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Tissue specificity, localization in brain, and cell-free translation of mRNA encoding the A3 isoform of Na^+,K^+-ATPase

(multiple isoenzymes/developmentally regulated expression/hybridization histochemistry)

JAY W. SCHNEIDER*, ROBERT W. MERCER†, MAUREEN GILMORE-HEBERT*, MANUEL F. UTSET*, CARY LAI‡, ADAM GREENE*, AND EDWARD J. BENZ, JR.*§

*Department of Human Genetics, Yale University School of Medicine, New Haven, CT 06510; †Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO 63110; and ‡Department of Biology, University of California, San Diego, CA 92037

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ABSTRACT **The isolation of multiple Na^+,K^+-ATPase cDNAs from rat brain has led to the discovery of a family of α-isoform genes. Using A1 (α), A2 (α^+), and A3 (αIII) Na^+,K^+-ATPase gene probes, we have analyzed the distribution of Na^+,K^+-ATPase mRNAs in adult and fetal rat tissues by RNA blot and hybridization histochemistry. A1 Na^+,K^+-ATPase mRNA was found ubiquitously among various tissues, with highest levels in transport epithelial and neural tissues. A2 mRNA was found in adult neural and muscle tissues, and A3 mRNA was found only in neural tissues and fetal heart muscle. Both A1 and A2 mRNAs were less abundant in fetal brain than in adult brain; in contrast, A3 mRNA was abundant at both stages. *In situ* mapping of brain areas that contain A3 mRNA suggests that this Na^+,K^+-ATPase isoenzyme is expressed predominantly by neural cells. Analysis of Na^+,K^+-ATPase proteins generated by cell-free translation of synthetic mRNAs suggests that the A3 protein has properties similar to A2 (α^+).**

Na^+,K^+-ATPase is a heterodimeric protein that transduces energy generated by ATP hydrolysis into the movement of Na^+ and K^+ in opposite directions across the plasma membrane of animal cells. This enzyme is responsible for the regulation of cell volume, for the generation of electrochemical gradients in contractile and neural tissues, and for the Na^+ gradients that drive transport of other solutes and ions, such as glucose, amino acids, and Ca^{2+}, across plasma membranes. Na^+,K^+-ATPase is widely distributed and known to exhibit structural and ouabain-binding heterogeneity in several tissues, including brain (1, 2), heart (3–5), fat, and skeletal muscle (6).

Previous studies have characterized two isoenzymes of Na^+,K^+-ATPase named α and α^+ (so designated because the catalytic subunit of α^+ has slower mobility on $NaDodSO_4$/PAGE) (2). These isoenzymes have been shown to possess distinctive molecular features. The α^+ enzyme can be distinguished from α by its higher affinities for ouabain and ATP (7), lower affinity for Na^+ (8), greater sensitivity to sulfhydryl reducing agents (2), specific inactivation by pyrithiamin (3), and higher resistance to trypsin digestion (2). The α and α^+ proteins also can be immunochemically distinguished by using isoform-specific antisera (9). The α^+ form is also selectively sensitive to insulin, which increases the affinity of the enzyme for Na^+ (6, 8). The distinct biophysical properties of these two enzymes suggest that each may be specialized to fulfill a different tissue or cellular requirement for ion transport.

Our group (10) and Shull *et al.* (11) have independently obtained evidence for additional heterogeneity of Na^+,K^+-ATPase. Both groups have isolated highly homologous rat brain cDNAs encoding three forms of the large subunit, Na^+,K^+-ATPases A1 (α), A2 (α^+), and a third form named A3 (αIII). The A3 cDNA encodes an isoform not previously appreciated by analysis of membrane protein or transport activities. Comparison of the deduced primary structures of A1, A2, and A3 proteins revealed ≈85% amino acid sequence homology and predicts no major differences in the secondary structures or membrane topologies of the isoform proteins (11).

