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## Differential Neuroendocrine Expression of Multiple Brain-Derived Neurotrophic Factor Transcripts

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Brain-derived neurotrophic factor (BDNF) is a neurotrophin with important growth-promoting properties. We report here the first characterization of a BDNF gene in an amphibian, *Xenopus laevis*, and demonstrate that environmental factors can activate this gene in a promoter-specific fashion. The *Xenopus* BDNF gene contains six promoter-specific 5'-exons and one 3'-protein-encoding exon. We examined the expression of promoter-specific transcripts in *Xenopus* neuroendocrine melanotrope cells. These cells make a good model to study how environmental factors control gene expression. In animals placed on a black background melanotrope cells more actively produce and release  $\alpha$ MSH than in animals on a white background. BDNF is cosequestered and coreleased with  $\alpha$ MSH and stimulates biosynthesis of proopiomelanocortin (POMC), the precursor protein for  $\alpha$ MSH. Our analysis of the expression of the BDNF transcripts revealed that there is differential use of some BDNF promoters in melanotrope cells, depending on the adaptation state of the frog. During black-background adaptation, stimulation of expression of BDNF transcript IV preceded that of the POMC transcript, suggesting the BDNF gene is an effector gene for POMC expression. The possible mechanisms regulating expression of the various transcripts are discussed on the basis of the potential calcium- and cAMP-responsive elements in the promoter region of exon IV. Finally, we show that the upstream open reading frames of BDNF transcripts I and IV markedly decrease BDNF translation efficiency, giving the first indication for a functional role of untranslated BDNF exons. (*Endocrinology* 150: 1361–1368, 2009)

**B**rain-derived neurotrophic factor (BDNF) is a neurotrophic factor that stimulates brain development (1, 2) and in adulthood promotes neuronal survival and synaptic activity (3, 4). BDNF binds the high-affinity full-length TrkB receptor (TrkB.FL), thereby initiating a wide range of downstream signaling events (5). The BDNF gene in mammals and zebrafish consists of multiple alternative first exons (6–8) and a single exon coding for the entire proBDNF protein. Multiple promoters drive rat BDNF gene expression, with regulated transcription directed by promoters I (9) and IV (10). Rat BDNF transcript IV is increased rapidly after neuronal stimulation indicating that the BDNF gene transcribed from exon IV promoter may be an immediate early gene (IEG) (11).

The neuroendocrine melanotrope cell in the pituitary pars intermedia of *Xenopus laevis* produces BDNF (12). This cell is a good model to study how physiological factors control gene expression in neuronal and endocrine cells (13, 14). In animals placed on a black background, melanotropes release  $\alpha$ MSH, which stimulates the dispersion of pigment in dermal melanophores, giving the animal a black appearance. In animals on a white background, the release of  $\alpha$ MSH is inhibited and consequently the animal becomes pale. The activation of melanotropes in black-adapted frogs is accompanied by a marked cell volume increase (15) and enhanced production of  $\alpha$ MSH and its precursor protein proopiomelanocortin (POMC) (16). In *Xenopus* melanotropes, BDNF is packaged into the same secretory gran-

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Abbreviations: AUG, Start codon; BDNF, Brain-derived neurotrophic factor; CRE, cAMP-responsive element; CaRE, calcium-responsive element; DRE, downstream regulatory element; Ct, cycle threshold; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IEG, immediate early gene; NIL, neurointermediate lobe; POMC, proopiomelanocortin; RACE, rapid amplification of cDNA ends; uORF, upstream open reading frame; UTR, untranslated region.

ule as  $\alpha$ MSH (17) and is presumably coreleased with the hormone. The melanotropes express the three major BDNF receptors, namely TrkB.FL, a C-terminal truncated form (TrkB.T) and the neurotrophin receptor p75<sup>NTR</sup> (18). BDNF stimulates melanotrope production of POMC (12), indicating that BDNF acts as an autocrine factor.

In this study we first identified the 5'-untranslated regions (UTRs) in BDNF transcripts of *X. laevis* by mapping the transcripts to the *X. tropicalis* genome. In this way we determined the exon-intron structure of the *X. laevis* BDNF gene. Then, using exon-specific primers, we studied the organ-specific occurrence of these transcripts and tested whether there is differential use of BDNF promoters to achieve the higher expression of BDNF observed in melanotropes of black-adapted frogs. We then examined the rate of change in the level of BDNF transcript IV during the process of black-background adaptation to determine whether BDNF acts as an IEG for POMC expression. The sequence of the promoter region of BDNF transcript IV was screened for potential responsive elements and we tested whether upstream open reading frames (uORFs) of the BDNF transcripts might affect the translation efficiency.

## Materials and Methods

### Animals

Young adults of the South African clawed toad *X. laevis*, aged 6 months, were reared in our laboratory under standard conditions, kept in tap water at 22 C, and fed beef heart and trout pellets (Touvit; Trouw, Putten, The Netherlands). Full-background skin adaptation was achieved by keeping the animals on a black or white background under continuous light for 3 wk. In the time-course adaptation experiment, 25 white-adapted animals that had been kept in white buckets (five animals per bucket) were placed on a black background (five animals per black bucket) for 1, 3, 6, or 24 h. The transfer time schedule was designed in such a way that all animals could be decapitated around the same time of the same day. To have similar handling, animals of the white- and black-adapted control groups were transferred from white to white and black to black buckets, respectively, 3 h before dissection. Animal treatment was in agreement with the Declaration of Helsinki and the Dutch law concerning animal welfare, as verified by the Committee for Animal Experimentation of Radboud University Nijmegen.

