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A nervous system-specific isotype of the β subunit of Na^+,K^+-ATPase expressed during early development of *Xenopus laevis*

(brain/β subunit isoforms/neural induction)

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ABSTRACT **We have previously described the isolation of several genes expressed exclusively in the nervous system of adult *Xenopus laevis* and activated in the embryo shortly after neural induction. The sequence of one of these cDNAs, 24-15, identifies the corresponding protein as an isotype of the β subunit of Na^+,K^+-ATPase [ATP phosphohydrolase (Na^+/K^+-transporting); EC 3.6.1.37]. This form is distinct from the previously described β1 subunit of *Xenopus*, and the protein sequence comparison suggests that it is not the frog homolog of the mammalian β2 subunit; therefore, we refer to the 24-15 protein as the β3 subunit of Na^+,K^+-ATPase of *Xenopus*. Antisera directed against β3-subunit fusion protein detected a protein in adult brain extracts with the size and properties expected for a Na^+,K^+-ATPase β subunit. In *Xenopus* the β1 and β3 subunits are expressed as maternal mRNAs at similar levels; during embryogenesis rapid accumulation of β3 mRNA begins at stage 14 (early neurula), and the rapid accumulation of β1 mRNA begins at stage 23/24. *In situ* hybridization of antisense RNA probes to tadpole brain sections indicates that β3 subunit is expressed throughout the developing brain. We suggest that β3 is a major Na^+,K^+-ATPase β subunit present during early nervous system development in the frog.**

Regulation of the ionic environment of a cell is important for cell function, in particular during embryogenesis and in the nervous system. The levels of Na^+ and K^+ ions in the cell are determined, in part, by the action of membrane-bound Na^+,K^+-ATPase [ATP phosphohydrolase (Na^+/K^+-transporting); EC 3.6.1.37] or the sodium pump. This enzyme is composed of two subunits, a 95,000–100,000 M_r α subunit and a 45,000–60,000 M_r β subunit (1, 2). The α or catalytic subunit contains the ion channel and the ion-, ATP-, and cardiac glycoside (i.e., ouabain)-binding sites characteristic of the enzyme. The β subunit has no known function, although it has been proposed to be important in synthesis and transport of the α subunit (3–5). Different isoforms of Na^+,K^+-ATPase have been identified in various tissues, such as brain and heart, in addition to a constitutive form present in most cells. All three α subunits (6, 7) are expressed in the mammalian nervous system (8–13), α2 being expressed preferentially in the nervous system in contrast to the nearly ubiquitous expression of α1 (8, 9, 12, 13). Analogously, a nervous system-specific β subunit (β2) has been described recently in addition to the β1 subunit present in most cells (14, 15).

We have previously characterized a series of cDNAs expressed in the developing nervous system of *Xenopus laevis* embryos (16). These cDNAs were isolated on the basis of two properties: (*i*) exclusive expression of the corresponding RNA in adult nervous system, and (*ii*) activation of RNA accumulation during gastrulation. One of these cDNAs§ encodes an isotype of the β subunit of Na^+,K^+-ATPase that is expressed in pregastrula embryos and is induced in the nervous system after gastrulation.

MATERIALS AND METHODS

Sequencing. Sequences were determined by using Sequenase (United States Biochemical) with double-stranded plasmid DNA templates. Templates were prepared either with the Erase-a-Base kit (Promega) or by specific restriction enzyme digestions. Where necessary, oligonucleotide primers were synthesized to complete the sequence of both strands. The sequence was assembled and analyzed with the University of Wisconsin Genetics Computer Group programs (17).

Nucleic Acid Probes. For the β3 subunit of *Xenopus* Na^+,K^+-ATPase an open reading frame probe for blots was constructed by restriction enzyme digestion. For the *Xenopus* β1 subunit of Na^+,K^+-ATPase, specific oligonucleotide primers near the 5′ and 3′ ends of the coding region were synthesized and used to polymerase-chain-reaction (PCR) amplify the cDNA from XTC-cell RNA (18). The amplified cDNAs were cloned into a plasmid vector, sequenced to confirm the identity of the insert, and used as probes.

Nucleic Acid Analyses. RNA blots were either as described by Richter *et al.* (16) or by Thomas (19); the blots were hybridized with random-primed probes and washed as described (16). Primer-extension analysis was performed with the primer described in the legend for Fig. 2.

