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Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system

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ABSTRACT **Here we describe two mammalian transcription factors selectively expressed in the central nervous system. Both proteins, neuronal PAS domain protein (NPAS) 1 and NPAS2, are members of the basic helix–loop–helix-PAS family of transcription factors. cDNAs encoding mouse and human forms of NPAS1 and NPAS2 have been isolated and sequenced. RNA blotting assays demonstrated the selective presence of NPAS1 and NPAS2 mRNAs in brain and spinal cord tissues of adult mice. NPAS1 mRNA was first detected at embryonic day 15 of mouse development, shortly after early organogenesis of the brain. NPAS2 mRNA was first detected during early postnatal development of the mouse brain. *In situ* hybridization assays using brain tissue of postnatal mice revealed an exclusively neuronal pattern of expression for NPAS1 and NPAS2 mRNAs. The human *NPAS1* gene was mapped to chromosome 19q13.2–q13.3, and the mouse *Npas1* gene to chromosome 7 at 2 centimorgans. Similarly, the human *NPAS2* gene was assigned to chromosome 2p11.2–2q13, and the mouse *Npas2* gene to chromosome 1 at 21–22 centimorgans. The chromosomal regions to which human *NPAS1* and *NPAS2* map are syntenic with those containing the mouse *Npas1* and *Npas2* genes, indicating that the mouse and human genes are true homologs.**

Molecular biological studies reported during the past decade have identified a family of transcription factors designated basic helix–loop–helix (bHLH)-PAS proteins. Members of this protein family contain a bHLH DNA binding domain located on the amino-terminal side of a PAS domain. PAS is an acronym derived from the initial three proteins observed to contain this polypeptide motif: the *period* gene product of fruit flies (1–3), the aryl hydrocarbon receptor nuclear transporter (4), and the *single-minded* gene product of fruit flies (5). The PAS domain is approximately 260 amino acids in length and contains two direct repeats of about 60 amino acids (5).

Biochemical studies of the aryl hydrocarbon (AH) receptor have provided evidence that it is directly regulated by xenobiotic compounds (reviewed in ref. 6). In its resting state, the AH receptor is retained in the cytoplasm in association with heat shock protein 90 (HSP90) (7). Upon exposure to xenobiotics, the AH receptor is released from HSP90 and dimerizes with the AH receptor nuclear transporter, a second bHLH-PAS domain protein critical to the function of the AH receptor (4, 8). The activated AH receptor/AH receptor nuclear transporter heterodimer enters the nucleus and activates a battery of genes, including those encoding P450 enzymes that facilitate detoxification (9, 10). The PAS domain of the AH receptor performs three biochemical functions in this regulatory pathway. In the latent state, the PAS domain binds HSP90 (7, 11). Upon activation, the PAS domain binds xenobiotic compounds, perhaps leading to release of HSP90 (7, 11, 12). Finally, upon association with AH receptor nuclear transporter, the PAS domain of each protein contributes a part of the dimer interface, thus facilitating formation of an active transcription factor (13, 14).

Although other bHLH-PAS domain proteins have not been studied in equivalent biochemical detail, they have been implicated in an interesting spectrum of biological pathways. Hypoxia inducible factor (HIF) and a related bHLH-PAS domain protein designated endothelial PAS domain protein (EPAS) 1 have been found to activate mammalian gene expression in response to hypoxia (15, 16). Both HIF and EPAS1 appear to function as obligate heterodimers with AH receptor nuclear transporter. HIF has been implicated in the activation of the gene encoding erythropoietin in hypoxic kidney tissue (17), whereas EPAS1 appears to control gene expression in vascular endothelial cells (16).

Two bHLH-PAS domain proteins, single-minded and trachealess, have been studied extensively in fruit flies. Recessive mutations in the gene encoding single-minded affect midline formation of the *Drosophila melanogaster* central nervous system (5, 18, 19). Loss of function mutations in the gene encoding trachealess impede tubulogenesis in the fly embryo (20, 21). Circadian rhythm in fruit flies is regulated by the product of the *period* gene, designated Per, which encodes a PAS domain protein devoid of a bHLH domain (1, 2, 5). Instead of acting in concert with another bHLH-PAS domain protein, Per interacts with the product of the *timeless* gene to synchronize fly behavior with the day/night cycle (22–24).

Recognizing that bHLH-PAS domain proteins regulate important biological processes in a variety of organisms, we set out to identify new members of this family of transcription factors. Two new bHLH-PAS domain proteins were identified in a search of publicly accessible data bases of expressed cDNA sequences. Here we provide the initial molecular characterization of neuronal PAS domain protein (NPAS) 1 and NPAS2, including (*i*) resolution of their primary amino acid sequences, (*ii*) assessment of their tissue distributions and temporal patterns of expression, and (*iii*) the map locations of their encoding genes in mice and humans.