### Molecular cloning of *X. laevis* BDNF transcripts

After decapitation, freshly dissected brains and neurointermediate lobes (NILs) of three black- and three white-adapted animals were collected in 500  $\mu$ l ice-cold Trizol (Life Technologies, Paisley, UK) and homogenized by sonification. After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 25  $\mu$ l ribonuclease-free H<sub>2</sub>O. Total RNA was measured with an Eppendorf Biophotometer (Vaudaux-Eppendorf AG, Basel, Switzerland). To clone the presumed different 5'-untranslated BDNF exons, 5'-rapid amplification of cDNA ends (RACE) was applied by using the GeneRacer kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RT-PCR amplification was performed with a high-fidelity PCR enzyme mix (Fermentas, Vilnius, Lithuania), a GeneRacer 5'-forward primer, and a reverse primer specific for the 3'-coding exon. To design the reverse primer, the known coding sequence of *X. laevis* BDNF (accession no. BC082887) was aligned with the *X. tropicalis* genome database (JGI version 4.1; Joint Genome Institute, scaffold 309), whose genome is almost fully known, in contrast to that of its close relative *X. laevis*. In this way the antisense primer 5'-ATAGTAAGGAAAAGGATGGTCAT-3' near the

5'-end of the coding exon could be constructed. Then nested PCR was performed to increase the specificity and sensitivity of RACE by using GeneRacer 5'-nested primer and BDNF nested primer, 5'-CACTCT-TCTCACCTGATGGAA-3'. RACE products were analyzed on a 2% agarose gel and cloned into the pCR4-Topo vector (Invitrogen). Exon sequences were analyzed at a commercial sequencing facility (Davis Sequencing, LLC, Davis, CA). Finally, we determined the genomic structure of the *X. laevis* BDNF gene by aligning the amplified *X. laevis* cDNA sequence with the *X. tropicalis* genomic sequence.

### RT-PCR

RNA was isolated from freshly dissected brains, NILs, and peripheral organs (see Results) of four black- and four white-adapted animals as described above and dissolved in 25  $\mu$ l ribonuclease-free H<sub>2</sub>O. First-strand cDNA synthesis was performed with 0.1  $\mu$ g RNA and 5 mU/ $\mu$ l random primers (Roche, Mannheim, Germany) at 70 C for 10 min, followed by first-strand buffer (Life Technologies), 10 mM dithiothreitol, 20 U Rnasin (Promega, Madison, WI), 0.5 mM deoxynucleotide triphosphates (Roche), and 100 U Superscript II reverse transcriptase (Life Technologies) at 37 C for 75 min and 95 C for 10 min. After RT-PCR using primers specific for transcripts I-VII5'ext, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; see Table 1), the amplified products from the various organs were run on a 2% agarose gel and visualized with ethidium bromide to check the length of the amplified cDNA.

Quantitative RT-PCR was performed in a total volume of 25  $\mu$ l in a buffer solution containing 5  $\mu$ l of template cDNA, 12.5  $\mu$ l SYBR Green master mix (Applied Biosystems Benelux, Nieuwerkerk aan den IJssel, The Netherlands), and 0.6  $\mu$ M of each primer. Primer pairs were designed for the different BDNF transcripts, GAPDH, POMC, and cFos, as shown in Table 1. Optimum cycling temperature was 95 C for 10 min, which was followed by 40 reaction cycles at 95 C for 15 sec and 60 C for 1 min, using a 7500 real-time PCR system (Applied Biosystems). For each mRNA, the cycle threshold (Ct) was determined, *i.e.* the cycle number needed to reach an arbitrary fluorescence value (0.2) where Ct values of all mRNAs to be compared were within the linear phase of their amplification. To determine the relative mRNA level of interest, the Ct values of the mRNA of interest were converted to  $\Delta$ Ct by subtraction of the corresponding Ct value of the mRNA of the housekeeping enzyme GAPDH. To determine relative differences in mRNA expression between different adaptation states, the 2<sup>- $\Delta$ Ct</sup> was applied (19), expressed in arbitrary units, where the average level of mRNA in white-adapted animals is defined as 1.0 and the mRNA level in black-adapted animals is relative to this value. The quantitative RT-PCR was performed in three independent experiments, which gave essentially the same results. To control for genomic DNA contamination, all primers were designed in such a way that they span an intron, except the primers for BDNF VII5'ext. For this transcript a deoxyribonuclease (Invitrogen) treatment was applied.

TABLE 1. Primers used for quantitative RT-PCR analyses

Name	Primer sequence (5'-3')	Accession no.
BDNF-I	GTTAGAGAGACTGGATTTTAAAG	EU 363494
BDNF-II	TGGAACAGGCTGCTTTCAGTG	EU 363495
BDNF-III	AGAACAAGAAAGGGTTACAG	EU 363496
BDNF-IV	CTATATTATCCAGAGTTTCAG	EU 363497
BDNF-V	TCTTGGCAGAGGATATTAGGAG	EU 363498
BDNF-VI	CAGAACTTTGGTCAGGTTACC	EU 363499
BDNF-VII5'ext	AGTCACACCACCACATTAAGT	EU 363500
BDNF reverse	CACTCTTCTCACCTGATGGAA	X61477
GAPDH forward	GCTCCTCTCGCAAAGGTCAT	U41753
GAPDH reverse	GGCCATCCACTGTCTTCTG	
POMC forward	AGGGAACGATGGAAGCAACA	X03843
POMC reverse	TGTCACCTGGAGCATTTCTGATC	
cFos forward	AAGAAGAGGAAAAATGGAGCAGCT	(35)
cFos reverse	AGTTTCCGCTTGAAGAGTGTCTG	

