

## JDD1, a Novel Member of the DnaJ Family, Is Expressed in the Germinal Zone of the Rat Brain

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**We identified a novel gene encoding a new member of the DnaJ family, JDD1 (J domain of DnaJ-like-protein 1), from the rat. The cloned JDD1 cDNA is 1689 bp in size and its deduced amino acid sequence consists of 259 amino acid residues. Immunoblot analysis revealed that JDD1 protein is approximately 30 kDa in size. JDD1 has a J domain that is unique to the DnaJ family but lacks the G/F region (a region that is rich in the amino acids glycine and phenylalanine) and the zinc finger region (also known as the cysteine-rich region)—both characteristic to the DnaJ. JDD1 mRNA is expressed heterogeneously *in vivo*. In the central nervous system, JDD1 mRNA expression is confined to the germinal (ventricular and subventricular) zone where, except for cells situated deepest in the ventricular zone, neurons and glias are generated and then differentiate during the embryonic period. Expression of JDD1 mRNA in the subventricular zone persists after birth. In addition to the brain, its robust expression is notable in the liver, lung, cortex of the kidney, and several other tissues in the embryo.** © 2001 Academic Press

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Members of the heat shock protein 40 (hsp40), or DnaJ family, defined by the presence of a highly conserved J domain, have been cloned from many species (1–7). Originally, DnaJ was cloned from *Escherichia*

The nucleotide sequence of the gene reported in this paper (*JDD1*) has been submitted to the DDBJ (DNA Databank of Japan) database with an Accession No. AB002591.

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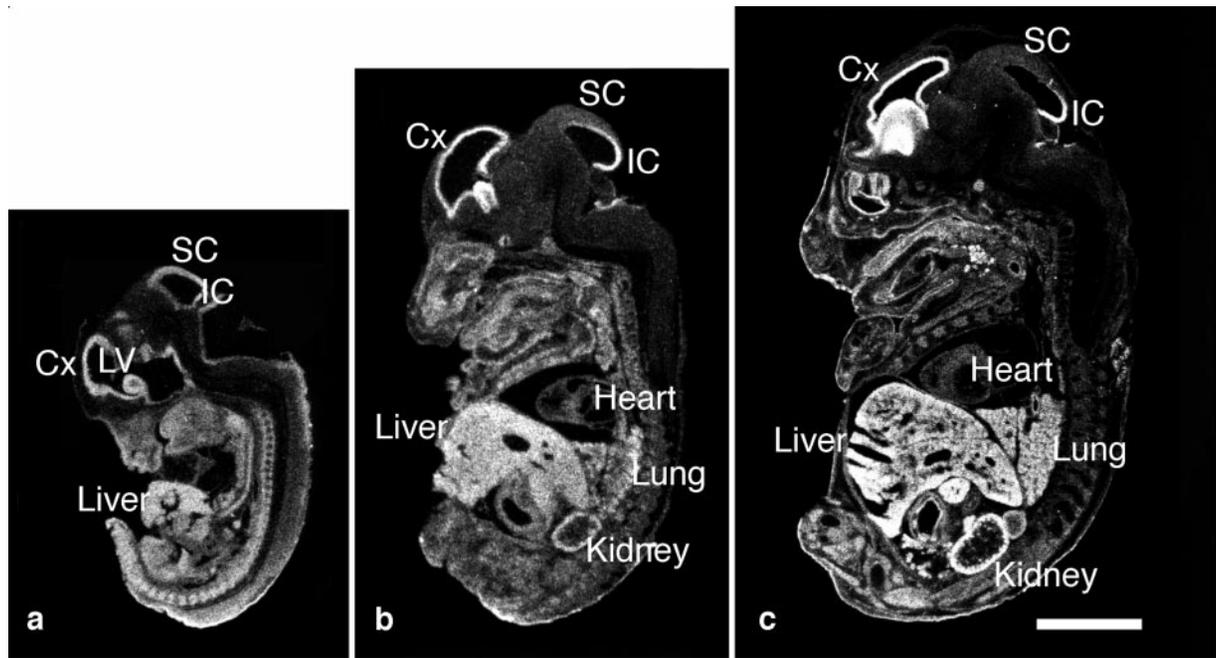
*coli* as one of the essential proteins for the replication of bacteriophage lambda (8, 9). The role of DnaJ as a molecular chaperone was later established; it is considered that, together with DnaK and GroEL, DnaJ is indispensable for protein folding in *Escherichia coli* (10, 11). To date, essential but various functions of DnaJ and DnaJ-related proteins have been demonstrated in many organisms, especially in the yeast, *Saccharomyces cerevisiae* (12–15). At present, a large number of mammalian members of the DnaJ family have been isolated. For example, hsp40, Hdj-2 and Mtj1 (1, 5, 7) are mammalian homologues of *S. cerevisiae* SIS1, YDJ1 and SEC63, respectively (16–18). Since the subcellular localization and the demonstrated function of these *S. cerevisiae* proteins are diverse but essential for normal cellular activity (13–21), it is likely that the individual mammalian homologues also play distinct but crucial roles *in vivo* (22–27).