To determine the tissue specificity of Na^+,K^+-ATPase isoenzyme expression, we measured A1, A2, and A3 mRNA levels in different adult and fetal rat tissues by RNA blot and hybridization histochemistry. We found that mRNA encoding each isoform was heterogeneously distributed among various rat tissues. Synthesis of A3 mRNA was restricted to neural tissues and fetal heart muscle. In adult brain and spinal cord, A3 mRNA represented a major transcript, similar in abundance to A1 and A2 mRNAs.

Since the A3 enzyme has never been identified in brain or fetal heart membranes *in vivo*, we used *in vitro* transcription and translation to synthesize A3 protein. The data presented in this paper suggest that the α isoform encoded by A3 cDNA represents a major component of Na^+,K^+-ATPase of neural cells.

MATERIALS AND METHODS

cDNA Clones. We have described (12) a rat brain cDNA (rb5) encoding a portion of the Na^+,K^+-ATPase A1 subunit that was obtained by antibody screening of an expression library. By using this cDNA to screen additional rat brain and heart libraries, we identified three distinct classes of Na^+,K^+-ATPase cDNA: A1 (α), A2 (α^+), and A3 (αIII). Rat brain cDNAs were isolated from a random-primed neonatal rat brain λgt11 library. Additional rat brain clones were isolated from an oligo(dT)-primed adult rat brain cDNA library constructed in pUC18 as described by Milner *et al.* (13). Several A2 cDNAs were also isolated from an oligo(dT)-primed rat heart λgt11 library (Clontech, Palo Alto, CA). Our sequence data agree exactly with those of Shull *et al.* (11), who established the primary structure of A1, A2, and A3 proteins from cloned rat brain and kidney cDNAs. Complementary DNAs containing the complete protein coding regions of A1, A2, and A3 were subcloned into the transcription vectors, pGEM-4 (Promega Biotec, Madison, WI) or Bluescript (Stratagene, San Diego, CA). These vectors were used to generate ^{35}S-labeled "antisense" RNA (complementary to mRNA) for hybridization histochemistry and "message-sense" RNA for use as cell-free translation template.

RNA Extraction and Blot Analysis. Total RNA was extracted from adult and ≈18-day-old fetal rat tissues by the

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§To whom reprint requests should be addressed.

guanidinium isothiocyanate/cesium chloride method of Chirgwin *et al.* (14). RNA blot analysis of electrophoretically separated RNA was performed as described (12) with ^{32}P-labeled cDNA probes generated by the multipriming system (Amersham). Full-length or near-full-length A1, A2, and A3 cDNA fragments were used as probes. Cross-isoform hybridization was eliminated by a high-stringency wash (>3 hr in 0.015 M NaCl/0.0015 M sodium citrate, pH 7/0.1% $NaDodSO_4$ at 68°C).

Hybridization Histochemistry. *In situ* hybridization was performed as described (15). Briefly, fetal whole-body or adult-brain frozen sections (10–20 μm thick) were mounted on gelatin-coated slides, fixed with 4% paraformaldehyde, and dehydrated. Sections were hybridized with single-stranded ^{35}S-labeled RNA probes prepared from transcription vectors containing full-length or near-full-length cDNA inserts encoding either the A1, A2, or A3 Na^+,K^+-ATPase. Hybridization was performed in 50% formamide at 55°C for 12–18 hr. Posthybridization treatments included RNase A digestion and high-stringency wash in 0.015 M NaCl/0.0015 M sodium citrate, pH 7/0.1% 2-mercaptoethanol at 60°C. Slides were dehydrated and then were exposed to x-ray film at room temperature for 3–5 days. For liquid emulsion autoradiography, slides were dipped into Kodak NBT-2 and exposed for 3 days. Control experiment with ^{35}S-labeled "message-sense" RNA probe yielded no specific labeling of fetal whole-body or adult-brain tissue sections. Identical results have been obtained at least three times with each isoform probe.