## Reporter gene constructs

A fragment containing the 5'-UTR of BDNF transcript I (I) with three upstream start codons (AUGs), and a shorter fragment with just one upstream in-frame AUG (I-s), were amplified by PCR from NIL cDNA using sense primers 5'-AGCTAAGCTTGCGGATATTAGATTCATA-AGTCAC-3' and 5'-AGCTAAGCTTATCTGTTAGAGAGGCTG-GATTTTA-3', respectively, and exon VII-specific antisense primer 5'-AGCTCCATGGCTCTTCTCACCTGATGGAAC-3'. Similarly, a fragment corresponding to the 5'-UTR of BDNF transcript IV (IV) with six upstream AUGs and a shorter fragment with just one upstream AUG (IV-s) were amplified by PCR using sense primers 5'-AGCTAAGCTTATTCA-CAGCTCCAGAGGGCTG-3' and 5'-AGCTAAGCTTGAGCCCAC-GAAGAGCCTGAAT-3', respectively, and the same exon VII-specific antisense primer. The PCR fragments were cloned into the *Hind*III and *Nco*I sites of pGL3-control (C) (Promega), resulting in plasmids pGL3-control-I, pGL3-control-I-s, pGL3-control-IV and pGL3-control-IV-s. Each plasmid was sequenced for verification.

## Tissue culture, transfections, and reporter gene assays

HeLa cells were cultured at 37 C in 5% CO<sub>2</sub> in high-glucose DMEM supplemented with 10% fetal calf serum (PAA Laboratories, Pasching, Austria) and penicillin/streptomycin (Roche). Transient transfections were performed using Fugene-6 (Roche) according to the manufacturer's instructions. Cells were seeded on 24-well plates and the next day transfected with a mixture of 175 ng luciferase plasmid and 25 ng  $\beta$ act- $\beta$ gal plasmid per well. Forty-eight hours after transfection, cells were lysed by incubation in 200  $\mu$ l reporter lysis mixture [25 mM bicine (pH 7.5), 0.05% Tween 20, 0.05% Tween 80] for 10 min. For the  $\beta$ -galactosidase assay, 20  $\mu$ l cell lysate were mixed with 100  $\mu$ l Galacton mixture [100 mM Na-phosphate (pH 8.2), 10 mM MgCl<sub>2</sub>, 1% Galacton-Plus (Tropix, Bedford, MA)]. After 30 min incubation at 20 C, 150  $\mu$ l accelerator II (Tropix) were added and luminescence was measured with the Lumat LB 9507 tube luminometer (Berthold Technologies, Bad Wildbad, Germany). For the luciferase assay, 20  $\mu$ l cell lysate were mixed with 50  $\mu$ l luciferin solution (Promega), and luminescence was measured with the Lumat luminometer. Each gene construct was transfected into three groups of HeLa cells and reporter gene assays conducted on each cell preparation.

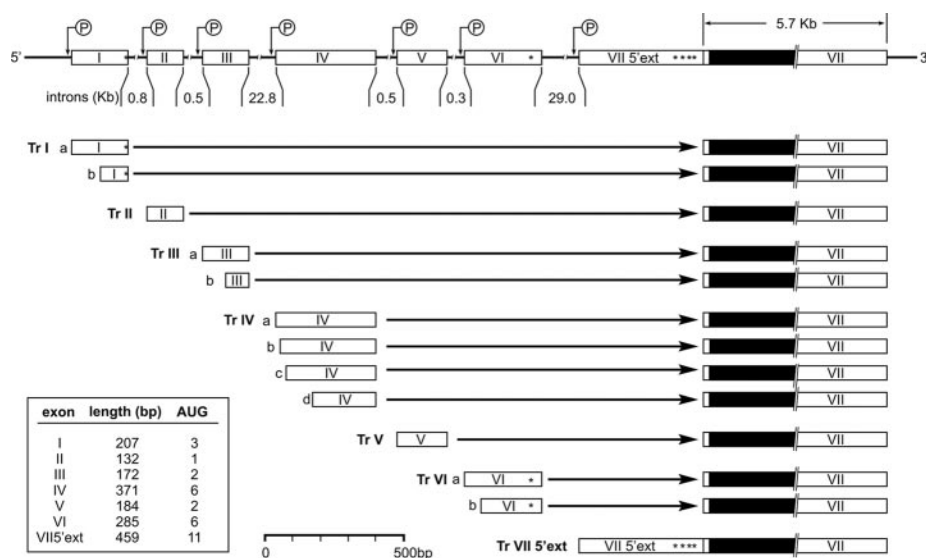
## Statistics

Quantitative data were expressed as mean and SEM. Values of the quantitative RT-PCR (see Fig. 4) were tested with Student's *t* test ( $\alpha = 5\%$ ), using Microsoft Excel software. The time course of expression of BDNF transcripts IV and VII5'ext, cFos, and POMC mRNA data (see Fig. 5) were tested by one-way ANOVA followed by Dunnett's *post hoc* test ( $\alpha = 5\%$ ) using SPSS (Chicago, IL). In the analysis of the translation efficiency of BDNF transcript I and IV (see Fig. 7B), data were analyzed by one-way ANOVA followed by Tukey's *post hoc* test ( $\alpha = 5\%$ ) for multiple comparisons using SPSS.

## Results

### Identification of *Xenopus* BDNF transcripts

To study whether transcription of the *X. laevis* BDNF gene is driven by multiple promoters, RACE-ready cDNAs from the *X. laevis* brain and NIL were screened following the GeneRacer protocol. This resulted in the amplification of various BDNF transcripts. Because the *X. laevis* genome sequence is only partly known, we aligned the transcripts to the *X. tropicalis* genomic database because there is strong sequence similarity between the two *Xenopus* species. This alignment allowed us to identify six alternative first exons (numbered I–VI) in addition to the known coding exon, designated exon VII (Fig. 1). In addition, a transcript with a 5'-extension of the protein coding exon (probably due to transcription initiation upstream of exon VII) was found and designated VII5'ext. There were also alternative transcription initiation sites for exons I, III, IV, and VI. Exons I and IV showed sequence homology with their respective counterparts in rat, human, and zebrafish (Fig. 2). Exon I contained an in-frame translation initiation site and might encode an N-terminal extension of the preproBDNF protein. Exons II, III, V, and VI did not show appreciable homology with mammalian or zebrafish BDNF exons.