We were searching for key proteins involved in biological events in the germinal zone of the central nervous system (CNS) during development in an attempt to elucidate the molecular mechanisms underlying neurogenesis. We identified a novel member of the DnaJ family, JDD1 (J domain of DnaJ-like-protein 1), in the rat. This newly identified protein is expressed strongly in the germinal zone of the CNS and its expression continues after birth.

### MATERIALS AND METHODS

**Animals.** Pregnant Wistar rats were purchased from a local vendor (Keari Japan, SLC Japan). Embryos at various stages and newborn pups were used as follows: embryonic day 11 (E11; 24 h from insemination, designated as E0) ( $n = 20$ ); E12 ( $n = 50$ ), E15 ( $n = 12$ ), E18 ( $n = 28$ ), newborn (postnatal day 0, P0,  $n = 2$ ) and P7 ( $n = 3$ ). Our protocol for animals was approved by the Animal-Care Committee of Fukui Medical University, and all experiments were carried out in accordance with its guidelines.

**Isolation of a rat cDNA fragment by differential display.** We employed the mRNA differential display (28) to search for genes



**FIG. 1.** *In situ* localization of #A81 clone mRNA in rat embryos. Strong hybridization signals are observed in the germinal zone of the brain, liver, lung, and cortex of the kidney. Parasagittal sections: localization of #A81 mRNA and *JDD1* mRNA is identical. (a) Embryonic day 12 (E12), (b) E15, (c) E18. Cx, cerebral cortex; IC, inferior colliculus; LV, lateral ventricle; SC, superior colliculus. Scale bar, 3 mm.

expressed transiently in the cortex of E11 animals. First, polyadenylated [poly(A)<sup>+</sup>] RNA was extracted using a microFast Track kit (Invitrogen, U.S.A.) from the telencephalon of 15 rat embryos at E11 and 15 at E18. We used a mixture of various primers [A(G)TCA(G, T, C)CCA(G, T, C)CG] to reverse-transcribe poly(A)<sup>+</sup> RNA and to isolate the cDNA fragments. Then we carried out polymerase chain reaction (PCR) using conventional two sets of primers in the presence of [<sup>35</sup>S]dATP (28). The products were then separated by electrophoresis on a DNA sequencing gel. Amplified cDNA fragments were visualized by autoradiography as bands in order of their molecular weights. Some of these bands appeared dominantly in the E11 lane but not the E18 lane. These E11-derived specific bands were considered to be partial cDNA fragments of genes expressed in the telencephalon of E11 but not expressed strongly in that of E18. We extracted these apparent E11-derived specific DNAs from the gel, subcloned them into the pGEM plasmid (Promega, U.S.A.) and determined their nucleotide sequences. Inquiry into a DNA databank using the Fasta program (29) revealed that we had identified a novel DNA fragment, which was temporarily designated #A81, among the clones we had obtained.