Embryo Culture Experiments. *Xenopus* embryos were staged according to Nieuwkoop and Faber (20). Animal caps were dissected from stage-8/9 embryos and treated with conditioned medium from XTC cells as described by Rosa *et al.* (21).

Antibodies and Immunoblots. A trpE fusion protein was constructed by cloning the extracellular domain of the C terminus of the β3 subunit into vector pATH3 (22). The appropriate DNA fragment was synthesized by PCR (23) with a sense synthetic primer (5′-CACGTCGACAGAGACAGAGTTTCTTCTCCA) encoding a *Sal* I site adjacent to the sequence starting at amino acid 63 and a vector primer; this fragment was inserted into pATH3 at the *Sal* I and *Hin*dIII sites. Production of antisera from gel-purified antigen, immunoblots, and affinity purification of antisera on immunoblots were done as described (24). Goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma) or ^{125}I-labeled protein A (Amersham) were used interchangeably for detection.

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Abbreviations: MIF, mesoderm inducing factor; nt, nucleotide(s); PCR, polymerase chain reaction.

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37788).

Membrane Protein Preparations. Membrane proteins were prepared by differential centrifugation. Dissected tissue was homogenized with a Dounce homogenizer in homogenization buffer (20 mM Hepes, pH 7.2/0.25 M sucrose/2 mM EDTA/1 mM phenylmethylsulfonyl fluoride/4 mM leupeptin/0.1 mM aprotinin) and centrifuged at 15,000 × *g* for 1 hr over a cushion of 50% sucrose in homogenization buffer. The supernatant and interphase were pooled and recentrifuged at 100,000 × *g* for 1 hr. The pellet was resuspended in solubilization buffer (150 mM NaCl/20 mM Hepes, pH 7.2/1 mM EDTA/1 mM EGTA/0.5% Nonidet P-40/5 mM phenylmethylsulfonyl fluoride/4 mM leupeptin/0.1 mM aprotinin) for 1 hr on ice and centrifuged for 15 min at 14,000 × *g*; the supernatant was collected as a crude membrane preparation. Protein concentration was determined with Pierce BCA reagent.

***In Situ* Hybridizations.** *In situ* hybridizations were performed with ^{35}S-labeled RNA probes on paraffin-embedded tissue sections from dissected stage-50/52 brains (25). Sense-strand hybridization was used as a background control.

RESULTS

Sequence and Identification of the 24-15 Insert. The insert from clone 24-15 is 1446 nucleotides (nt) long with the longest open reading frame encoding a protein of M_r 31,500. The predicted 24-15 protein product is related to the β subunit of Na^+,K^+-ATPase as shown by alignment with the *Xenopus* β1 and rat β1 and β2 subunits (Fig. 1). All proteins share a single predicted transmembrane domain in the N-terminal region; for the β1 subunit, the N terminus and C terminus are known to be the intracellular and extracellular domain, respectively (28). All β subunits contain six conserved cysteine residues in the extracellular domain involved in forming three intramolecular disulfide bonds (29). At least three possible glycosylation sites (NXS/T) are present, all of which are used in β1 subunits (30, 31).

The 24-15 protein is more closely related to the rat β2 subunit than the β1 subunit (Table 1), but identity between rat β2 and 24-15 protein (56%) is lower than that between the β1 subunits of *Xenopus* and rat (67%). This fact and additional differences (see *Discussion*) imply that the predicted 24-15 protein may not be the *Xenopus* homolog of the rat β2 subunit. Intending not to preempt the β2 designation for a *Xenopus* subunit more closely related to rat β2 that may be discovered in the future, we propose to name the protein encoded by clone 24-15 the *Xenopus* β3 subunit of Na^+,K^+-ATPase.

Table 1. Pairwise comparison of the protein sequence of different β subunits of Na^+,K^+-ATPase

	Subunit identities, %		
	Rat β1	Rat β2	Xl β1
Rat β2	41		
Xl β1	67	39	
24-15	42	56	43

Protein sequences were aligned using the GAP program of the University of Wisconsin Genetics Computer Group programs as in Fig. 1.