**FIG. 1.** Schematic representation of the *X. laevis* BDNF gene (top panel) and its seven transcripts (Tr). Exons are shown as boxes and introns as thick lines. The lengths of introns (*X. tropicalis*) are given in kilobases. UTRs of the exons are depicted as open boxes and the coding preproBDNF as black boxes. Each of the six 5' untranslated exons is spliced (arrow) to the common 3' protein-coding 1 exon VII. Additionally, a transcript with a 5'-extension of the protein-coding exon gives rise to transcript VII5'ext. For exons I, III, IV, and VI, one or more transcript variants (a–d) are generated as a result of alternative transcription initiation sites or possible truncated RACE products. Asterisks mark the position of in-frame AUG and therefore possible coding regions for an N-terminally extended precursor of BDNF. Exon numbers are shown in Roman numerals. The length of each exon is given in a separate box in base pairs (bp), and the total number of upstream AUGs (AUG) within each exon is also presented. P, Location of the promoters.

**A Exon I**

```

xExon1      -TTTTCT--GTCTTCA----GTAGTCACCAAGGTGGTGTAGAGTTTAAAGCTT-GCGGAT  51
mExon1      GTTGCCTTT--GTCTTCT----GTAGTCGCCAAGGTGGATGAGAGTTGAAGCTTTGCGGAT  54
hExon1      GTTGCCTTT--GTCTTCT----GTAGTCGCCAAGGTGGTGTAGAGTTTAAAGCTT-GCGGAT  53
zExon1a'    GTTCTATTGTCCTCGGCACAGTCGCCAAGGTGTAAGAGTTTAAAGCAC-GAGAT  59
            *** ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExon1      AT-----TAGATTATAAGTACACCAAGTGGTGGGCGATTCACTGAGCAAA  98
mExon1      ATTGCCAAGGGTTATTAGATTATAAGTACACCAAGTGGTGGGCGATCCACTGAGCAAA  114
hExon1      ATTGCCAAGGGTTATTAGATTATAAGTACACCAAGTGGTGGGCGATCCACTGAGCAAA  113
zExon1a'    ATTGCCAAGGGTTATTAGATTATAAGTACAC-CCAAGTGGTGGGCGATCTTCTGAGCACG  118
            ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExon1      GCAGAAGTCTTCAATGATGACTTCAAACAAGACGCATTACCTTCCTGCATCTGTTAGAG  158
mExon1      GCCGAACCTTCTCAATGATGACTTCAAACAAGACACATTACCTTCCTGCATCTGTTGGGG  174
hExon1      GCCGAACCTTCTCAATGATGACTTCAAACAAGACACATTACCTTCAGCATCTGTTGGGG  173
zExon1a'    GCAGAAGTCTTCAATGATGACTTCAAACAAGACACATTACCTTACCAGCATCTGTTGGAG  178
            * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExon1      AGGCTGGATTTTAAGACACTTGTGTCTCCAGGACAGGA-AAGAAACAATG  207
mExon1      AGACAAGATTTTAAGACACT-GAGTCTCCAGGACAGCA-AAGCCACAATG  222
hExon1      AGACGAGATTTTAAGACACTTGTGTCTCCAGGACAGCA-AAGCCACAATG  222
zExon1a'    TGTGTGGATTTTAAGACACTTCTATCTCCAGGACAGCATAGGGGAAAATG  228
            * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
    
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**B Exon IV**

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xExonIV     ATTACAGCTCCAGAGGGCTGATCAGCCAGTGTGTAACCCCAT-TGA---GTTACACTAG  56
mExonIV     ATTCAACCGAGGAGAGGA-CTGCTCTCGCTGCCGCTCCCCACCACCCCGGGCAGCTAG  60
hExonIV     ATTCAACCGGAGAGGG-CTGCTCTCGCTGCCGCTCCCCCC-----GGCGAAGCTAG  50
zExon1c     ATTCAAGCTGGAGAGCTCT-CTCAATGCCACTACACTGC-----GAGCGCTCAC  50
            *** ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExonIV     TATGAAATCTCTCTCTCAACAAGATCAATGGAGCCATTTATGAAGA---CATGCC  113
mExonIV     CATGAAATCTCCAGCCTCTGCTAGATCAATGGAGCTTCTCGCTGAAGGCGTGGCAGT  120
hExonIV     CATGAAATCTCCCTGCTCTGCCGAGATCAATGGAGCTTCTCGCTGATGGGGTGGCAGT  110
zExon1c     CATGCTATCCAACTGCTTCAACTCAACTCCAAGGATCCGCTCAGTATGG---GAGTCC  107
            *** ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExonIV     ATTACGTCCACCATGCAGCTTCACTATCAATAATTTAAATATCACTCGCTGAGAGCCCA  173
mExonIV     ATTACCTCCGCCATGCAATTTCCACTATCAATAATTTAACT--TCTTTGCTGCAGAACAG  178
hExonIV     ATTACCTCCGCCATGCAATTTCCACTATCAATAATTTAACT--TCTTTGCTGCAGAACAG  168
zExon1c     ATTACCTCAACATGCAATTTCCACCATCAATAATTTAACT--TATTTGCTCAAAGCTG  165
            ***** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExonIV     CGAAGAGCCTGAATTATTTTTTTTCTCCCGCTGTTTATTGTTTGTAGCCTTTTAATT  233
mExonIV     ---GAGT---ACATAT-----CGGCCACCAAGACTCGC---CCCTCCCCC  216
hExonIV     AAGGAGT---ACATAC-----CGGGCACCAGACTCGCGCCCCCTCCCCC  211
zExon1c     ---AAG---AGAC-----ACTTGCA-----GCTGTG  186
            * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExonIV     TTTTCCAAGGAAAGCTGGAAGAAAATCTCACCCCAACAAACAAAAACCCAAACCT  293
mExonIV     TTTTAACTGAAGA---GAAGGGGAAAT-ATA-----TAGTAA--GAGTCTAGAACCT  262
hExonIV     CTTTAATT-AAGC---GAAGGGAACGTGAAA-----AAATAATAGAGTGTGGGAGT  259
zExon1c     GCTTGGCGAAGAG---CGGACGAATATCGCA-----GAATAGTTGCCGCGAGGTCTT  235
            ** * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExonIV     ATATTATCCAGAGTTTCAGCTGAGATCCCAATAAAGTGTGTTG---ATGTGTTCTTT  349