**Tissue preparation.** Animals were deeply anaesthetized by hypothermia (for newborn pups) or sodium pentobarbital (for pregnant rats; 40 mg/kg by intraperitoneal injection). Whole embryos (E11–E18) or brains (P0–P7) were immediately frozen with powdered dry ice. Parasagittal sections or frontal sections were cut at a 15 μm thickness with a cryostat. Sections were then thaw-mounted onto glass slides coated with TESTA (3-amino-propyltriethoxysilane) (Sigma, U.S.A.) and stored at –80°C until use.

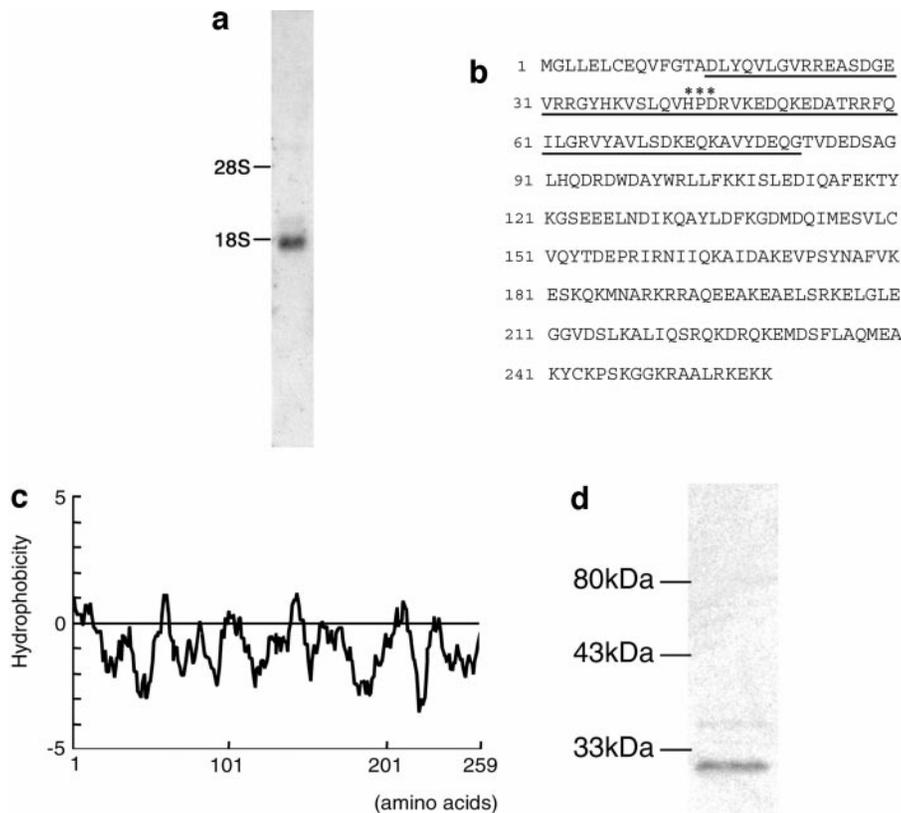
***In situ* hybridization histochemistry.** The conventional protocol was used for *in situ* hybridization histochemistry (30). [<sup>35</sup>S]UTP-labeled single-strand RNA was synthesized from the *Nco*I-digested pGEM template plasmid containing the rat #A81 fragment, with SP6 RNA polymerase as an antisense probe, and from the *Sa*II-digested template plasmid with T7 RNA polymerase as a sense probe (Fig. 1). After cloning the full-length of *JDD1*, we carried out *in situ* hybridization with a probe designed to avoid cross-reaction with other

genes containing the J domain (*JDD1*-specific probe). Nucleotides 599–1279 of *JDD1* were amplified by PCR then inserted into pGEM; a probe was then synthesized using RNA polymerases. Results were identical to those achieved with #A81. Hybridization and washing procedures and the criteria for identification of positive signals were the same as those published previously (30). Hybridization signals were visualized by macroautoradiography using New-RX X-ray film (Fuji, Japan) and by microautoradiography using emulsion (Ilford, UK).