The β3 Subunit mRNA Has Multiple 5′ Ends That Do Not Vary During Development. Primer-extension analysis of RNAs from different embryonic stages detected three predominant bands (Fig. 2), suggesting multiple initiation sites for this gene. The two largest bands could encode the 24-15 cDNA insert, whereas the smaller band could not. A similar heterogeneity of 5′-end location has been seen for the human and rat β1 subunit mRNAs (26, 32). At all stages tested, with both maternal and zygotic RNA, the locations and relative amounts of the 5′ ends were the same and were proportional to the RNA levels detected by Northern blots. Thus, maternal β3 subunit mRNA appears capable of being translated, and 5′-end formation is not developmentally regulated.

Developmental Expression of the β1 and β3 Subunit mRNAs. ATPase β3 mRNA is present as a maternal RNA and increases ≈5- to 10-fold by stage 14/15 (16). The β1 mRNA also is present as a maternal RNA at a similar level as β3 mRNA, but β1 mRNA begins to increase only by stage 18/19 (midneurula) and accumulates rapidly after stage 23/24 (Fig. 3). Thus, β1 and β3 mRNAs are available at similar levels in pregastrula embryos for synthesizing the corresponding proteins, but during early neurula stage the β3 mRNA predominates, suggesting that β3 subunit protein has a function at these developmental stages.

Inductive Activation of β3 Subunit mRNA. The rise of β3 subunit mRNA in gastrula/neurula stages suggested that gene activation occurs as a result of induction. To test this possibility we assayed β3 subunit mRNA levels in ectoderm

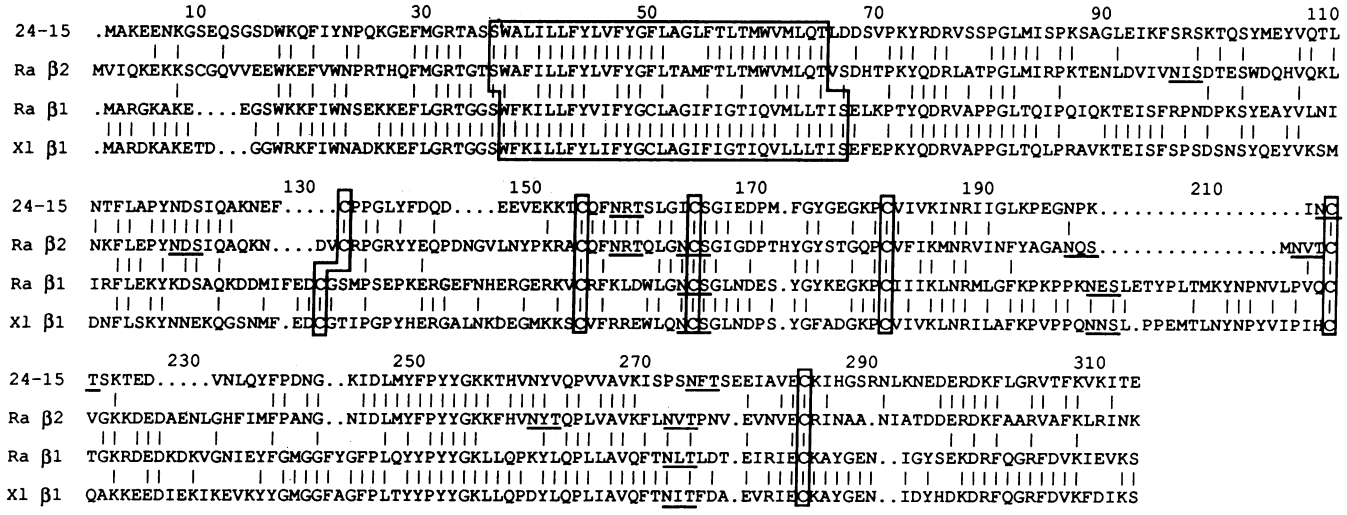

FIG. 1. Comparison of the protein sequences of different β subunits of Na^+,K^+-ATPase. The predicted protein encoded by the 24-15 cDNA insert (derived from the nucleotide sequence in GenBank accession no. M37788) was aligned with sequences of rat β2 (Ra β2) (14), rat β1 (Ra β1) (26), and *Xenopus* β1 (Xl β1) (27) subunits. Lines represent amino acid identities, and dots indicate a gap introduced for alignment. The box indicates the shared hydrophobic transmembrane domain. Conserved cysteine residues also are boxed, and possible N-linked glycosylation sites (NXS/T) are underlined.