mExonIV     TGGGGACCG-GTC-----TTCCCAGAGCAGCTGCCTTG---ATGTTTACTTT  306
hExonIV     TTGGGGCCGAAGTC-----TTCCCAGAGCAGCTGCCTTG---ATGTTTACTTT  305
zExon1c     ---ATCAAAAAC-----ATCCCAGATGACACTGTCTGCTGAATGGTCTCTCTT  280
            * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *

xExonIV     GACAAGTACTAACTAAAATAAG  371
mExonIV     GACAAGTAGTGACTGAAAA-AG  327
hExonIV     GACAAGTAGTGACTGAAA--AG  325
zExon1c     TACGACTGGACAGTAA---AG  298
            * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
    
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**FIG. 2.** Alignment of the nucleotide sequences of *X. laevis* BDNF exons I (A) and IV (B) with those of the respective transcripts of human (accession no. EF125679), rat (accession no. EF674521), and zebrafish (Heinrich, G., unpublished results). Asterisks indicate identical nucleotides in all four species. The ATGs are indicated in bold, and conserved uORFs are underlined.

**Organ-specific expression of BDNF transcripts containing different 5'-exons**

We studied whether the transcripts found are expressed in an organ-specific fashion by determining expression levels in the brain, NIL, and peripheral organs with RT-PCR (Fig. 3). The various BDNF transcripts appeared to be expressed in a clearly organ-specific fashion. The majority showed highest expression in the brain, but several BDNF transcripts also had high expression levels in the NIL and peripheral organs. For example, expression of transcripts from exons IV, VI, and VII5'ext was high in the NIL, whereas that

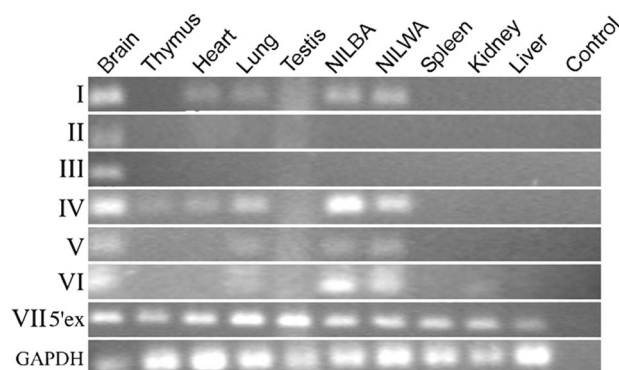
of exon VI was moderate in lung and kidney. A low expression of exon I transcript was observed in heart, lung, and NIL, and exon V transcript in lung and NIL. BDNF transcript VII5'ext was found at a high level in all organs studied.

**Quantitative RT-PCR of BDNF transcripts**

Because transcripts IV, VI, and VII5'ext appeared to be strongly expressed in the NIL and low amounts of transcripts I and V were also present in this organ, the levels of these transcripts were studied for possible regulation by the external light condition, using quantitative RT-PCR. Transcript IV was expressed at a 130 times higher level in NILs from black-adapted animals than white-adapted ones (Fig. 4A). Also transcripts I and V were expressed at a higher level in black- than white-adapted animals, in which these transcripts were essentially undetectable (the number of PCR cycles was above 37 or undetectable). The minimum number of PCR cycles required to detect transcripts in black-adapted NILs was 33 for exons I and V but 24 for exon IV, which indicates that the expression of exons I and V is much lower than that of exon IV. The levels of exon VI and VII5'ext transcripts were not significantly different between black- and white-adapted animals (Fig. 4, B and C).

**Time course of BDNF IV, VII5'ext, POMC, and cFos transcript induction by transfer from a white to a black background**

To determine whether the BDNF transcript IV is more rapidly induced by black background adaptation than POMC gene transcription, white-adapted *X. laevis* were time dependently transferred to a black background, and the accumulation of transcript IV was measured. BDNF exon VII5'ext was included as a negative control because the expression of this transcript did not change between the background adaptation states (Fig. 4C). Because cFos protein is known to be up-regulated in the *X. laevis* NIL during black background adaptation (20), the transcript of this established IEG was used as a positive control. *X. laevis* were killed from 1 to 24 h after transfer to a black background. After 6 h, the level of exon IV transcript was 13 times higher than the control level (fully white adapted animals) and continued to rise thereafter (Fig. 5A). As expected, the level of exon VII5'ext transcript did not change throughout the 24-h time course (Fig. 5B; note difference in scale). The POMC mRNA level rose slowly and only became elevated (3 times above control value) 24 h after transfer to the black back-



