosynthesis was thus much higher than that of GFP expression. Semi-quantitative analysis by densitometric scanning and taking into account the number of methionines and cysteines in the two proteins, indicated that the transgene POMC promoter fragment is ~15-fold less active in the melanotrope cells than the endogenous promoter. Southern blot analysis of genomic DNA isolated from this transgenic frog showed that there was only one copy of the transgene present in the genome. Immunoprecipitation analysis using an anti-GFP antibody revealed that newly synthesized GFP was produced in the intermediate pituitary as a 32-kDa protein (Fig. 7, lane 5). Following a long exposure of the gel, GFP was also detected in the transgenic AL lysate

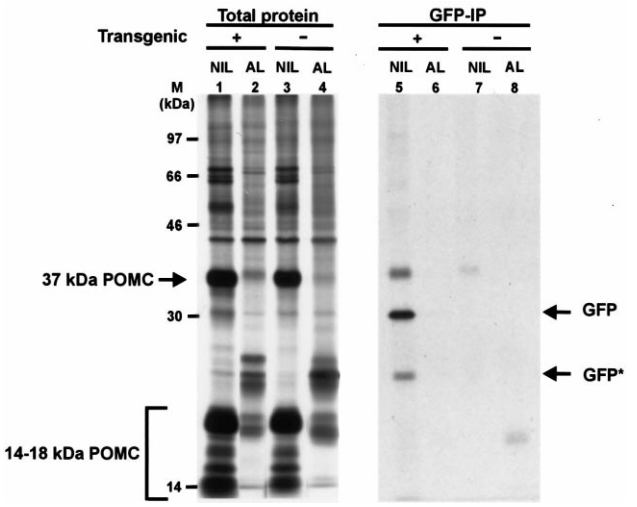

Fig. 7. Analysis of newly synthesized proteins produced by pituitary cells from pPOMCGFP transgenic *Xenopus*. Transgenic (+) and wild type (−) NILs and ALs were pulsed in the presence of ^{35}S methionine and cysteine for 3 h. 10% of the total lysates was directly analyzed (lanes 1–4). The remainder was immunoprecipitated with an anti-GFP antibody (lanes 5–8). Total lysates and immunoprecipitates were subjected to SDS–PAGE and radiolabelled proteins were visualized by fluorography (exposure times for NIL and AL total lysates were 4 and 16 h, respectively; exposure time for NIL and AL GFP-IP was 16 h). The product indicated with GFP* (lane 5) likely results from GFP cleavage since it is absent from the control lane (lane 7), even in a longer exposure of the gel. The low amount of co-immunoprecipitating 37-kDa POMC (lanes 5 and 7) is due to non-specific binding of this sticky protein.

(data not shown). Densitometric scanning revealed that the amount of GFP produced in the AL is ~1–2% of the amount produced in the NIL, indicating that the transgene POMC promoter fragment is not very active in the corticotrope cells.

4. Discussion

In the era of genomics and functional genomics, researchers are constantly exploring ways to analyze the physiological function of proteins. We use the melanotrope cells of the South African clawed toad frog *X. laevis* as a model for functional studies. These cells can be easily activated to transcribe the POMC gene to high levels and to secrete large amounts of the POMC-derived hormone α-MSH simply by placing the animal on a black background [4]. Recently, the technique of stable *Xenopus* transgenesis has been developed [2] and simplified [3] which allowed, for the first time, the stable insertion of a transgene into the genome of *X. laevis*, and spatial and/or temporal transgene expression. In this study, we combined the benefits of our model system with the possibility to target transgene protein expression to specific tissues by stable *Xenopus* transgenesis.

In order to target proteins to the melanotrope cells in the pituitary, we cloned a 529-bp fragment of the POMC gene promoter and used GFP as a marker protein. We found the transgene to be successfully integrated into the genome of *Xenopus*. In most cases more than one copy of the transgene had integrated as concatemers, at up to six different sites. This is in line with earlier studies on the use of other transgenes where 1–8 integration sites containing single copies or short concatemers of the transgene were found [2,3].