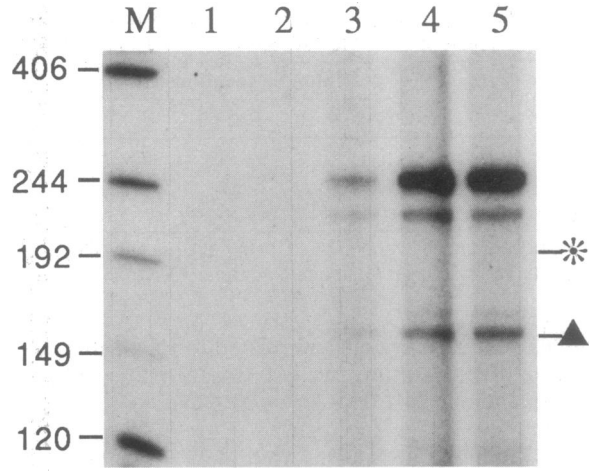

FIG. 2. Primer-extension analysis of β3 mRNA. A labeled antisense oligonucleotide (5′-GAGTCCGGCGAGGAATCCAT, nt 195–176 of the cDNA sequence, where the coding region starts at nt 37) was hybridized to 10 μg of RNA and extended with reverse transcriptase. The extension products were electrophoresed on urea/6% polyacrylamide gel and exposed to x-ray film. Lanes: 1, control reaction with only carrier tRNA; 2, kidney RNA; 3, stage 8 RNA; 4, stage 19 RNA; and 5, stage 25 RNA. Migration of molecular size markers (lane M) is indicated at left. Asterisk and triangle at right represent locations of the endpoint of cDNA 24-15 and the AUG initiation codon, respectively.

after induction with a mesoderm inducing factor (MIF). In this assay, isolated stage-8 to -9 ectodermal explants ("animal caps"), which would form epidermis in the absence of induction, are induced with MIF contained in conditioned medium from XTC cells (XTC-MIF) to generate various mesodermal derivatives (33). At high concentrations of XTC-MIF, neural tissue is induced as well (34, 35), presumably from secondary interactions between induced mesoderm and residual ectoderm (for reviews, see refs. 33 and 36).

A Northern blot of RNA from induced and control explants is shown in Fig. 4. In induced animal caps the level of β3 subunit mRNA increased 5-fold between equivalent stages 9 and 19, similar to the increase seen in whole embryos.

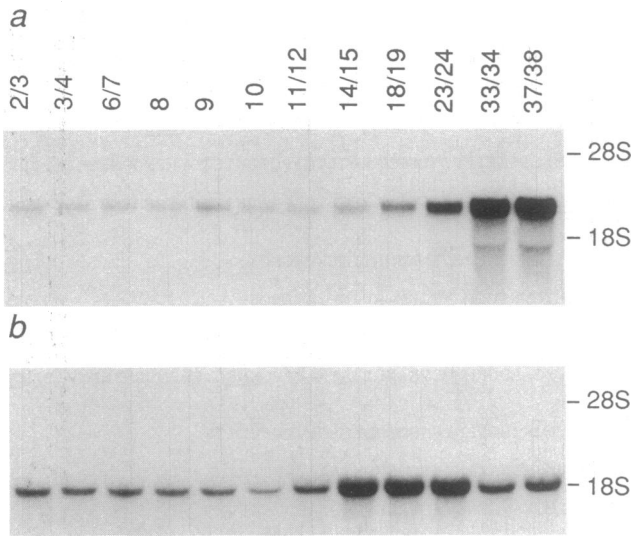

FIG. 3. RNA accumulation of β1 and β3 mRNAs during early development. Total RNA (10 μg) isolated from staged embryos was analyzed by Northern blots probed either with a *Xenopus* β1 (*a*) or β3 (*b*) coding region probe. The lanes are labeled with the stage, and migration of 28S and 18S rRNA is indicated at right. Both films were exposed for 12 hr.