Cell-Free Protein Synthesis. Templates for *in vitro* translation were generated by transcription of vectors that contained the entire protein-coding regions of A1, A2, or A3 cDNAs. The transcription reactions were performed with reagents provided in the Riboprobe Gemini II System (Promega Biotec). For translations, 3 μl of a 20-μl transcription reaction were directly added to a 30-μl rabbit reticulocyte lysate translation reaction (Promega Biotec) supplemented with [^{35}S]methionine (Amersham). For membrane association, translation reactions were supplemented with dog pancreatic microsomes according to directions provided by the supplier (Amersham). Immunoprecipitations were performed with antiserum "K1" (9) as described (16). The proteins were analyzed by $NaDodSO_4$/PAGE (5% polyacrylamide) and enhanced fluorography.

RESULTS

Differential Expression of Na^+,K^+-ATPase Isoform mRNAs in Adult and Fetal Rat Tissues. Adult brain, diaphragm, intestine, heart, kidney, and spinal cord RNAs yielded informative differences in isoform mRNA expression (Fig. 1). The A1 cDNA hybridized to a single major mRNA, ≈3.7 kilobases (kb) long, which was found in varying abundance in all adult tissues examined (Fig. 1*a*). The A2 probe showed tissue-specific mRNA expression (Fig. 1*b*). In adult rat tissues, A2 transcripts were confined to brain, diaphragm, heart, and spinal cord. In these tissues, A2 cDNA hybridized to two discrete mRNAs, one ≈3.4 and the other ≈5.3 kb long. The A3 mRNA was also ≈3.7 kb long and was found only in adult brain and spinal cord (Fig. 1*c*). A3 mRNA was present in abundance similar to that of A1 and A2 mRNAs in total RNA prepared from adult rat brain, judging by the relative intensities of hybridization (Fig. 1).

Blot-hybridization analysis of RNA isolated from fetal rat brain, heart, and kidney revealed both tissue and developmental specificity of isoenzyme mRNA expression (Fig. 1). A1 mRNA was found in fetal kidney and heart and was present at low levels in brain (Fig. 1*a*); A2 mRNA was present at low levels in fetal brain and was not present in heart or kidney (Fig. 1*b*); and A3 mRNA was abundant in fetal brain,

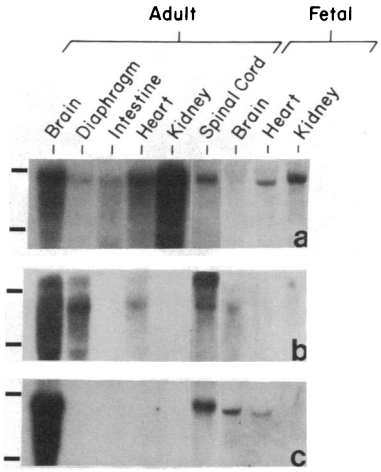

FIG. 1. RNA blot analysis of Na^+,K^+-ATPase isoform mRNAs in adult and fetal rat tissues. Total RNAs isolated from various adult and ≈18-day-old fetal rat tissues were denatured, subjected to electrophoresis in a 1% agarose/2.2 M formaldehyde gel, transferred to nitrocellulose, and hybridized to A1 (*a*), A2 (*b*), or A3 (*c*) cDNA probes. Filters were washed at high stringency (0.015 M NaCl/0.0015 M sodium citrate, pH 7/1% $NaDodSO_4$ at 68°C) for several hours and then exposed to film for 24 hr at −70°C in the presence of an enhancing screen. Twenty-five micrograms of RNA was added per lane. The two horizontal bars at the left of each panel indicate the positions of 28S and 18S ribosomal RNAs. The α-isoform mRNA size estimates used in the text are from ref. 17.

was present in low abundance in heart, and was not present in kidney (Fig. 1*c*). Thus, both A1 and A2 were minor mRNAs in fetal brain, whereas A3 was the major Na^+,K^+-ATPase transcript. In addition, A3 appeared to be a fetal-specific form of rat heart Na^+,K^+-ATPase mRNA.

Anatomic Distribution of Na^+,K^+-ATPase mRNAs in Fetal Rats. The distribution of Na^+,K^+-ATPase mRNAs in fetal rat organs was further investigated by *in situ* hybridization to sagittal sections of ≈18-day-old fetuses. Fig. 2 *A–C* represents photographs of stained fetal sections, and Fig. 2 *D–F* are the corresponding autoradiographs obtained after hybridization with ^{35}S-labeled single-stranded RNA probes.