MATERIALS AND METHODS

Gene Isolation and Sequencing. The National Center for Biotechnology Information GenBank data base was searched for expressed sequence tags (ESTs) bearing sequence similarity to the PAS domain of the AH receptor. ESTs designated R67292 and R58054 were identified and used to generate oligonucleotide primers for PCR amplification. Mouse and human cDNA clones containing these two ESTs were obtained

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Abbreviations: bHLH, basic helix–loop–helix; AH, aryl hydrocarbon; HIF, hypoxia inducible factor; EPAS, endothelial PAS domain protein; EST, expressed sequence tag; RH, radiation hybrid; cM, centimorgan.

by hybridization probing of bacteriophage λ cDNA libraries. Individual clones were sequenced, allowing conceptual translation of the reading frames encoding NPAS1 and NPAS2.

RNA Blotting and *in Situ* Hybridization. Total RNA samples were purified from dissected mouse organs or staged mouse embryos and subjected to electrophoresis on 1.2% agarose gels run in the presence of formaldehyde (25). Poly(A)$^+$ RNA samples were purified from total RNA and subjected to the same analysis as for total RNA. Fractionated RNA was transferred to nitrocellulose filters, cross-linked to the filter by UV light, and probed by hybridization using ^{32}P-labeled DNA derived from mouse cDNAs encoding either NPAS1 or NPAS2.

The templates used to generate *in situ* RNA probes for NPAS1 and NPAS2 were cloned into a pGEM-T vector (Promega). For NPAS1, the probe corresponded to the cDNA sequence encoding amino acid residues 142–266. For NPAS2, the probe corresponded to the cDNA sequence encoding amino acid residues 92–234. Each labeling reaction used 1 μg of linearized template, 50 μCi of α-[^{35}S]thio-UTP (1 Ci = 37 GBq) (Amersham), and was transcribed using T7 RNA polymerase (Ambion, Austin, TX).

Mice (C57BL/6 × SJL F_1) at 11 days of age were anesthetized with metofane and perfused via the left ventricle with heparinized saline followed by 4% paraformaldehyde. The brain was dissected and immersed in 4% paraformaldehyde overnight at 4°C. The tissue was placed in 70% ethanol, dehydrated through graded ethanol solutions, cleared in xylene, and infused with paraffin. Coronal and parasagittal sections were cut at 4-μm intervals and mounted on Vectabond-treated slides (Vector Laboratories). Contiguous sections were probed with sense or antisense transcripts of NPAS1 or NPAS2, or stained for Nissl granules to identify individual neurons.

In situ hybridization was performed to determine the expression patterns of the *Npas1* and *Npas2* genes in the mouse brain. Paraffin was removed from the sections with xylene, followed by graded ethanol hydration, post-fixation in 4% paraformaldehyde, Pronase digestion (20 μg/ml Pronase for 7.5 min), and acetylation [0.1 M triethanolamine·HCl, pH 7.5/0.25% acetic anhydride for 5 min]. Hybridization was conducted for 12 hr at 55°C in a solution containing 50% formamide, 0.3% dextran sulfate, 1× Denhardt's solution, 0.5 mg/ml tRNA, and 7.5×10^6 cpm/ml riboprobe. After hybridization, the slides were washed in 5× SSC at 55°C for 40 min followed by a wash in high stringency buffer (50% formamide/2× SSC supplemented by 10 mM DTT) at 65°C for 30 min. K.5 nuclear emulsion (Ilford) was applied to the slides before exposure at 4°C for 21 (NPAS2) or 28 (NPAS1) days.

Genetic Mapping. The human *NPAS1* and *NPAS2* genes were localized to specific chromosomes using a panel of 17 human × Chinese hamster cell hybrid lines (26). The murine *Npas1* and *Npas2* genes were mapped by analyzing a panel of 16 mouse × Chinese hamster cell hybrid lines and 2 mouse × rat somatic cell hybrid lines (27). PCR primers used to amplify human NPAS2, murine Npas1, and murine Npas2 sequences were derived from the 3′ untranslated region. PCR primers for human NPAS1 were derived from its coding region. The PCR primers used were: human NPAS1, 5′-GAGAGCAGAGTCAGCGACCAC-3′ and 5′-TCCAGCAAGTCCACGTGGCTC-3′; mouse Npas1, 5′-CCTCGCTTCCAGAGGCCT-3′ and 5′-GAGGGAGCCCCTTAGGGC-3′; human NPAS2, 5′-CGCAGGCATCCAGGGAGATG-3′ and 5′-CAACACCTCTCCAAACAATTC-3′; mouse Npas2, 5′-GCACACCTGTGCACAAGG-3′ and 5′-GGAAGAATCCAGTGTTCTG. PCR conditions were 94°C, 3 min; then 35 cycles of 94°C, 30 sec; 55°C, 30 sec; 72°C, 60 sec; followed by 72°C, 7 min.

