The presence of both negative and positive elements in the 5'-flanking sequence of the rat Na,K-ATPase $\alpha 3$ subunit gene are required for brain expression in transgenic mice

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ABSTRACT

The Na,K-ATPase is an integral plasma membrane protein consisting of α and β subunits, each of which has discrete isoforms expressed in a tissue-specific manner. Of the three functional α isoform genes, the one encoding the $\alpha 3$ isoform is the most tissue-restricted in its expression, being found primarily in the brain. To identify regions of the $\alpha 3$ isoform gene that are involved in directing expression in the brain, a 1.6 kb 5'-flanking sequence was attached to a reporter gene, chloramphenicol acetyltransferase (CAT). The $\alpha 3$ -CAT chimeric gene construct was microinjected into fertilized mouse eggs, and transgenic mice were produced. Analysis of adult transgenic mice from different lines revealed that the transgene is expressed primarily in the brain. To further delineate regions that are needed for conferring expression in this tissue, systematic deletions of the 5'-flanking sequence of the $\alpha 3$ -CAT fusion constructs were made and analyzed, again using transgenic mice. The results from these analyses indicate that DNA sequences required for mediating brain-specific expression of the $\alpha 3$ isoform gene are present within 210 bp upstream of the transcription initiation site. $\alpha 3$ -CAT promoter constructs containing scanning mutations in this region were also assayed in transgenic mice. These studies have identified both a functional neural-restrictive silencer element as well as a positively acting *cis* element.

INTRODUCTION

The Na,K-ATPase (E.C. 3.6.1.37) is a plasma membrane enzyme that maintains transmembrane gradients of Na⁺ and K⁺ utilizing ATP. The maintenance of these gradients is important

for a variety of cellular functions in all cells, such as regulating cell volume, producing the resting potential and transporting various nutrients, i.e. sugars, amino acid, etc. (1). In addition, the enzyme is of particular importance in certain specialized cell types, such as excitable muscle and nerve cells, and in fluid movement in epithelial cells of kidney and intestine. Na,K-ATPase is also the receptor for cardiac glycosides, such as digoxin and ouabain (2).

The protein consists of two subunits, a large catalytic α subunit (112 kDa) and a smaller glycosylated β subunit (45–60 kDa). The α subunit has three isoforms $\alpha 1$, $\alpha 2$ and $\alpha 3$ that exhibit differences in cardiac glycoside binding affinity, response to insulin, and affinity for sodium ions (reviewed in 3,4). In addition, the isoforms are expressed in a distinct tissue- and developmental stage-specific manner. The $\alpha 1$ isoform is expressed in almost all tissues while the $\alpha 2$ and $\alpha 3$ isoforms are expressed primarily in electrically excitable tissues. The $\alpha 3$ isoform has the most limited distribution of all three isoforms, being expressed specifically in the nervous system (5,6) as well as in fetal and neonatal heart and pineal gland in rat (6,7) and fetal and adult human heart (8). The α isoforms are encoded by separate genes whose genomic structure and DNA sequence have been characterized in different species (9–19).

We are particularly interested in identifying molecular determinants that specify expression of the $\alpha 3$ isoform in the brain. To address this question we have previously cloned and characterized the 5'-flanking region of the $\alpha 3$ isoform gene (15). The present study explores expression of the $\alpha 3$ isoform gene using transgenic mice. This system was chosen as it utilizes whole animals and therefore represents a more accurate description of specific gene function, and because tissue culture models for neuronal expression of the $\alpha 3$ isoform gene have not been identified. To define regions of the gene responsible for brain-specific expression, a 1.6 kb 5'-flanking region of the rat $\alpha 3$

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isoform was fused to a bacterial chloramphenicol acetyltransferase (CAT) gene and this construct as well as deletions and mutations in the flanking region were analyzed using transgenic mice.

MATERIALS AND METHODS

Construction of plasmids

A 1.6 kb *Ava*I–*Ava*I fragment containing the 5'-flanking region and transcription initiation sites of the rat $\alpha 3$ isoform gene was fused with a bacterial CAT gene in the Bluescript plasmid (Stratagene Inc.). This construct was used to initiate these studies and has been described previously (20). The various deletion constructs were made using restriction enzyme sites to cleave the rat $\alpha 3$ isoform gene, remove a fragment, and ligate with convenient restriction sites in the polylinker of the vector.

Production of transgenic mice

DNA fragments from the various constructs were cleaved with appropriate restriction enzymes to release the $\alpha 3$ –CAT fusion gene, free of vector sequences. These fragments were isolated in an agarose gel, electroeluted (21), and purified by ultracentrifugation over CsCl (22). The DNA was microinjected into pronuclei of single cell embryos obtained from FVB/N parents and the injected embryos were transplanted into the oviduct of pseudo-pregnant foster mothers (22). Transgenic offspring were identified by PCR amplification of the CAT gene. Positives thus identified were checked for the integrity of the transgene and copy number by performing Southern blot analysis of the DNA obtained from their tails (21).