As anticipated, the A1 probe detected abundant mRNA in ion- and water-transporting epithelia such as kidney (ki in Fig. 2*B*), gastrointestinal tract (gi), bladder (bl), rectum (rm), and nasotracheal respiratory epithelium (ne) (Fig. 2*E*). Abundant A1 mRNA was also found in fetal heart (ht in Fig. 2A) and neural structures such as the dorsal root ganglia (dg in Fig. 2*B*) and cranial nerve ganglia (cg) (Fig. 2*E*).

Analysis of fetal sections with the A2 probe repeatedly failed to demonstrate labeling of fetal brain or peripheral tissues (Fig. 2*D*). This result suggests that, in contrast to RNA isolated from adult neural and muscle tissues (Fig. 1*b*), A2 mRNA, if present, is at minimal abundance in ≈18-day-old fetal rat tissues. This result is consistent with the result obtained by blot analysis of RNA isolated from fetal rat tissues (Fig. 1*b*).

Expression in fetal rat tissues of mRNA encoding the A3 Na^+,K^+-ATPase contrasted markedly with the patterns seen for A1 and A2. A3 mRNA was found abundantly in the nervous system of fetal rats. Fig. 2*F* shows the autoradiograph of a fetal rat section that was hybridized to the A3 probe. Heavy A3-labeling is present in brain tissue, along the entire length of the spinal cord, and over the trigeminal ganglion. These results confirm the brain- and spinal cord-specificity of A3 Na^+,K^+-ATPase expression that had been suggested by blot analysis of RNA from adult tissues (Fig. 1*c*).

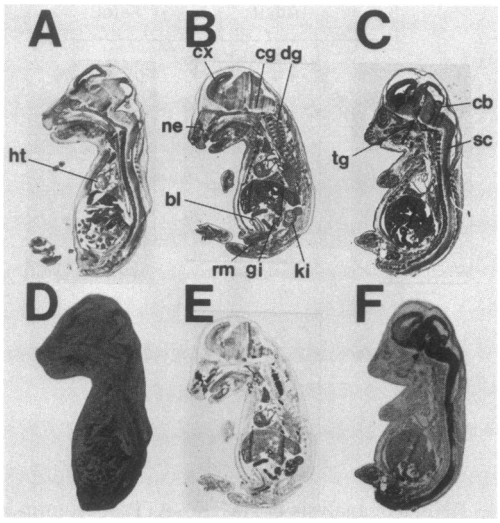

FIG. 2. Localization of Na^+,K^+-ATPase isoform mRNAs in fetal rat tissues by hybridization histochemistry. (*A–C*) Photographs of Giemsa-stained saggital sections of ≈18-day-old embryonic rats. These sections were hybridized with either A2 (*A*), A1 (*B*), or A3 (*C*) ^{35}S-labeled RNA probes complementary to Na^+,K^+-ATPase mRNAs. (*D–F*) Corresponding autoradiographs obtained after exposure of the hybridized sections in *A–C*, respectively, to film for 3 days. The apparent signals in the head region of *E* and in the liver of both *E* and *F* are due to folds in the sections at those positions. The apparent labeling in the caudal region of *F* is due to edge artifact and nonspecific bone-labeling. *In situ* hybridization of fetal rat sections with a "sense"-strand probe prepared from the A2 cDNA demonstrated no specific hybridization (data not shown). bl, Bladder; cb, cerebellum; cg, cranial nerve ganglion; cx, neocortex; dg, dorsal root ganglia; gi, gastrointestinal tract; ht, heart, ki, kidney; ne, nasotracheal epithelium; rm, rectum; sc, spinal cord; tg, trigeminal ganglion.