**Cloning of full-length cDNA of rat *JDD1*.** A rat E12 forebrain cDNA library (about 1 × 10<sup>6</sup> plaques) constructed in lambda ZAP II was screened using the #A81 DNA fragment as a probe. Thirty-seven positive clones were obtained. Identified clones were subcloned into p-Bluescript (II) KS– for sequencing and further analyses. An apparent full-length cDNA was not obtained at first. We then screened a rat liver lambda genomic library (Stratagene) with the *JDD1*-cDNA-specific fragment (which was designed to skip the J domain, corresponding to nucleotides 599–1279 of the finally obtained cDNA). A single clone 18 kb in size was obtained, then several PCRs targeting the exons were run against the cDNAs taken from the E18 Wistar rat brain. Finally, an apparent full-length cDNA containing the #A81 fragment was obtained. We termed this newly cloned molecule *JDD1* (details are described in the 'Results and Discussion' section). The nucleotide sequence and the predicted amino acid sequence were then compared with the DNA and protein databases (DDBJ, EMBL, GenBank, PIR and SWISS-PORT).

**Northern blot analyses.** Conventional protocol for Northern blot analyses was used for poly(A)<sup>+</sup> RNA (mRNA, 1 μg) extracted from the brains of E18 Wistar rats (30). Part of the *JDD1* cDNA fragment (nucleotides 599–1279, the same region selected for *in situ* hybridization) was labeled with [<sup>32</sup>P]dCTP using a MegaPrime labeling kit (Amersham).

**Preparation and characterization of the antibody.** Polyclonal antisera were generated against the partial amino acid sequence of the *JDD1* protein. Synthetic peptide (sequence: H-GVRREASDGEV-



**FIG. 2.** Rat JDD1 molecule. (a) *JDD1* mRNA is approximately 1.7 kb in size. Northern blot analysis: 18S and 28S indicate the size of 18S and 28S ribosomal RNA, respectively. (b) Predicted amino acid sequence of JDD1. A possible J domain is underlined and the HPD motif is shown with asterisks. (c) Hydrophobicity profile of JDD1. Hydrophobicity scores are calculated based on Kyte and Doolittle (33). (d) JDD1 is approximately 30 kDa in size. Immunoblot analysis. Samples were taken from E12 rat embryos.

RRGYC-NH<sub>2</sub>) conjugated to carrier protein of diphtheria toxoid-MCS (6-maleimidocaproic acyl *N*-hydroxysuccinimide ester) was purchased from Chiron Mimotopes Pty Ltd (Australia). Each 200 μg of conjugated peptide emulsified in Freund's complete adjuvant was injected into three young New Zealand white rabbits (SLC, Japan) subcutaneously. Two weeks after an initial injection, boost injections were administered at intervals of 10 days using 200 μg of peptide emulsified in Freund's incomplete adjuvant. Antisera were initially tested for their ability to react with the peptide by dot blotting. Antisera were purified by affinity column chromatography of the synthetic peptide conjugated to FMP (2-fluoro-1-methylpyridinium toluene-4-sulfonate)-activated Cellulofine (Seikagaku, Japan).