DNA of an interspecies mapping panel, BSS panel 2, was obtained from The Jackson Laboratory (28). Parental strains of C57BL/6JEi (B6) and *Mus spretus* (SPRET/Ei) were screened for DNA variants by PCR amplification and single-stranded conformation analysis. To distinguish the PCR products from amplification of these two DNA samples, single-stranded conformation analysis was carried out (29). The DNA was denatured by mixing 2 μl of PCR product with 10 μl of 90% formamide and 20 mM EDTA, and incubated at 95°C for 5 min followed by cooling on ice. The mix was loaded onto a nondenaturing 12% polyacrylamide gel containing 1× TBE (90 mM Tris–borate/2 mM EDTA). Electrophoresis was performed in a Tris–glycine buffer at 200 V for 3 hr at 4°C. The PCR primers for amplifying mouse Npas2 were the same as those used for SCH mapping. PCR primers for amplifying mouse Npas1 were from exonic sequences: 5′-CGCATCAAAGTGGAGGCC-3′ and 5′-CAACGGGTGGCAGGAAGC-3′. Amplification with both sets of PCR primers allowed detection of strain-specific variation between parental strains (C57BL/6JEi vs. SPRET/Ei). The mouse Npas1 and Npas2 distribution patterns were sent to The Jackson Laboratory backcross service for comparison to the existing typing data base (L. Rowe and M. Barter, personal communication).

Two human radiation hybrid mapping panels, GeneBridge 4 (Whitehead Genome Center) and Stanford G3 (Stanford Genome Center) were used to define the localization of the human *NPAS1* and *NPAS2* genes. Typing was carried out using primers and PCR conditions described above.

RESULTS

Identification of Two New bHLH-PAS Domain Proteins. Conceptual translation of two ESTs found in the GenBank data base, designated R67292 and R58054, revealed sequences related to the PAS domain of the AH receptor. cDNAs corresponding to EST R67292 are hereafter designated NPAS1, whereas cDNAs corresponding to EST R58054 are designated NPAS2. cDNAs encoding mouse and human versions of NPAS1 and NPAS2 were isolated and sequenced. Fig. 1*A* shows the conceptually translated amino acid sequences of human and mouse NPAS1. Fig. 1*B* shows the sequences of human and mouse NPAS2.

The mouse and human NPAS1 proteins share 86% identity and specify, respectively, polypeptides of 63.7 and 62.7 kDa. The mouse and human NPAS2 proteins share 87% identity and specify polypeptides of 90.9 and 91.8 kDa. Having observed amino acid sequence similarity between NPAS1, NPAS2, and established bHLH-PAS proteins, we provisionally identify these as new members of this family of transcription factors.

Although a formal test of this prediction will require functional studies of the NPAS1 and NPAS2 proteins, several features of their primary amino acid sequences support the prediction that they are *bona fide* members of the bHLH-PAS domain family. Each of the functionally defined regions of the bHLH-PAS domain rely on defined arrangements of amino acids to specify function. The top of Fig. 2 provides an alignment of the bHLH domains of nine members of the bHLH-PAS domain family of proteins (4, 15, 16, 20, 21, 30, 31). Eighteen residues were observed to be conserved within the bHLH domain of at least seven of the nine proteins analyzed. The bHLH domain of NPAS1 contained the consensus amino acid at 17 of these 18 positions. The bHLH domain of NPAS2 appeared to diverge from the bHLH consensus more substantially than NPAS1. NPAS2 contained the consensus amino acid at only 9 of the 18 positions. NPAS2 likewise appeared to be missing three residues in the loop region separating helix 1 from helix 2. However, given that loop size is known to vary among other bHLH proteins (reviewed in ref. 32), and that the majority of the NPAS2 variant amino acids represent conservative changes, it likely specifies a functional bHLH domain.

The lower part of Fig. 2 provides an alignment of the two PAS domains of the same set of bHLH-PAS domain proteins. The PAS-A domains contained 18 residues conserved among at least seven of the nine proteins analyzed. The putative PAS-A domain of NPAS1 contained conserved amino acids at 16 of these 18 positions, whereas that of NPAS2 contained identities at 12 of the most highly conserved residues. Similar