To more closely examine the hybridization signal obtained with the A1 probe, a second fetal rat section (Fig. 3*e*) was examined by liquid emulsion autoradiography after hybridization to the A1 probe. Fig. 3*g* is a bright-field photomicrograph of a dorsal root ganglion (DG) and spinal vertebrae (V) (identified by the arrowhead in Fig. 3*e*) that shows abundant silver grains localized over the ganglion body. The trigeminal ganglion (identified by the arrow in Fig. 3*e*) also demonstrated heavy A1 labeling. Fig. 3*f* is a dark-field photomicrograph that shows the fibrillar distribution of silver grains observed along the body of this structure. In contrast to neural ganglia, fetal brain and spinal cord tissues exhibited low levels of A1 labeling (Figs. 2*E* and 3*e*).

Mapping of Brain Regions That Contain Cell Bodies Expressing Na^+,K^+-ATPase A3 mRNA. To determine whether the cells expressing the transport enzyme encoded by A3 mRNA map to specific regions within brain, we performed *in situ* hybridization to rat brain sections. Fig. 4 shows adult rat brain sections after hybridization with a probe complementary to A3 mRNA. The right- and left-hand halves of each photograph are mirror images; the right half has been stained with hematoxylin/eosin, and the left half is the corresponding autoradiograph.

The A3 probe intensely labeled major neuronal formations of rat brain, including the hippocampus (hi in Fig. 4*B*), dentate gyrus (de in Fig. 4*A*), neocortex (cx), and subiculum (sb), whereas white matter was not appreciably labeled (Fig. 4). The areas of brain tissue labeled by A3 are all densely populated by neuron cell bodies. As an example, an intense signal was obtained in hippocampus with the A3 probe (arrow in Fig. 4*B*). Liquid emulsion autoradiography confirmed the neural cell-specificity of this hybridization. The dark field photograph in Fig. 3*a* shows that the A3 labeling (bright spots) found in hippocampus (hi) maps to the pyramidal cells.

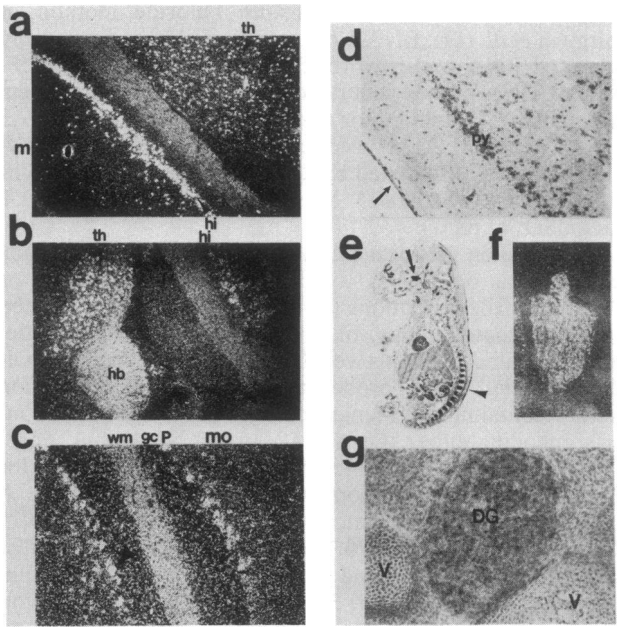

FIG. 3. Localization of Na^+,K^+-ATPase A3 mRNA to neural cells of adult rat brain and A1 mRNA to fetal neural structures. (*a–c*) Dark-field photomicrographs showing A3 hybridization (white spots) in hippocampal (*a* and *b*), thalamic (*a* and *b*), habenular (*b*), and cerebellar neurons (*c*) (the arrowhead in *c* shows the position of a Purkinje neuron). White matter (wm) stains artifactually bright in dark-field photomicrographs. (*d–f*) Bright-field photomicrograph showing silver-grain clusters over the pyramidal cells of neocortex (*d*), autoradiograph of a fetal rat section after hybridization with the A1 probe (this section was exposed for 3 days in liquid emulsion) (*e*), photomicrograph of the trigeminal ganglion indicated by the arrow in *e* (*f*), and photomicrograph of the dorsal root ganglion (DG) and spinal vertebrae (V) indicated by the arrowhead in *e* (*g*). gc, Granule cell layer of cerebellum; hb, habenula; hi, pyramidal neurons of hippocampus; m, molecular layer of hippocampus (A) and cerebellum (C); mo, molecular layer of cerebellum; P, Purkinje cell layer of cerebellum; py, pyramidal cell layer of neocortex; th, thalamus; V, spinal vertebrae; wm, white matter of cerebellum.