**FIG. 3.** RT-PCR analysis of BDNF and control GAPDH mRNAs in different *Xenopus* organs. Roman numerals on the left indicate the BDNF transcripts, and Control indicates the RT-PCR without template. NILBA, neurointermediate lobe of black-adapted animal; NILWA, neurointermediate lobe of white-adapted animal.

ground (Fig. 5C), whereas the level of cFos mRNA was strongly increased already 1 h after transfer and declined thereafter (Fig. 5D). In this experiment the white control group underwent a transfer to a white bucket 3 h before dissection, and the black control group was transferred to a new black bucket 3 h before dissection. Because c-Fos was so rapidly up-regulated (within 1 h), we conducted a separate experiment to determine whether white-to-white or black-to-black transfers had any effect on expression of this gene 1 h after transfer. No significant effects were found (data not shown).

#### Identification of potential regulatory elements in promoter IV

In view of the strong induction of transcript IV during black-background adaptation, we searched in the *X. tropicalis* BDNF promoter IV sequence region for regulatory elements. We found a sequence, 50 bp upstream from the *Xenopus* exon IV transcription initiation site, with high homology to an identified calcium/cAMP-responsive element (CRE) in the rat and human promoter IV (Fig. 6). In a region spanning 84 and 63 bp upstream of the initiation site, sequences resembling identified calcium-responsive elements (CaRE) 1 and 2 in human and rat were also found (Fig. 6). In addition, in the region of CRE, a sequence resembling downstream regulatory element (DRE) was identified (48 bp upstream). This site is also conserved in the human and rat BDNF promoter region IV (Fig. 6). We could not find

sequences representing these potential responsive elements in the promoter region of any of the other exons.

#### Translation efficiency of transcripts I and IV

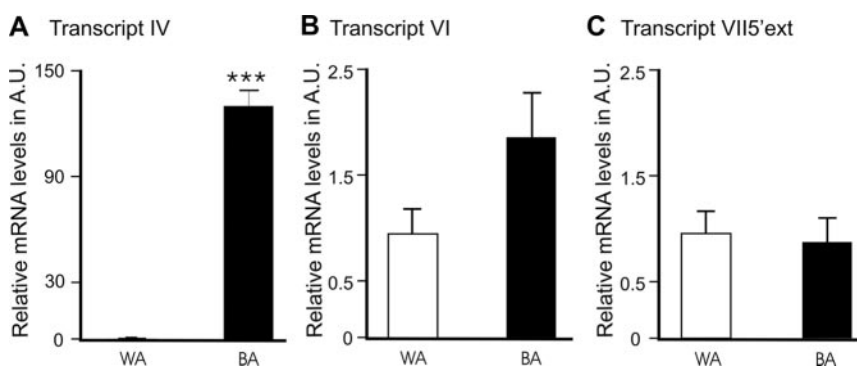
All *Xenopus* BDNF transcripts contain uORFs (Fig. 1). The underlined uORFs in exons I and IV (Fig. 2) are almost perfectly conserved among mammals, *Xenopus* and zebrafish, suggesting that they are of functional importance. To test whether the conserved uORFs of exons I and IV influence the expression level of a downstream ORF, reporter constructs were generated in which the luciferase-coding sequence is preceded by the exon I or IV sequence, either with (Tr I, Tr IV) or without the conserved uORFs (Tr I-s and Tr IV-s). Tr I, which contains three AUGs (Fig. 7A), inhibited the luciferase expression 3.5-fold, whereas Tr I-s, which contains only one (in-frame) upstream AUG, showed no inhibition (Fig. 7B). Tr IV, which contains six AUGs, resulted in a 10-fold inhibition of luciferase expression, whereas Tr IV-s, containing only one (out of frame) upstream AUG, showed only a 2-fold inhibition (Fig. 7B). These data suggest that the multiple upstream AUGs inhibited translation of the downstream luciferase-coding sequence.

#### Discussion

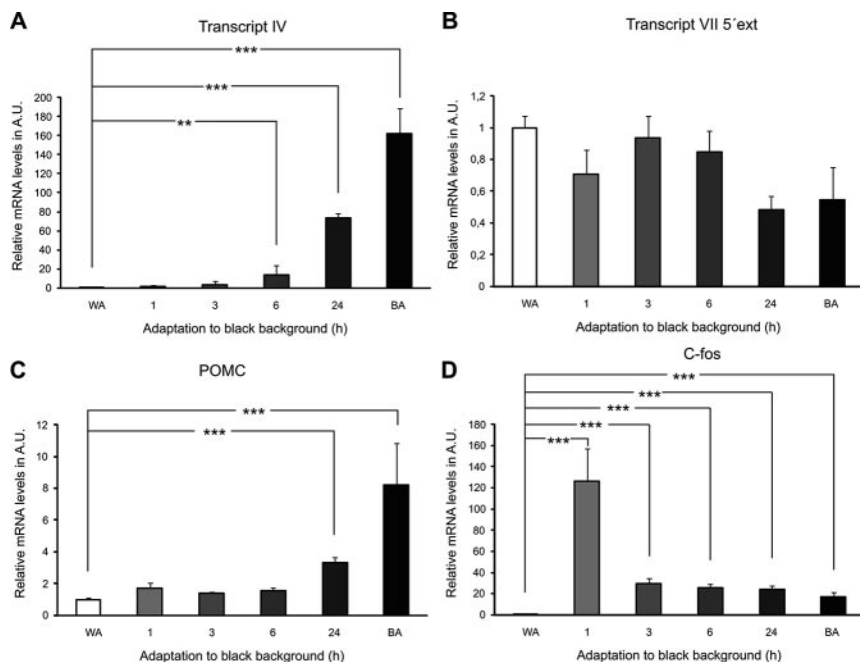
This study concerns the first characterization of the BDNF gene in an amphibian, and demonstrates that environmental factors can activate the transcription of this gene in a promoter-specific fashion. The *Xenopus* BDNF gene consists of at least six alternative 5'-exons and one 3'-protein-coding exon. Analysis of the BDNF transcripts shows that all alternative first exons (I–VI) are spliced to the protein-coding exon VII. The BDNF gene in rodents has been studied extensively and until recently was assumed to contain four alternative first exons (21) but is now known to contain eight exons (7). Recently new first exons were also discovered in the human BDNF gene, bringing their total number to 10 (8). Because of these new discoveries, the nomenclature of BDNF exons has changed, and we will adhere to this change. This means that what previously was known as exon III is designated by us as exon IV.

The *X. laevis* BDNF exons I and IV have strong sequence homology with exons I and IV, respectively, of the rat, mouse, and human BDNF gene. These exons also show homology with exons 1a and 1c, respectively, in zebrafish (6). Exon I has an in-frame translation start site, which potentially leads to a BDNF preproprotein with extended N-terminal. This AUG codon is conserved in human, rat, mouse, *Xenopus* and zebrafish, which suggests that it is of strong functional significance.

We show that the distribution of the various BDNF transcripts in *X. laevis* is organ-specific with two transcripts (II and III) being exclusively expressed in the brain. The transcript from exon IV was also expressed in the NIL and at low levels in heart and