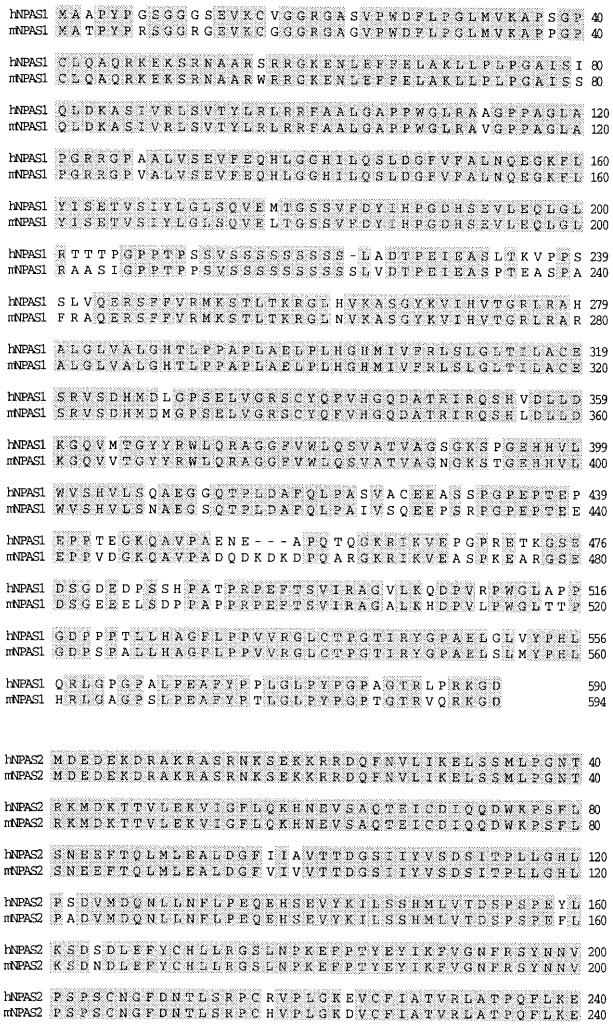

FIG. 1. Primary amino acid sequences of NPAS1 (*A*) and NPAS2 (*B*). Each protein sequence was conceptually translated from the sequences of mouse and human cDNA clones. Amino acid identities between mouse and human proteins are indicated by shading. Gen-

analysis of the PAS-B domains revealed 12 highly conserved residues, 8 of which were found in NPAS1 and 11 in NPAS2. The conservation of these signature amino acid residues in the putative bHLH, PAS-A, and PAS-B domains of NPAS1 and NPAS2 favor the interpretation that these proteins represent a functional member of the bHLH-PAS family of transcriptional regulatory proteins.

NPAS1 and NPAS2 mRNAs Are Enriched in Neuronal Tissues. The distribution of mouse tissues that express NPAS1 and NPAS2 mRNAs was evaluated by RNA blotting. Seventeen tissues were dissected from adult mice and evaluated for NPAS1 mRNA abundance. Brain and spinal cord tissues contained a 2.4-kb RNA that hybridized to the NPAS1 cDNA probe, whereas the remaining 15 tissues failed to show a detectable hybridization signal (Fig. 3*A*). NPAS2 mRNA abundance was evaluated in 16 tissues dissected from adult mice, including 14 that were tested for NPAS1 mRNA abundance and 2 additional tissues (colon and pancreas). The highest level of the 2.6-kb NPAS2 mRNA was observed in brain tissue (Fig. 3*B*). Less robust hybridization was observed in spinal cord, small intestine, uterus, and colon. Although the tissue distribution of NPAS2 mRNA was less selectively restricted to neuronal tissue than that of NPAS1, ethidium bromide staining of RNA samples showed relative consistencies in both abundance and integrity of 18S and 28S ribosomal RNA. Thus, relative to these structural RNAs, NPAS2 mRNA appears to be more enriched in brain than in any of the 16 other tissues that were tested.

Developmental Appearance of NPAS1 and NPAS2 mRNAs. The temporal appearance of NPAS1 and NPAS2 mRNA was examined by RNA blotting using samples obtained from mouse embryos and early postnatal animals. Embryos staged between embryonic days 10 and 13 were dissected to retrieve the anterior one-third of the embryonic mass. Later, staged embryos and postnatal animals were dissected to isolate the brain from other tissues.

NPAS1 mRNA was detected on blots prepared using total RNA. As shown in Fig. 4*A*, NPAS1 mRNA was first observed between embryonic day 15 and 16. Its relative abundance appeared to increase during late embryogenesis and appeared to be maintained during postnatal development. The apparent enrichment of NPAS1 mRNA at postnatal day 3 may represent a *bona fide* event, yet it is partially offset by a slight, relative increase in the amount of total RNA that was present in that particular sample.

NPAS2 mRNA was detected on blots prepared using poly(A)$^+$ RNA from late-stage mouse embryos, postnatal animals, and adults. In all cases, brain tissue was dissected and retrieved for RNA isolation. NPAS2 mRNA was first observed 3 days after birth. The apparent abundance of NPAS2 mRNA increased slightly through postnatal day 9, yet was substantially elevated in brain tissue obtained from adult mice. As a control for RNA integrity and loading, the same filter that was used for detection of NPAS2 mRNA was stripped and blotted using a cDNA probe specific to β-actin.

***In Situ* Expression Patterns of NPAS1 and NPAS2 mRNA.** Brain tissue was dissected after anesthetization and perfusion of 11-day-old mice. After immersion fixation, the material was embedded in paraffin, sectioned, and applied to glass microscope slides. Sense and antisense probes specific to the mouse genes encoding NPAS1 and NPAS2 were used under standard conditions of *in situ* hybridization (*Materials and Methods*). Both NPAS1 and NPAS2 mRNA expression was restricted to large, cytoplasm-rich neurons having pale-staining nuclei and prominent nucleoli. Their identity was confirmed as neurons

Bank accession nos. are: mouse NPAS1 (U77967), human NPAS1 (U77968), mouse NPAS2 (U77969), and human NPAS2 (U77970).