In addition, scattered neurons in the dorsomedial nucleus of the thalamus (th) and in the molecular layer of the hippocampus (m) show A3 hybridization (Fig. 3 *a* and *b*). Intense A3 hybridization was also observed in the habenula (hb), shown adjacent to the thalamus (th) in Fig. 3*B*. This epithelamic nucleus is a neural-cell-body-rich structure of rat brain.

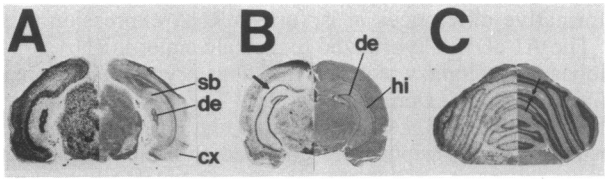

FIG. 4. Localization of Na^+,K^+-ATPase A3 mRNA in rat brain by hybridization histochemistry. The right-hand halves of *A*, *B*, and *C* are photographs of hemotoxylin/eosin-stained coronal sections of adult rat brain. These sections were hybridized with a ^{35}S-labeled RNA probe complementary to A3 mRNA. The left-hand halves are the corresponding autoradiographs obtained after exposure of each hybridized section to film for 3 days. Thus, the stained sections and corresponding autoradiographs are positioned as mirror images of each other. The arrow in *B* shows position of pyramidal neurons of hippocampus, and the apposed arrows in *C* show the position of the Purkinje cell layer of the cerebellum. *In situ* hybridization of adult brain sections with a "sense"-strand probe prepared from A2 cDNA revealed no specific hybridization. cx, Neocortex; de, dentate gyrus; hi, hippocampus; sb, subiculum.

Abundant A3 mRNA also was found in the pyramidal cells of rat brain neocortex. The A3 labeling obtained in the neocortex (cx of Fig. 4*A*) suggests that the signal intensity corresponds to the distribution of neuron cell bodies within the layers of the neocortex. Fig. 3*d* is a light-field photograph of rat brain neocortex after hybridization with the A3 probe. The arrow in Fig. 3*d* shows the pia membrane, which showed only background labeling. In contrast, silver grains are seen clustered over the large pyramidal neurons of the neocortex (py).

In cerebellum, A3 mRNA is largely restricted to the Purkinje cell layer, which consists of a single row of cell bodies of large Purkinje neurons. Fig. 4*C* shows a section through the cerebellar folia of rat brain after hybridization with the A3 probe. The arrows in Fig. 4*C* show the position of the Purkinje cell layer, located between the granule cell and molecular layers of the cerebellar cortex. The Purkinje cell layer clearly showed A3 hybridization. The localization of this signal to Purkinje cell bodies was confirmed by liquid emulsion autoradiography, as shown by the arrowhead in Fig. 3*c*. These results indicate that mRNA encoding the A3 Na^+,K^+-ATPase is most abundantly expressed by specific major neurons of adult rat brain.

Cell-Free Synthesis of Na^+,K^+-ATPase Proteins. Based on its high degree of homology with the protein sequences encoded by A1 and A2 cDNA, the protein derived from Na^+,K^+-ATPase A3 cDNA is proposed to be an α isoform of Na^+,K^+-ATPase. However, in the absence of N-terminal peptide sequence information of the mature A3 protein *in vivo*, unequivocal assignment of the initiator methionine or identification of the mature N terminus and the chain length from the cDNA sequence is not possible. We have demonstrated by cell-free transcription and translation that A3 mRNA generates a stable protein that can associate with microsomal membranes and possesses α^+-like $NaDodSO_4$/PAGE mobility.