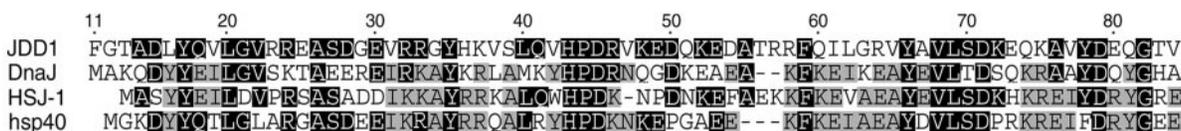
**Western blot analyses.** Homogenized protein extracts were prepared from the Wistar rat embryos (E12) with the sample buffer (125 mM Tris-HCl, pH 6.8, 5% mercaptoethanol, 2% SDS, 0.1% glycerol). Aliquots (50 μg) were resolved by electrophoresis on 12% SDS-polyacrylamide gels using a Mini-PROTEAN II Electrophoresis Cell (200 V, 45 min) and transferred to a nitrocellulose membrane (Trans-

Blot Transfer Medium, Bio-Rad). The membrane was blocked by incubation in blocking solution, 3% bovine albumin in Tris-buffered saline (20 mM Tris, 500 mM NaCl, pH 7.5) for 5 min, followed by overnight incubation at 4°C in anti-JDD1 antisera (1:1000 dilution). After washing twice with TTBS (20 mM Tris, 500 mM NaCl, 0.05% Tween 20, pH 7.5), incubation with the secondary antibody (goat anti-rabbit IgG-alkaline phosphatase, 1:1000 dilution, Bio-Rad) was carried out for 1 h at room temperature. Signals were visualized by the alkaline phosphatase color reagent.

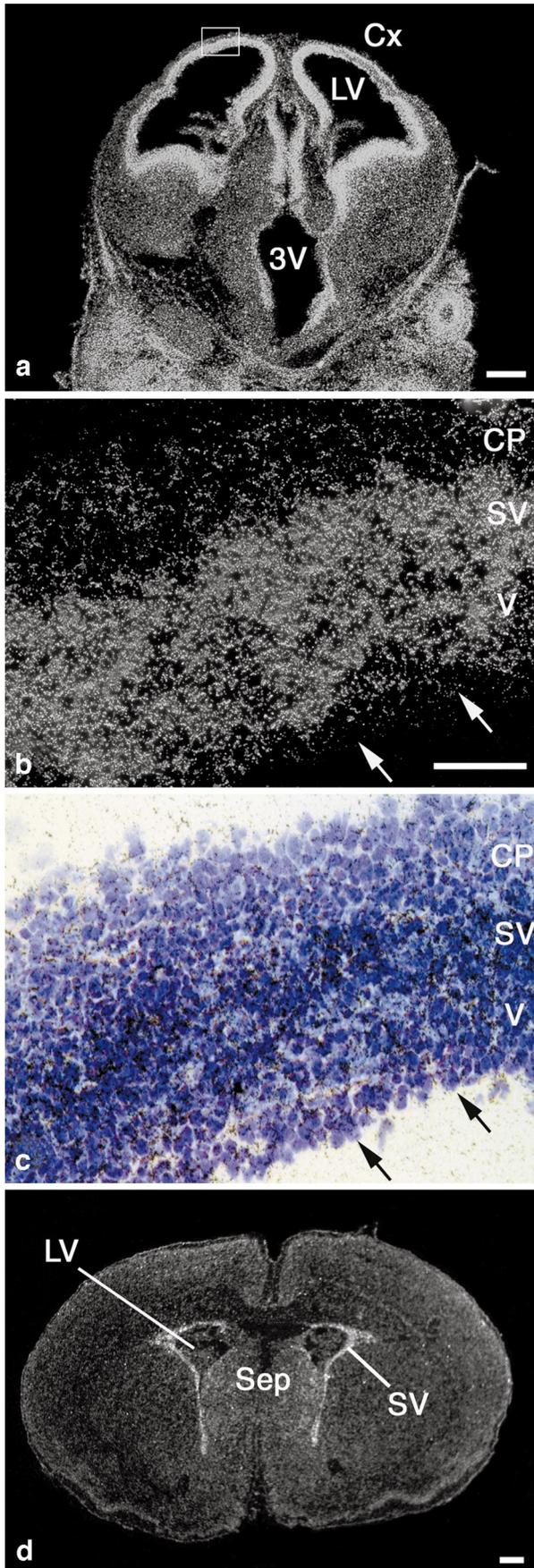
**RESULTS AND DISCUSSION**

*JDD1 Was Identified as a Gene with Robust Expression in the Germinal Zone of the Brain*