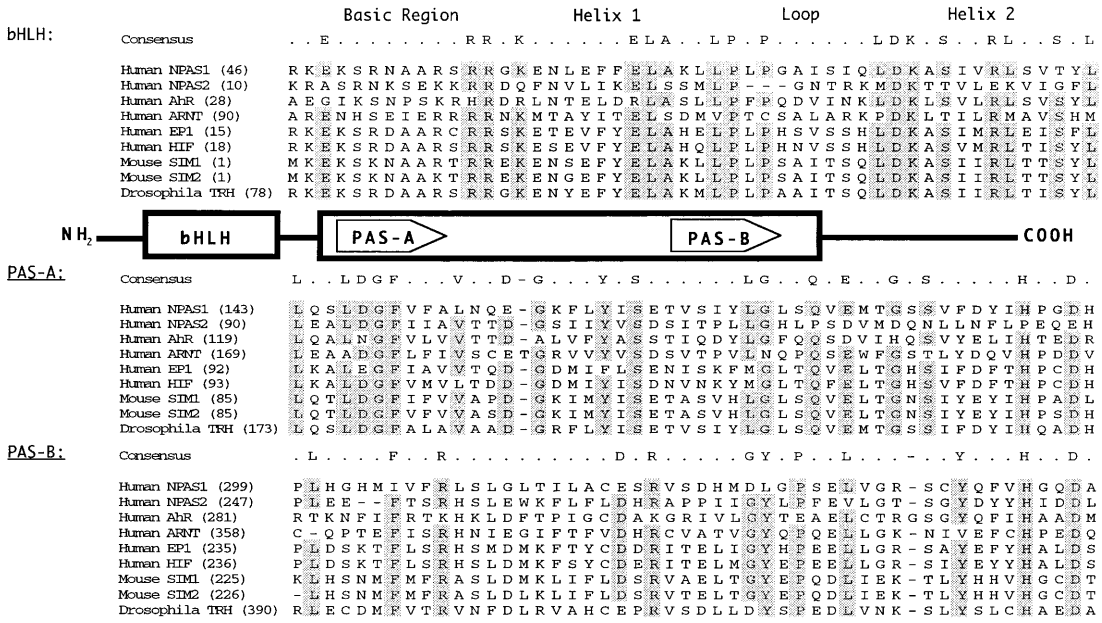

FIG. 2. Sequence comparison of nine bHLH-PAS domain proteins. The schematic diagram (*Middle*) shows the canonical organization of bHLH-PAS domain proteins. Amino acid sequences shown above correspond to the bHLH domains, sequences shown below correspond to the PAS-A and PAS-B domains. Individual amino acid sequences were obtained using the Entrez sequence retrieval system (National Center for Biotechnology Information). Shaded amino acids, designated consensus, represent residues identical among seven or more of the nine proteins analyzed. Numbers in parentheses after designation of each protein (*Left*) represent the residue number at which each sequence starts relative to the initiator methionine of the relevant protein.

by cresyl echt violet staining for Nissl substance on contiguous sections (Fig. 5 *I–L*).

The distributions of NPAS1 and NPAS2 mRNA in the mouse brain appeared to be broad, complex, and largely nonoverlapping. In general, the NPAS1 hybridization signal tended to be more discrete and intense than that of NPAS2, whereas the NPAS2 hybridization probe appeared to stain a higher proportion of neurons than NPAS1 (Fig. 5, compare *J* with *K*).

As shown in Fig. 5 *B* and *F*, NPAS1 expression in the neocortex was observed in deep pyramidal cell layers, whereas only rare neurons in the superficial layers were positive. Small numbers of intensely expressing neurons were observed in the polymorph layer of the hippocampus and dentate gyrus. In the basal ganglia, expression was restricted to the amygdala complex. NPAS1 mRNA also appeared in the ventroposterior area of the thalamus, as well as in neurons in the medial nuclei of the hypothalamus. In the mesencephalon, the intermediate grey area of the superior colliculus was positive, as was the sensory trigeminal nucleus of the pons. No expression of NPAS1 was observed in the cerebellum, caudoputamen, or inferior colliculus.