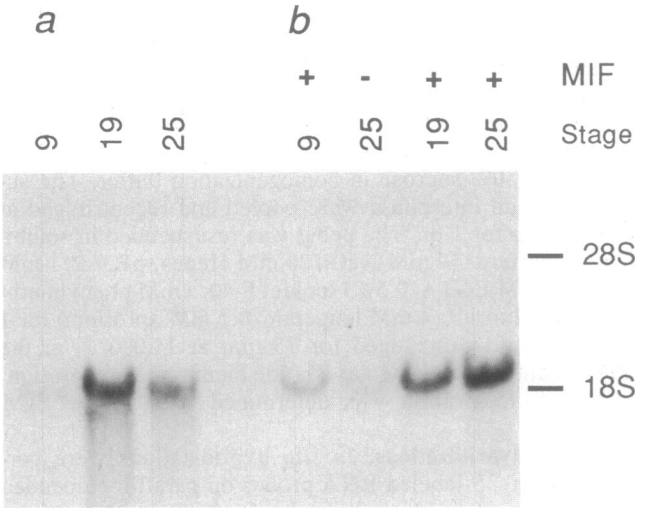

FIG. 4. Induction of *Xenopus* β3 mRNA in animal caps. Animal cap explants were isolated from stage-8 to -9 embryos and treated with XTC-conditioned medium containing MIF. RNA was isolated and analyzed by Northern blotting for expression of β3 mRNA. As control, one set of caps was treated with fresh medium; in addition, total-embryo RNA was isolated at corresponding stages. (*a*) RNA from treated animal caps. Lanes are labeled with the equivalent stage of RNA isolation; + and − refer to treatment with MIF or control medium, respectively. (*b*) RNA from whole embryos at indicated stages. Location of 28S and 18S rRNA is indicated at right.

Therefore, expression of the β3 gene responds to the inducing factor, suggesting that this RNA begins to accumulate in the neural plate during normal development as a consequence of neural induction. Lack of localization in neural plate-stage embryos seen earlier (16) presumably is due to the high level of unlocalized maternal RNA. Eventually, the induced tissue-specific accumulation restricts β3 subunit mRNA to the nervous system in later tadpoles and adults.

Detection of β3 Subunit Protein by Antibody Staining. β3 subunit is predicted to be a membrane glycoprotein restricted to the nervous system in adult *Xenopus*. To identify the protein, we constructed a fusion of *trpE* and the DNA encoding the presumed extracellular domain of β3 to produce an antigen. Antisera produced in rabbits against this fusion protein reacted with a broad band of 50–60 kDa found only in membrane preparations from brain but not from liver and kidney of adult *Xenopus* (Fig. 5). The higher-than-predicted molecular size and the broad band characterize glycoproteins in general and the β subunit of Na^+,K^+-ATPase in particular.

Localization of β3 Subunit mRNA in Tadpole Brain. To answer whether the β3 subunit gene is expressed differentially in brain regions we examined β3 mRNA distribution in stage-50/52 tadpole brain by *in situ* hybridization. Control hybridization with sense-strand probes revealed no significant accumulation of grains (data not shown). Fig. 6 illustrates that antisense probes hybridized effectively, showing a nearly ubiquitous brain distribution. The resolution of these *in situ* hybridizations does not allow us to determine whether particular cell types, such as glia or neurons, express this gene.

DISCUSSION

A Nervous System-Specific β Subunit of Na^+,K^+-ATPase in *Xenopus*. Our results indicate that the gene represented by cDNA 24-15 encodes a β subunit of Na^+,K^+-ATPase. This conclusion is based on sequence similarities with all known β subunits and on common structural features, including a transmembrane domain, at least six cysteine residues, and at least three glycosylation sites (Fig. 1). Further, the protein