Fig. 5 shows the results of an experiment in which synthetic A1 (lane a), A2 (lane b), and A3 (lane c) isoform mRNAs were separately added to a rabbit reticulocyte lysate in the presence of dog pancreatic microsomes. After 60 min of incubation, the reaction mixture was centrifuged and the microsomal pellets were collected. The microsome-associated proteins were immunoprecipitated with an antiserum raised against purified rat kidney α subunit. The immunoprecipitants were analyzed by $NaDodSO_4$/5% PAGE.

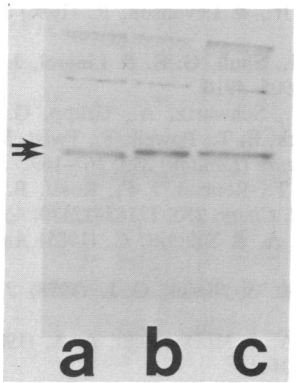

FIG. 5. Cell-free synthesis, membrane-association, and immunoprecipitation of Na^+,K^+-ATPase isoform proteins. ^{35}S-labeled Na^+,K^+-ATPase polypeptides were generated by translation of synthetic mRNA in rabbit reticulocyte lysate supplemented with rough microsomes. The protein associated with the membrane pellet was immunoprecipitated with an anti-Na^+,K^+-ATPase antibody and was analyzed on $NaDodSO_4$/5% PAGE. Lanes: a, Na^+,K^+-ATPase A1; b, Na^+,K^+-ATPase A2; c, Na^+,K^+-ATPase A3.

Pure radiolabeled rat brain A1, A2, and A3 proteins each associated with the microsomal pellet, which suggests insertion into the membrane. All three isoform proteins were immunoprecipitated with the Na^+,K^+-ATPase antibody. The Na^+,K^+-ATPase A3 protein was also immunoprecipitated by an α^+-specific antiserum (M.G.-H., unpublished data; antiserum "A2," ref. 9). Both A2 and A3 proteins, synthesized *in vitro*, migrated slower than did A1 in this gel system. Thus, the A3 protein has structural features similar to both A1 and A2 proteins and appears to possess mobility similar to that of α^+.

DISCUSSION

Our results indicate that the A1, A2, and A3 isoform mRNAs have distinct, but partially overlapping, distributions in brain and peripheral tissues. These results suggest that the patterns of α-isoform mRNA expression may reflect important differences in the physiologic functions of the individual isoenzymes.

In rat brain, each of the two enzymes was initially assigned to a different cell type: α to nonneuronal cells (e.g., glia) and α^+ to neuronal axolemma (myelinated axons) (2). This model was supported by ouabain-binding studies in brain, which identified a neuron-specific high-affinity ouabain-binding site, presumably residing on the α^+ Na^+,K^+-ATPase enzyme (18). However, several observations have complicated this simple model. First, retinal ganglion neurons synthesize and axonally transport both α and α^+ proteins (19), whereas unmyelinated superior cervical ganglion neurons synthesize only α (2). The neuronal expression of α^+ is not strictly dependent upon the presence of myelin, however, since α^+ protein is synthesized in the prenatal, premyelinated rat brain (20). In addition, the presence of α^+ protein and high-affinity ouabain-binding sites in peripheral tissues, such as fat, skeletal muscle, and heart, suggests a wider extraneuronal distribution of the α^+ isoenzyme. Finally, the data presented here suggest that some of the properties previously attributed to either α or α^+ enzymes in rat neural or muscle tissues may actually belong to A3.

The ubiquitous distribution of A1 mRNA in rat tissues suggests that the A1 gene may be expressed in all rat cells. The highest levels of A1 mRNA were observed in transport epithelia, especially kidney, gastrointestinal tract, and nasotracheal epithelium (Figs. 1–3). High specific activities of Na^+,K^+-ATPase in intact transport epithelial tissues has been amply documented in the literature (21–25). Our data, obtained with nucleic acid probes, shows that embryonic transport epithelial tissues abundantly synthesize A1 subunit mRNA, but not A2 or A3 subunit mRNAs (Figs. 1–3).