The distribution of NPAS2 mRNA detected by *in situ* hybridization ranged broadly throughout all layers of the neocortex with the exception of layer I (Fig. 5 *C* and *G*). The signal extended into the subiculum and the pyramidal neurons of the CA1 area of the hippocampus. Small numbers of neurons in the superficial aspect of the pyramidal layer of the inner and outer blades of the dentate gyrus also appeared to express NPAS2 mRNA. More abundant numbers of neurons

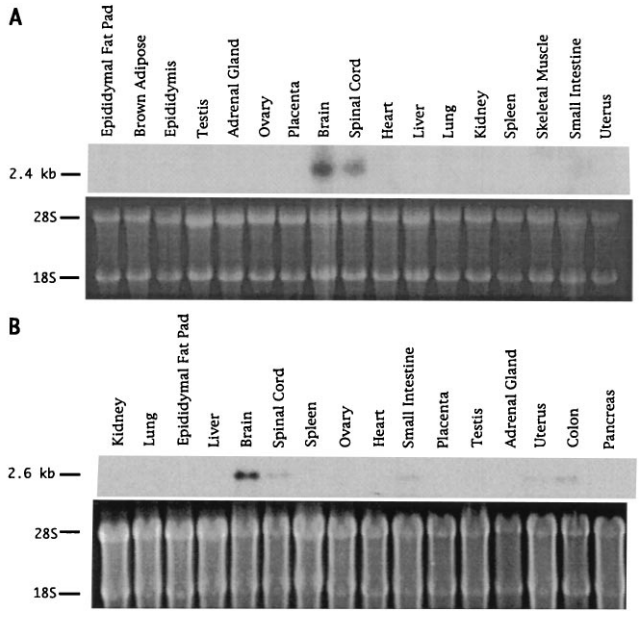

FIG. 3. Tissue distributions of NPAS1 (*A*) and NPAS2 (*B*) mRNA in adult mice. (*A* and *B Top*) Northern blot images developed using hybridization probes specific to NPAS1 and NPAS2 mRNA. (*A* and *B Bottom*) Ethidium bromide staining patterns of RNA samples used for Northern blotting.

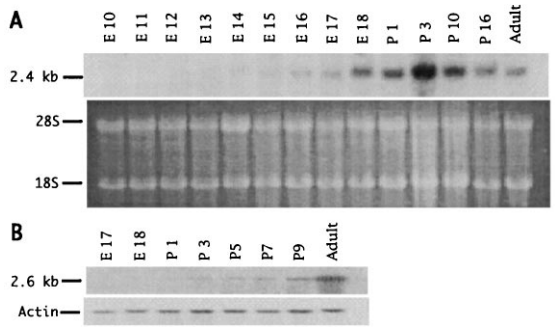

FIG. 4. Temporal patterns of appearance of NPAS1 (*A*) and NPAS2 (*B*) mRNA in developing mice. (*A Top*) Northern blot image developed using a hybridization probe specific to NPAS1 mRNA. (*A Bottom*) Ethidium bromide staining pattern of RNA samples used for Northern blotting. (*B Top*) Northern blot image developed using a hybridization probe specific to NPAS2 mRNA. (*B Bottom*) Image developed using a probe specific for a β-actin mRNA.