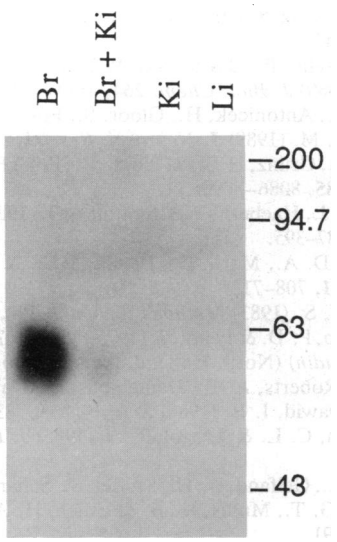

FIG. 5. Immunoblot of membrane proteins treated with anti-*Xenopus* Na^+,K^+-ATPase $\beta 3$ subunit fusion-protein antisera. Membrane proteins (40 μg) from different tissues were electrophoresed on SDS/10% polyacrylamide gel and transferred to Immobilon-P membrane (Millipore). After incubation with rabbit antisera and ^{125}I-labeled protein A, immunoreactive proteins were visualized by autoradiography. Br, brain; Ki, kidney; Li, liver. The lane labeled Br + Ki contained a 1:10 mixture of brain and kidney proteins. Migration of prestained molecular mass markers is indicated at right.

encoded by 24-15 cDNA was visualized by immunoblotting with fusion-protein antisera as a band migrating with a mobility similar to that of other β subunits of Na^+,K^+-ATPase (Fig. 5). Finally, 24-15 cDNA encodes a protein capable of associating with a Na^+,K^+-ATPase α subunit in mRNA-injected *Xenopus* oocytes. (P. Jaunin, P. Good, I. Corthésy, K. Geering, unpublished observations).

Whether the Na^+,K^+-ATPase we describe is the *Xenopus* homolog of the $\beta 2$ subunit of mammals preferentially expressed in the nervous system (14, 15, 37) or a distinct isotype is uncertain. Although the frog protein, which we named the $\beta 3$ subunit, resembles mammalian $\beta 2$ more than the $\beta 1$ subunit, differences in size and sequence appear to distinguish between $\beta 2$ and $\beta 3$ isotypes. The rat and *Xenopus* $\beta 1$ subunits show 67% sequence identity and differ in size by only one amino acid, whereas the rat $\beta 2$ and *Xenopus* $\beta 3$ subunits differ in size (290 vs. 277 amino acids) and are only 56% identical (Fig. 1 and Table 1). Furthermore, the mammalian $\beta 2$ subunits have eight to nine potential glycosylation sites, but the *Xenopus* $\beta 3$ subunit has only four. Sequence comparisons in the 3′-untranslated region support these relationships. While the known $\beta 1$ isotypes (including those from rat and *Xenopus*) and $\beta 2$ isotypes (human, rat, and mouse) share striking sequence similarities in the 3′-untranslated region within a particular isotype (3, 14, 27, 37), there is no above-random similarity between the 3′ regions of rat $\beta 2$ (14) and *Xenopus* $\beta 3$ subunits. Thus, we prefer the interpretation that the gene described here encodes a distinct subunit rather than a widely diverged form of the $\beta 2$ subunit, although the latter interpretation cannot be excluded.

The existence of additional β subunits of Na^+,K^+-ATPase in mammals has been postulated to explain the lack of $\beta 1$ or $\beta 2$ subunits in specific cell types that contain α subunits. In both the liver (9, 12) and pineal glands (38) of rats, no β subunit could be detected with probes for $\beta 1$ and $\beta 2$, despite the presence of α subunits. Although the role of the β subunit is unknown, the functional enzyme always has an α and β subunit (1, 2, 5).

Localized Expression of $\beta 3$ Subunit mRNA. The $\beta 3$ mRNA is expressed in the nervous system of tadpole and adult *Xenopus*. Our previous experiments revealed a maternal store of $\beta 3$ mRNA that is distributed throughout the egg and is still not localized in the early neurula stage (16). At this time $\beta 3$ mRNA begins to accumulate as a consequence of induction, as demonstrated by the suppression of its accumulation in UV-irradiated embryos (16) and its induction in MIF-treated animal cap explants (Fig. 4). This induced accumulation eventually leads to nervous system-restricted expression of $\beta 3$ mRNA in the later tadpole and adult.