We searched for genes which were transiently expressed in the germinal zone of the nervous system



**FIG. 3.** Sequence comparison of JDD1 with other members of the DnaJ family. Amino acid residues common to JDD1 and the any listed members of the DnaJ family are shown with white letters on black background, while those common to more than two members (excluding JDD1) are shown with gray background. DnaJ is *Escherichia coli* DnaJ (9). HSJ-1 and hsp40 are members of DnaJ family of human (7, 34).



during the middle stage of embryogenesis in order to identify any proteins basic for neurogenesis. With an mRNA differential display, two hundred gene fragments were obtained that were recognized as dominant on E12 but not E18–20. Neurogenesis in the germinal zone is almost complete by E18–20, whereas many neurons (and precursors) are about to be generated on E11–12 (31). These candidates were sequenced to exclude overlap, then further selection was performed by *in situ* hybridization histochemistry. Among 80 independent clones, a novel clone, #A81, which was later designated as *JDD1*, showed restricted expression in the germinal zone of the cortex in the CNS during the embryonic period (Fig. 1). In addition to the CNS, #A81 mRNA was widely but heterogeneously expressed elsewhere in the embryo; notably in the developing liver, lung and cortex of the kidney (Fig. 1).

#### Full-Length *JDD1* cDNA Was Cloned

A #A81 cDNA comprising 1431 bp was obtained by screening a rat cDNA library. Although a coding sequence of 259 amino acid residues was recognized in the DNA obtained, no nonsense codon was noticed in its 5' terminus, raising the possibility that the authentic cDNA of *JDD1* is longer than that obtained. After an additional cloning strategy was taken by screening a genomic DNA library, a cDNA 1689 bp in size, which was almost the same size as that estimated by Northern blot analysis, was obtained (GenBank Accession No. AB002591, Fig. 2a). Its deduced amino acid sequence consists of 259 amino acid residues and its calculated molecular weight is 30061 Da (30 kDa) (Fig. 2b). No typical signal peptide sequences, membrane spanning regions or membrane anchoring regions were identified in its hydrophobicity profile (Fig. 2c) (32, 33). Western blot analysis revealed that *JDD1* protein is approximately 30 kDa in size (Fig. 2d). The accordance of the molecular weight obtained by immunoblot analysis with that estimated suggests that *JDD1* is not highly processed (cleaved) or highly glycosylated after translation.

**FIG. 4.** *In situ* localization of *JDD1* mRNA with a *JDD1*-specific probe. (a) Frontal section of E15 (embryonic day 15) embryo. In the cortex, expression of *JDD1* mRNA is recognized in the germinal zone, which lines the ventricle. Cx, cerebral cortex; LV, lateral ventricle; 3V, 3rd ventricle. Scale bar, 500  $\mu$ m. (b) High magnification of the rectangular area shown in a. Expression of *JDD1* mRNA in the germinal zone [ventricular zone (V) and subventricular zone (SV)] is notable. Arrows indicate the border of the ventricular zone. CP, cortical plate; SV, subventricular zone; V, ventricular zone. Scale bar, 100  $\mu$ m. (c) The same region in b, counterstained with thionin. Arrows indicate the border of the ventricular zone. (d) *In situ* localization of *JDD1* mRNA in the cortex of P7 (postnatal day 7) rat. Frontal section. Persistent expression of *JDD1* mRNA in the subventricular zone is noticeable. Sep, septum. Scale bar, 500  $\mu$ m.