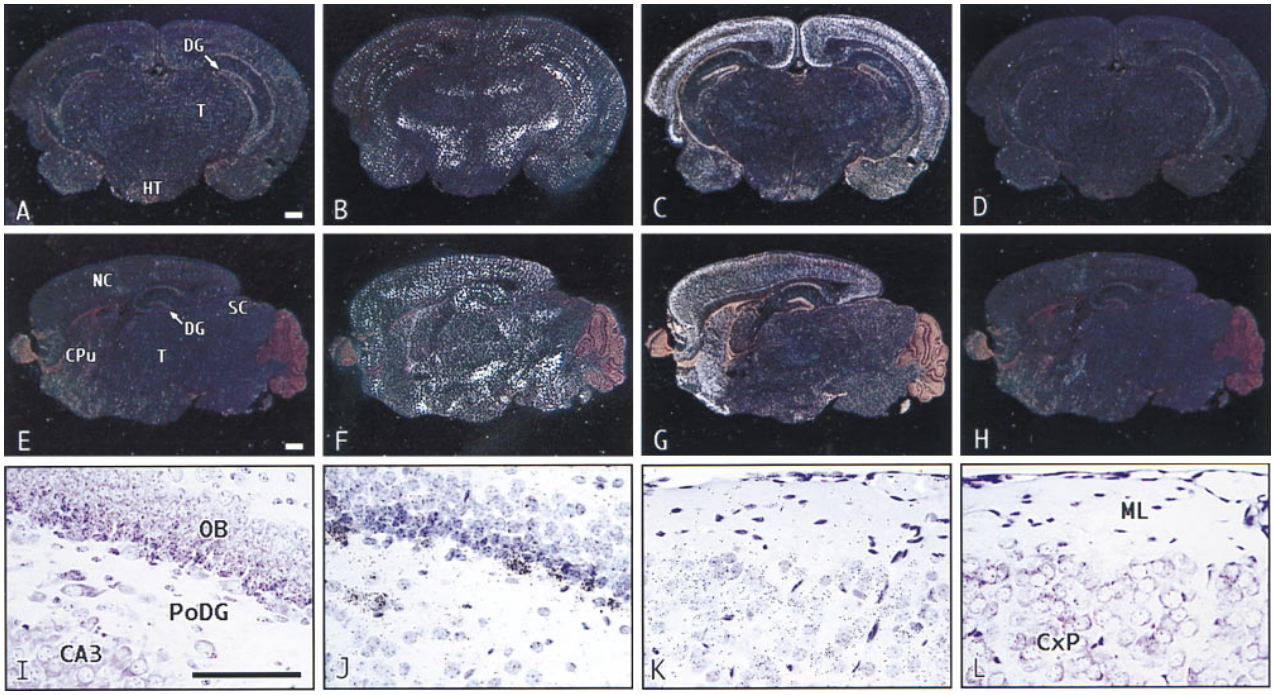

FIG. 5. *In situ* localization of NPAS1 (*A, B, E, F*, and *J*) and NPAS2 (*C, D, G, H*, and *K*) transcripts in coronal (*A–D*) and sagittal (*E* and *L*) sections of brain from an 11-day-old postnatal mouse. Images are shown of *in situ* hybridization patterns developed using antisense probes (*B, C, F, G, J*, and *K*) and control, sense probes (*A, D, E*, and *H*). Nissl-stained sections (*I* and *L*) correspond to the fields shown in *J* and *K*. CA3, CA3 region of the hippocampus; CPu, caudate putamen; CxP, cortical plate; DG, dentate gyrus; HT, hypothalamus; ML, molecular layer; NC, neocortex; OB, outer blade of the dentate gyrus; PoDG, polymorph layer of the dentate gyrus; SC, superior colliculus; T, thalamus. (Bars: *A–H* = 500 μm, *I–L* = 250 μm.)

diffusely distributed throughout the caudoputamen and pallidum of the basal ganglia were NPAS2-positive, as were nuclei of the anteriolateral thalamus. No signal was observed in the medulla, pons, superior or inferior colliculi, cerebellum, or olfactory bulbs.

Chromosomal Map Locations of the Genes Encoding NPAS1 and NPAS2. Somatic cell hybrids were used to assign the human *NPAS1* gene to chromosome 19, mouse *Npas1* to chromosome 7, human *NPAS2* to chromosome 2, and mouse *Npas2* to chromosome 1 (*Materials and Methods*). A regional mapping panel for human chromosome 2 was typed by NPAS2-specific primers that placed the *NPAS2* gene in region 2q13–q33 (data not shown).

Mouse backcross mapping panels were used to more closely define the locations of the genes encoding NPAS1 and NPAS2. DNA from the BSS panel 2 (The Jackson Laboratory) was typed by PCR single-stranded conformation analysis using primers specific to the mouse *Npas1* and *Npas2* genes (*Materials and Methods*). The *Npas1* gene fits a proximal chromosome 7 map position in a large cluster of nonrecombining loci that included *D7Mit56*, *D7Mit75*, and *D7Bir6*. Thus, the *Npas1* gene was placed on the linkage map of mouse chromosome 7 around 2 centimorgans (cM) from the centromere. The *Npas2* gene was found to be closely linked to two markers, *D1Bir8* and *D1Hun 31*, on chromosome 1. It was mapped between these two markers with one recombination occurring between *Npas2* and each marker. Thus, the *Npas2* gene was placed on the linkage map of mouse chromosome 1 between 21 and 22 cM from the centromere.

To further define the location of the human *NPAS1* gene, two radiation hybrid (RH) mapping panels were typed by PCR amplification using NPAS1-specific primers. In the Stanford G3 RH mapping panel, 12 of the 83 RH cell lines were positive for the human-specific *NPAS1* gene signal. By maximum likelihood analysis, the *NPAS1* gene was found to be closely linked to sequence-tagged site markers *D19S851*, *D19S985*, *D19S995*, *D19S1000*, *D19S1096*, *D19S412*, and *D19S1105*. These markers are clustered as chromosome 19 Bin no. 23 in the Stanford Genome Center RH map. In the GeneBridge 4 mapping panel, 23 of 93 RH cell lines were positive for the human-specific *NPAS1* gene signal. By maximum likelihood analysis, *NPAS1* was placed 0.9 centiRay (cR3000) from the chromosome 19 marker *WI-9028*. The order for placement of *NPAS1* was *D19S408 - WI-9028 - NPAS1 - D19S412*. Since *D19S408* and *D19S412* were respectively mapped to 19q13.2 and 19q13.3, *NPAS1* is most likely located at 19q13.2–q13.3.