To get insight into the activity and specificity of the POMC gene promoter fragment during early embryonic development, we followed GFP expression in vivo. Since we observed the presence of GFP, albeit at low levels, from stage 25 onwards, we conclude that the promoter fragment is already active at that stage. Previous in situ hybridization studies on the localization of endogenous POMC expression during *Xenopus* development showed the presence of POMC mRNA in the pituitary primordium in stage 28 and in the forebrain in stage 29/30 embryos [8]. The fact that we found GFP expression in stage 25 embryos in the total prosencephalon indicates the occurrence of inhibitory elements in the endogenous POMC promoter that are not present in the fragment that was used to drive transgenic GFP expression. Alternatively, the previously used technique of in situ hybridization may not have been sensitive enough to detect any low endogenous POMC promoter activity at an early developmental stage, whereas in our present study GFP fluorescence was readily detectable. Using the more sensitive technique of RT-PCR, POMC mRNA has been detected already in neural plate stage 13 [9]. Apparently, the activity of the POMC gene promoter fragment is not high enough to detect transgene-driven GFP expression in these young embryos. Of further interest is the presence of GFP in the olfactory placode, in line with the fact that the POMC-derived peptide α-MSH is also found in this organ in *X. laevis* (Kramer and Roubos, manuscript in preparation) as well as in *Rana esculenta* [20]. During further development the expression of GFP became restricted to the pituitary. In some cases, cells in the anterior part of the diencephalon of stage 45 tadpoles were found to express GFP as well. Since we and others have observed multiple integration sites and different copy numbers of transgene integration among various transgenic *Xenopus* [2,3], the variations in GFP expression patterns may be due to the sites of integration of the transgene.

In the intact animal, we thus observed that GFP expression becomes restricted to the pituitary region and we therefore decided to analyze brains with pituitaries of juvenile transgenic frogs to study the expression in more detail and to compare GFP expression driven by the POMC promoter fragment with endogenously produced POMC. Although in *Xenopus* the major sites of POMC synthesis are the anterior and, in particular, intermediate pituitary, also other brain areas such as the hypothalamus, the olfactory bulb, the medial septum, the amygdala and the epiphysis, have been shown to produce POMC or its cleavage products [21]. Analyzing brain cryosections of the transgenic frogs, we did not detect any GFP expression outside the pituitary, not even in the ACTH-producing corticotrope cells of the anterior pituitary or in other brain areas which produce endogenous POMC, such as the VH.

Metabolic labelling of the pituitaries provided insight into the level of expression of the newly synthesized transgene GFP protein as well as into the site of transgene expression. Also at the level of the newly synthesized protein, GFP was almost exclusively present in the intermediate pituitary of the transgenic frogs, albeit at a much lower level than newly synthesized POMC. Since only after a long exposure time some GFP was detected in the lysate of the anterior pituitary, the melanotrope cells in the intermediate pituitary are indeed the major site of transgenic GFP expression. The fact that minor expression of GFP was found in the anterior pituitary after

the metabolic labelling and not at the fluorescence level is probably due to differences in the sensitivities of the detection methods used. Furthermore, the metabolic cell labelling showed that the introduction of the transgene did not affect endogenous POMC biosynthesis and processing, since the amounts of 37 and 14–18 kDa POMC were similar in the transgenic and wild type intermediate pituitaries. This finding is in line with the observation that the transgenic frogs adapt normally to a black background.

The fact that steady state and newly synthesized GFP was almost exclusively present in the melanotrope cells and not or at very low levels in the corticotrope cells of the pituitary or in POMC-producing brain cells indicates that the 529-bp POMC promoter fragment contains transcriptional elements that are primarily activated in the melanotropes. Alternatively, the promoter fragment may lack DNA elements that have an inhibitory effect on POMC gene transcription in the intermediate pituitary. Previous studies by others using a 300- to 400-bp mouse POMC promoter fragment to express a reporter protein in transgenic mice have also shown that such a small promoter region is sufficient for transgene expression in the pituitary, but not enough to express the transgene in neuronal cells [22,23]. It has been shown that to target the expression of transgenes into the central nervous system of the mouse in a POMC-neuron specific manner, distal 5′-sequences (between 2 and 13 kb upstream of the POMC gene) are necessary [24].

In conclusion, we have shown that the melanotrope cells of the intermediate pituitary can be specifically targeted for transgene expression using stable *Xenopus* transgenesis and a 529-bp POMC gene promoter fragment. Hence, this unique and well-defined system represents an attractive tool for cost-effective and efficient functional studies, and can now be used for functional neurogenomics close to the in vivo situation.

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