We also found high levels of A1 mRNA in adult neural tissue, especially adult brain (Fig. 1). The dorsal root and cranial nerve ganglia of fetal rats abundantly expressed A1 mRNA (Fig. 3 *e–g*). Avian dorsal root ganglia have been suggested to possess two different types of Na^+,K^+-ATPase, one of which is restricted to ganglion neurons (26). Our results show that fetal rat neural ganglia express high levels of both A1 and A3 mRNAs but do not express A2 mRNA (Figs. 2 and 3).

Abundant expression of A2-isoform mRNA appears to be restricted to muscle and neural tissues in adult rat (Fig. 1). It has been reported that adult rat kidney poly(A)$^+$ RNA and adipose, stomach, and lung total RNAs contain low levels of A2 mRNA (17). The relative abundance of the two A2 mRNAs in muscle versus neural tissues (Fig. 1b) is reminiscent of our previous finding of multiple Na^+,K^+-ATPase β subunit mRNAs that exhibit tissue-specific patterns of predominance (27). The heterogeneity of Na^+,K^+-ATPase β mRNA species was shown to result from tissue-specific use of multiple transcription initiation and polyadenylylation

sites (28). The tissue-specific patterns of abundance of multiple β and A2 mRNAs suggests that their noncoding sequences may contain functional information that may be important for regulation of enzyme biosynthesis in different tissues.

Rat heart has two classes of "receptors" that are responsible for the positive inotropic effect of ouabain in this tissue (29, 30). However, the identity of these receptor sites as Na^+,K^+-ATPase enzymes has remained controversial, especially since only one form of enzyme protein (α) has been detected in rat and guinea pig cardiac muscle (3, 31). However, both α and α^+ protein forms of Na^+,K^+-ATPase have been demonstrated in canine heart and isolated canine cardiomyocytes (3, 5). Our data show that A2 mRNA is expressed in adult rat heart and diaphragm muscle (Fig. 1). Furthermore, the relative abundance of A1 to A2 mRNA in total heart, estimated from the intensities of hybridization (Fig. 1), is consistent with the report that high-affinity ouabain-binding sites (presumably α^+) account for 25% of the total Na^+,K^+-ATPase enzyme molecules present in ventricular muscle of rat heart (4).

Studies of the biosynthesis of Na^+,K^+-ATPase enzyme in developing rat brain have shown that total Na^+,K^+-ATPase enzyme activity of brain membranes increases 10-fold during the transition from pre- to postnatal life (32). The α^+-isoform protein first appears in rat brain after day 14 of gestation (2, 20). Furthermore, the abundance of α and α^+ polypeptides increases 10-fold between day 18 of gestation and day 20 of postnatal life (33). Using RNA blot and *in situ* hybridization, we have demonstrated that both A1 and A2 mRNAs are present at low abundance relative to A3 mRNA in ≈18-day-old fetal rat brain (Figs. 1 and 2). In contrast, A1, A2, and A3 transcripts are all abundant in adult rat brain RNA (Fig. 1). These data suggest that transcription of Na^+,K^+-ATPase isoenzyme genes is subject to separate developmental controls in rat brain.

In heart, the developmental specificity of Na^+,K^+-ATPase mRNA expression suggests an A3-to-A2, fetal-to-adult isoform transition. By RNA blot, A1 and A3 mRNAs predominate in fetal heart, whereas A1 and A2 mRNAs predominate in adult heart (Fig. 1). Since A3 and A2 Na^+,K^+-ATPase isoenzymes appear to be the products of separate genes, this suggests the possibility of a program of gene switching during heart development. In addition, ouabain-binding studies have shown that Na^+,K^+-ATPase of newborn rat heart has different kinetic properties than those of the enzyme of normal adult heart but similar to those of hypertrophic adult heart (34). We speculate that this difference may result from the additional presence of A3 isoenzymes in newborn and hypertrophic adult heart membranes. These data suggest that differential regulation of the Na^+,K^+-ATPase gene family may be an important feature of both neural and muscle tissue development.

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