Two RH mapping panels were used to further define the location of the human *NPAS2* gene on chromosome 2. In the G3 RH mapping panel, 23 of 83 RH cell lines were positive for the human-specific *NPAS2* gene signal. By maximum likelihood analysis, *NPAS2* was found to be closely linked to sequence-tagged site markers *D2S2886*, *D2S2776*, *D2S2311*, and *D2S2187*, which are clustered as chromosome 2 Bin nos. 44 and 45 in the Stanford Genome Center RH map. In the GeneBridge 4 mapping panel, 33 of the 93 RH lines were positive, and NPAS2 was placed 6.2 centiRay (cR3000) from the chromosome 2 marker *D2S373*. *D2S2311*, *D2S2187*, and *D2S373* are known markers in the WC2.8 contig (Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research). Thus, the *NPAS2* gene is likely present in this yeast artificial chromosome contig. Since there is no cytogenetic localization of the known sequence-tagged site markers and genes in WC2.8 or of chromosome 2 Bin nos. 44 and 45, more distantly located flanking markers were examined. According to Bray-Ward *et al.* (33), the marker *D2S139* (WC2.5) maps to 2p11.2-12. The marker *D2S135* (WC2.9) was mapped to 2q11.2-12, *D2S160* (WC2.9) to 2q12-13, and *D2S114* (WC2.11) to 2q21-22. Since the RH data placed the *NPAS2* gene at 2p11.2-2q13, whereas the somatic cell hybrid data suggest 2q13–q33, the most likely map location for this gene is chromosome 2 band q13.

DISCUSSION

This report describes two mammalian genes encoding newly defined members of the bHLH-PAS family of transcription factors. RNA blotting revealed the expression of both genes in

brain and spinal cord tissues of adult mice. Therefore, we provisionally designate these as NPAS1 and NPAS2. NPAS1 mRNA was observed exclusively in brain and spinal cord tissues. NPAS2 mRNA was observed in the spinal cord, colon, small intestine, and uterus, albeit at a reduced level relative to the brain. Although we do not know the identity of the cell types responsible for NPAS2 expression in nonneuronal tissues, *in situ* hybridization assays clearly showed that both NPAS1 and NPAS2 mRNAs are expressed in neurons of the mouse brain. Hybridization signal was observed to coincide with large, Nissl-positive cells bearing prominent cytoplasm and distinctive nucleoli. Thus, it appears, at least in the context of the mouse brain, that NPAS1 and NPAS2 function in neurons.

A shortcoming of the present work is the lack of functional data addressing the biochemical or biological roles of NPAS1 and NPAS2. The primary amino acid sequences of NPAS1 and NPAS2 contain signature amino acids that specify the bHLH, PAS-A, and PAS-B domains of other members of this family of transcription factors. As such, we predict that both NPAS1 and NPAS2 will function as gene regulatory proteins. Pending validation of this prediction, a number of questions arise. For example: Will NPAS1 and/or NPAS2 function as homodimers, or will they depend on heterotypic interaction with existing or new members of the bHLH-PAS domain family? Will NPAS1 and/or NPAS2 activity be regulated by extrinsic ligands, as in the case of the AH receptor, or physiological perturbations as in the cases of HIF and EPAS1? Finally, what program of gene expression might these proteins regulate in neurons of the central nervous system?

Although difficult to assess, the descriptive information provided in this report may help frame potential roles for NPAS1 and NPAS2. The temporal patterns of expression of both mRNAs, especially in the case of NPAS2, initiate subsequent-to-overt organogenesis of the brain. As such, these proteins might be expected to help execute the function of fully differentiated neurons, rather than specifying neuronal cell fate. Moreover, *in situ* hybridization assays showed that most neurons of the mouse brain do not express either NPAS1 or NPAS2. It is therefore unlikely that either protein is strictly required for the specification of this cell lineage. A more appealing interpretation of the potential function of NPAS1 and NPAS2 is that they might play protective or modulatory roles once the central nervous system begins to operate in earnest during late embryogenesis and postnatal development. This interpretation stems both from the timing of their expression and the fact that other bHLH-PAS domain proteins have been implicated in protection against physiological stress (HIF and EPAS1) and xenobiotic toxins (AH receptor).

In this regard, we pay special attention to the results of chromosomal mapping of the genes encoding NPAS1 and NPAS2. We have assigned the *NPAS1* gene to human chromosome 19q13.2–q13.3 and mouse chromosome 7 at 2 cM. Examination of the mouse genome surrounding the *Npas1* locus revealed two neurological mutant loci, *nv* and *lnd*, close to *Npas1*. *nv*, Nijmegen waltzer, is a recessive mutation that has been mapped around 0–4.2 cM of chromosome 7 (34). Homozygous *nv* animals show circling behavior, head shaking, and hearing defects. *lnd*, lumbosacral neuroaxonal dystrophy, is recessive and has been mapped around 2 cM of chromosome 7 (35). *lnd* homozygous animals have dystrophic axons in the low lumbar and sacral spinal cord levels. Mutants can be identified by 3 weeks of age by a slight tremor of the head. They later develop wobbly gait, mild head tremors, nervous behavior, and a tendency to drag their hind limbs. It will be of special interest to determine whether the *Npas1* gene is disrupted in either of these mouse mutants.

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