**FIG. 4.** Effect of adapting *X. laevis* to a black- or white-background on the relative mRNA levels of BDNF transcripts (A) IV, (B) VI, and (C) VII 5'ex in the NIL, as studied by quantitative RT-PCR. Data are presented with the SEM ( $n = 4$ ) of white- (WA) and black (BA)-adapted frogs. Asterisks indicate statistically significant difference from the white group (\*\*\*,  $P < 0.0005$ ).



**FIG. 5.** Effect of transfer of white-adapted *X. laevis* to a black background for 1, 3, 6, and 24 h on the amount of transcript of BDNF exon IV (A), BDNF exon VII5'ext (B), POMC (C), and cFos (D) in neurointermediate lobes as studied by quantitative RT-PCR. White-adapted (WA) and black-adapted (BA) *Xenopus* were included as controls. Data are presented with the SEM (n = 5), and asterisks indicate statistically significant difference from the white group. \*\*, P < 0.005; \*\*\*, P < 0.0005.

lung, and exon VI transcript was expressed in the NIL, lung, and kidney. Organ-specific expression also holds for most BDNF exons in zebrafish and mammals (6–8, 22). We also have found that *Xenopus* expresses a 5'-extended version of the protein-coding exon (VII5'ext). This transcript was seen in all organs studied. In human, the 5'-extended protein coding exon transcript (IXabcd) also shows a wide pattern of expression (8).

One of the aims of this study was to determine whether there is promoter-specific expression of BDNF in *Xenopus* melanotropes during the process of background adaptation. We show that the expression of exon IV was 130-fold up-regulated in the NIL of black-adapted animals compared with that of white-adapted ones. The exon I and V transcripts were also higher expressed in black frogs. However, the expression levels of these exons were low compared with that of exon IV, and in white animals they were extremely low. In contrast to the dynamic picture of expression of exons I, IV, and V, expression levels of the transcripts from exons VI and VII5'ext did not differ between the two background illumination conditions. Therefore, we conclude that the expression pattern of BDNF transcripts is strongly promoter-specific, with BDNF

transcript IV being the most strongly regulated during background adaptation.

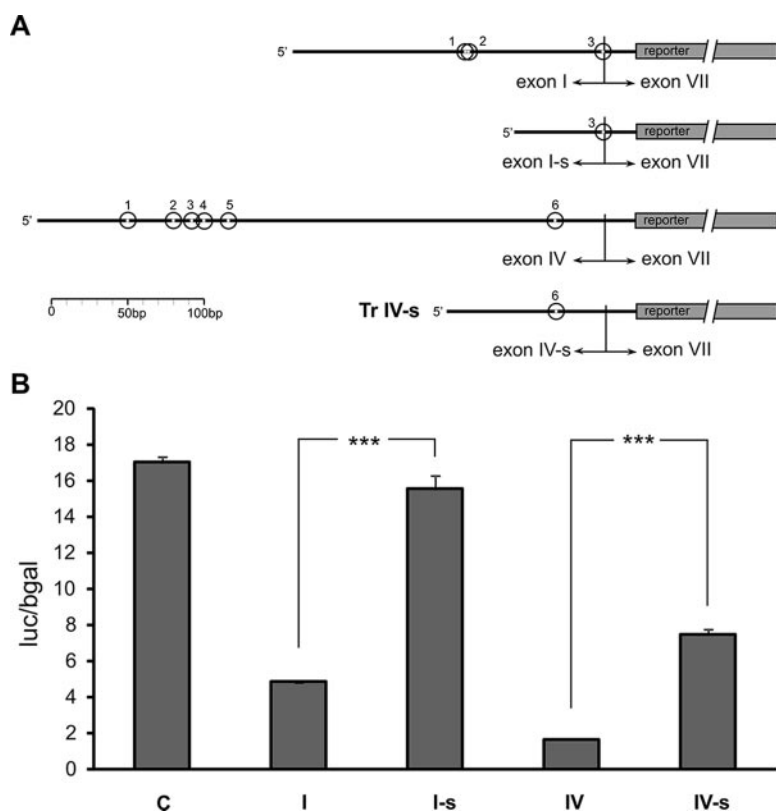
The extreme up-regulation of BDNF transcript IV as a result of black background adaptation prompted a detailed look at the promoter region upstream of exon IV. This region contains three potential responsive elements, namely CaRE1, CaRE2, and CRE. These elements function as responsive elements in BDNF exon IV of rat cortical neurons (10, 23, 24). Because in *Xenopus* melanotropes Ca<sup>2+</sup> oscillations and cAMP play an important role in regulating the cell's secretory activity (13, 14), it seems likely that the intracellular cascades initiated by these second messengers are also driving the expression of BDNF transcript IV through the CRE-like and CaRE-like responsive elements in its promoter. In addition to the responsive elements, there is also a potential repressing element in the promoter region of exon IV, namely a DRE, which in rat is a binding site for the calcium-binding protein DRE antagonist modulator (DREAM) (25). In mammals DREAM is involved in the Ca<sup>2+</sup>-dependent regulation of BDNF promoter IV (26). In the Ca<sup>2+</sup>-bound form,

DREAM lifts from the DRE site. Interestingly, in *Xenopus* melanotropes, the Ca<sup>2+</sup> oscillations are generated at the plasma membrane and travel through the cytoplasm to enter the nucleus (27, 28). We suggest that intranuclear Ca<sup>2+</sup>, acting on DREAM, might, at least in part, be responsible for the high expression of BDNF transcript IV seen in black-adapted *Xenopus*.

In view of the importance of cAMP and Ca<sup>2+</sup> in regulating the activity of the *X. laevis* melanotrope cell, it may seem surprising that analysis of the *Xenopus* POMC gene reveals a lack of CaREs and CREs within the gene's promoter region (29). The structure of the *Xenopus* BDNF gene, particularly the presence of multiple Ca<sup>2+</sup>- and cAMP-sensitive regulatory elements upstream of exon IV, gives rise to the idea that BDNF might act as an IEG to drive POMC expression. Although on transfer to a black background the BDNF transcript IV expression did precede POMC mRNA expression, the increase in BDNF mRNA started only after 6 h and remained elevated throughout adaptation. In contrast, cFos mRNA displayed a rapid (within 1 h) and transient pattern of expression, typical of an IEG. Therefore, BDNF transcript IV may not act *per se* as an IEG for POMC expression. Because *in vitro* treatment of melanotropes with BDNF induces POMC biosynthesis (12) and black background strongly up-regulates POMC mRNA expression as well as POMC protein biosynthesis (16, 30), we suggest that BDNF, although not acting as a typical IEG, does act as an effector gene for POMC expression. BDNF signaling in *Xenopus* melanotropes is associated with an