*JDD1 Possesses a J Domain Typical of the DnaJ Family but Not the G/F Region or the Zinc Finger Region*

DNA and protein database inquiries revealed that the cloned cDNA is novel and shows high sequence homology with DnaJ and members of the DnaJ family (Figs. 2b and 3). Since this newly cloned gene has a structure typical of the J domain of the DnaJ family (Figs. 2b and 3), we termed this novel gene *JDD1* (J domain of DnaJ-like-protein 1). We compared the protein sequence of the prospective J domain of *JDD1* (amino acids 11–90, shown in Fig. 3) with others in the database. DnaJ and its homologues were found to have the most similar amino acid sequences to the possible J domain of *JDD1*, e.g., DnaJ itself, HSJ-1, hsp40 (HDJ-1) (Fig. 3) (7, 9, 34). The J domain is the most highly conserved region in the DnaJ family and is known to be essential for interaction with DnaK (bacterial hsp 70) (14). In addition, *JDD1* has the HPD (histidine-proline-aspartate) sequence that is the most conserved motif in the J domain over species and is crucial for interaction with DnaK or its homologues (Figs. 2b and 3) (17, 27, 35). Conversely, *JDD1* bears little resemblance to DnaJ outside its J domain. *JDD1* lacks the G/F region (a region rich in both glycine and phenylalanine) and the zinc finger region (also known as a cysteine-rich region), both characteristic to DnaJ (12, 14, 27). However, some other potential members of the DnaJ family also do not possess either the G/F region or the zinc finger region (1, 2, 14). A typical example of this type is auxilin, which was identified as a cofactor for hsp70 in uncoating of clathrin-coated vesicles (2). Auxilin lacks the zinc finger region thought to be the site of attachment of unfolded polypeptides, but possesses a clathrin-binding domain instead (36). This raises the possibility that a binding site for some specific protein may also exist in *JDD1*. Although we have searched for potential protein binding sites in *JDD1* by comparing the regions outside the J domain of *JDD1* (amino acids 91–259) to database sequences, we could not identify any significant motifs or proteins homologous to this region.

*JDD1 mRNA Is Expressed in the Germinal Zone during Development and Its Expression Persists into Adulthood*

It is well known that brain cells are generated and differentiate in the ventricular zone (neuroepithelium), a germinal matrix lining the ventricle, then migrate up out of the ventricular zone toward the pial surface (31). The subventricular zone, which is situated outside the columnar neuroepithelium, is also considered another component of the germinal matrix (31). Therefore, robust but confined expression of *JDD1* mRNA in the germinal zone (ventricular and subventricular zone)

suggests that the function of *JDD1* in the CNS is related to cell generation and/or differentiation there. The fact that *JDD1* mRNA was already expressed at an early stage of neurogenesis, on E11, when cells have begun to be generated and/or differentiate in the ventricular zone (31), supports this assumption (Fig. 1). On the other hand, no obvious expression of *JDD1* mRNA was noticed outside the germinal zone in the CNS (Figs. 1 and 4). Interestingly, cells situated deepest in the ventricular zone (cells lining the ventricle) were devoid of any apparent *JDD1* mRNA signals (Figs. 4b and 4c). It is well known that such cells are in the mitotic stage (31). In addition, *JDD1* expression in the subventricular zone on P7 was notable (Fig. 4d). Recent studies have revealed that neurons and astrocytes are generated in the subventricular zone then migrate toward the olfactory bulb after birth, even in the adult (37, 38). The persistent expression of *JDD1* in the subventricular zone and its specific expression pattern in the ventricular zone raise the possibility that it is related to neurogenesis and/or gliogenesis but not to mitosis. Additionally, the specific expression pattern of *JDD1* mRNA suggests that *JDD1* could be an important molecular marker of the germinal zone in the CNS. So far, several marker molecules with different expression patterns in the germinal zone have been identified, and become essential tools for studies on neurogenesis (39, 40).

Outside the CNS, *JDD1* mRNA showed identical distribution to #A81 (Fig. 1). *JDD1* mRNA was widely but heterogeneously expressed elsewhere in the embryos: its expression was notable in the developing liver, lung and cortex of the kidney (data not shown).

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