Functions of Na^+,K^+-ATPase During Development. The regulation of Na^+ and K^+ ions by Na^+,K^+-ATPase is important during early neural development. In neurula-stage *Xenopus* embryos, cells in the neural plate have membrane potentials that differ from those of cells in adjacent ectoderm. This difference is abolished by treatment with inhibitors of Na^+,K^+-ATPase (39). Further, inhibition of Na^+,K^+-ATPase activity reduces the ability of cells from the presumptive neural plate of *Xenopus* embryos to differentiate

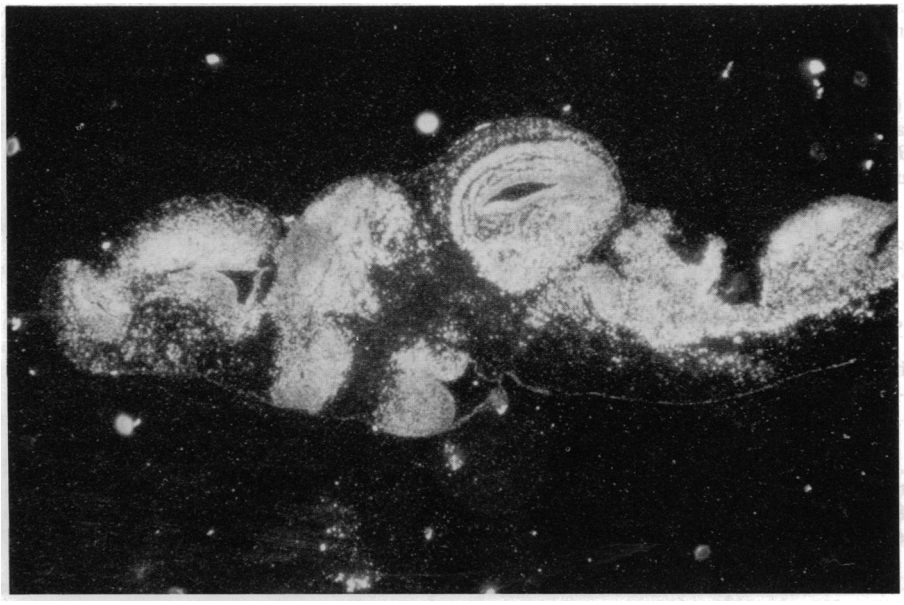

FIG. 6. *In situ* hybridization of antisense Na^+,K^+-ATPase $\beta 3$ subunit mRNA to a sagittal section of tadpole brain viewed with dark-field optics; anterior is at left, and dorsal is at top.

into neurons in an *in vitro* assay (40, 41). Neurula-stage embryos have a transepithelial potential generated, in part, by transport of Na^+ ions (42); this potential has been postulated to regulate differentiation and migration of neural cells (43, 44). Finally, Barth and Barth (45) have proposed that certain levels of Na^+ ions mediate neural induction in *Rana pipiens*.

The presence of maternal RNA components for the β1 and β3 subunits suggests that both are components of Na^+,K^+-ATPase in pregastrula embryos and have a function in these stages. The level of β1 RNA remains constant until late neurula stage, whereas the β3 RNA level increases soon after gastrulation (Fig. 3). Therefore, changes in Na^+,K^+-ATPase protein levels in the early neurula stage probably reflect the rise in β3 mRNA and the corresponding protein.

The β subunit of Na^+,K^+-ATPase may have a function apart from its role as an enzyme subunit. The adhesion molecule on glia (AMOG), which is important for neuron–astrocyte interaction, is the mouse β2 isotype of Na^+,K^+-ATPase (15, 37, 46). As discussed above, the *Xenopus* β3 subunit is more closely related to the mammalian β2 than the β1 sequence and might, therefore, function in neuronal and glial cell interactions. In a different system, the β subunit of Na^+,K^+-ATPase has been found in parts of the cell where no Na^+,K^+-ATPase activity or α subunit protein was detected (47). Thus, β subunits may have dual functions in general, or certain β subunits may not be part of the Na^+,K^+-ATPase at all but have an entirely separate role.

If the *Xenopus* β3 protein has a function in cell adhesion, its induction during neural-plate formation would present a second example in addition to neural cell adhesion molecule (NCAM) (48, 49) for the increase of an adhesion molecule at this stage. The similarity with the Na^+,K^+-ATPase β3 subunit is extended by the fact that NCAM mRNA also has a maternal component. At later stages of development NCAM is also expressed in muscle (50), where the Na^+,K^+-ATPase β3 subunit mRNA has not been detected (16). Thus, if the Na^+,K^+-ATPase has a function in cell adhesion, it is probably restricted to the nervous system, at least beyond the neurula stage.

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