**FIG. 6.** Alignment of four regulatory elements in the promoter of transcript IV of *X. tropicalis* (JGI version 4.1; Joint Genome Institute, scaffold 309). The calcium-responsive elements 1 and 2 (CaRE1 and CaRE2) and the Ca<sup>2+</sup>/CRE (boxed) and DRE (underlined), aligned with the homologous human (accession no. EF125679) and rat (accession no. EF674521) elements, are indicated. Identical nucleotides are shaded.



**FIG. 7.** The effect of the conserved uORFs of exons I and IV on expression of a downstream ORF. **A**, Schematic view of the reporter constructs that include the full lengths of exons I or IV or shorter versions of these exons (I-s and IV-s, respectively). The locations of AUGs are indicated by white boxes (and highlighted by circles). The third AUG of exon I is in frame with the reporter gene; all other AUGs are not in frame with the reporter. **B**, Expression levels of the reporter constructs in transfected HeLa cells, determined by dividing luciferase (luc) values by the corresponding  $\beta$ -galactosidase values to correct for varying transfection efficiencies. The control (C) had no BDNF sequence upstream of the reporter translation initiation site. The results are the average of three independent transfections. Data are presented with the SEM, and asterisks indicate statistically significant difference; \*\*\*,  $P < 0.0005$ .

increase in the amount of phosphorylated ERK (Jenks, B. G., unpublished results). In corticotrope cells phosphorylated ERK activates the Nur family of transcription factors to stimulate POMC gene expression (31). Possibly, BDNF activates this pathway in *Xenopus* melanotropes to promote POMC gene expression.

Our sequence analysis of the 5'UTRs of BDNF has revealed the existence of multiple uORFs in *X. laevis*. uORFs are also present in 5'UTRs of BDNF transcripts of human, rat, mouse, and zebrafish. Genes that encode potent regulatory proteins, such as cytokines, growth factors, and kinases, often produce mRNAs containing uORFs that are translated inefficiently (32). Because the uORFs of exons I and IV are almost completely conserved among mammals, *Xenopus* and zebrafish, we studied their effect on translation efficiency. The presence of either the exon I or exon IV sequence significantly inhibited the expression of a downstream reporter gene, and this inhibition depended on the presence of the conserved uORFs. This could reflect the upstream AUGs interfering with the ribosome scanning mechanism in the search for the appropriate AUG or, alternatively, the generation of an upstream peptide through

alternate AUG usage. That the uORFs could lead to inefficient BDNF translation in *Xenopus* melanotropes is suggested by the observation that BDNF mRNA is up-regulated by about 135-fold in neurointermediate lobes of animals on black background, whereas an earlier study (12) shows that there is only about a 3.5-fold increase in immunoreactive proBDNF in black-adapted animals. The transcripts VI and VII5'ext have the most uORFs, indicating that these constitutively expressed mRNAs represent a quiescent pool of mRNAs waiting to be used under appropriate conditions. The presence of uORFs may reflect a protective mechanism whereby potent proteins are inefficiently produced to avoid harmful overproduction (cf. 33).

In conclusion, it would seem that the well-conserved, complex organization of the BDNF gene is necessary for a tight control of BDNF production. This notion is supported by our observation that there is a highly selective promoter-specific expression of BDNF during physiological adaptations and by our demonstration that the 5'UTR of BDNF transcripts has a dramatic effect on translation efficiency. Such a notion is also consistent with the finding that in transgenic *X. laevis*, in which BDNF is under control of the POMC promoter, the overexpression of BDNF led to atypical and irreversible structural changes of the pituitary intermediate lobe on prolonged black-background adaptation (34).

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