RNA isolation and Northern blot analysis

Total RNA was isolated from adult mouse brain, ovaries and testes using the method of Chomczynski and Sacchi (23). Northern blots were prepared as follows. Total cellular RNA (10 μ g) from each of the tissues was denatured with glyoxal, fractionated in 1% agarose gels, and blotted onto Nytran membranes. The blot was baked, prehybridized and hybridized according to the manufacturer's protocol. A subunit-specific DNA restriction endonuclease fragment for the $\alpha 3$ subunit of the rat Na,K-ATPase (*Pst*I–*Sma*I, nt 51–331) was isolated as described previously (5). The DNA fragment was radiolabeled with [α - 32 P]dCTP using an oligolabeling kit (Pharmacia LKB Biotechnology Inc.). The radiolabeled DNA fragment ($\sim 1 \times 10^9$ c.p.m./ μ g) was added to the hybridization solution (2×10^6 c.p.m./l) and used as a probe. After completion of hybridization, the membrane was washed in $3 \times$ SSC, 0.1% SDS at 25°C for 30 min and then in $2 \times$ SSC, 0.1% SDS at 50°C for 15 min. Autoradiography was performed at –70°C for 48 h with Kodak XAR-5 film (Eastman Kodak, Rochester, New York) using a DuPont Cronex Lightning Plus intensifying screen (DuPont–New England Nuclear).

Chloramphenicol acetyltransferase (CAT) assay

Extracts were prepared from various tissues by homogenization using a Polytron homogenizer in 250 mM Tris–HCl, pH 7.8. These were heat inactivated at 65°C for 5 min and centrifuged at 12,500 r.p.m. for 2 min. Protein concentration of the supernatant was determined by the method of Bradford (24).

Appropriate amounts of extract were used in a 150 μ l assay containing 1.6 mM acetyl CoA and 0.05 mCi [14 C]chloramphenicol (54 mCi/mmol, Amersham Corp.) in 250 mM Tris–HCl, pH 7.8, and incubated at 37°C for 3 h. The assay was

Figure 1. (A) Genomic organization of the 5' region of the rat $\alpha 3$ isoform gene. Exon I is indicated as a box. The transcription initiation site is indicated with an arrow. The numbers refer to nucleotide positions relative to the transcription start site. The positions of the putative TATA and CCAAT boxes are indicated. The letters indicate restriction enzyme sites. They are B, *Bam*HI and A, *Ava*I. (B) Deletion analysis of the rat $\alpha 3$ isoform gene promoter. The open box indicates a CAT reporter gene. Various deletion constructs were made with convenient restriction sites and relative to the 5' *Ava*I site.

terminated and extracted with 250 μ l of ethyl acetate. The organic phase was dried, suspended in 10 μ l of ethyl acetate and spotted onto a silica gel thin layer plate. The products were resolved in a chloroform/methanol (95:5) TLC system. Quantitation was done using the PhosphorImager system (Molecular Diagnostics).

Site-directed mutagenesis

Site-directed mutagenesis was performed by the method of Kunkel(25). A *Bam*HI–*Hind*III cassette (–210 to +77) of the rat $\alpha 3$ isoform gene was cloned into M13mp19 and single-stranded uracil-rich template was prepared. Synthetic oligonucleotides containing the mutated sequence were phosphorylated using kinase buffer (0.1 M Tris–HCl, pH 7.9, 10 mM MgCl₂, 5 mM DTT) in the presence of ATP. Circular single-stranded uracil-rich template (0.1 pmol) was annealed to 2 pmol of each kinased oligonucleotide in annealing buffer (0.1 M Tris–HCl, pH 7.9, 10 mM MgCl₂, 50 mM NaCl) and allowed to cool from an initial temperature of 95°C to room temperature over a period of 2 h. To this the following were added: reaction buffer (10 mM Tris–HCl, pH 7.9, 5 mM MgCl₂, 5 mM DTT), 3 mM dNTPs, and 5 mM ATP, T4 DNA polymerase (3 units) and T4 DNA ligase (2 units). The mixture was incubated for 5 min at 4°C, 5 min at room temperature and 2 h at 37°C. An aliquot of this mixture was used to transform DH5 α F' host bacterial cells, and clones identified by standard molecular biological techniques.

RESULTS

Expression of a transgene containing 1.6 kb of the 5'-flanking region of the rat $\alpha 3$ isoform gene

The 5'-flanking region of the rat $\alpha 3$ isoform gene (nt –1596 to +77 relative to the transcription start site, Fig. 1A) was fused to a CAT gene (20), and used for microinjection into fertilized mouse eggs to create transgenic mice. A total of seven founders

Figure 2. Expression of the various deletion constructs in transgenic mice. Tissue extracts (containing 50 mg total protein) from various tissues of a transgenic mouse from the relevant line were used to measure CAT activity. The lines shown are as follows: (i) pRAT α 3CAT Δ AvaI(23-7), (ii) pRAT α 3CAT Δ XbaI(X18-7), (iii) pRAT α 3CAT Δ KpnI(K9-35), (iv) pRAT α 3CAT Δ ApaI(A9-33), (v) pRAT α 3CAT Δ BamHI(B9-7), (vi) pRAT α 3CAT Δ PvuII(P1-14). The unacetylated substrate is the bottom-most band, while the two acetylated products are the bands seen above. The different tissues are labeled above as follows: Br, brain; He, heart; Li, liver; Lu, lung; Ki, kidney; Spl, spleen; St, stomach; SI, small intestine; LI, large intestine; Tes, testis; Ov, ovary; Ut, uterus; Ad, adipose.

were identified by Southern blot analysis of tail DNA and used to establish independent lines. Six of these transmitted the transgene to their progeny. Examination of tissues from adult mice of the F1 generation for expression of CAT activity revealed that the transgene is expressed predominantly in the brain, although the level of expression is variable among lines (Fig. 2, Table I). In some lines, expression was observed in certain other tissues (Table II). However, this expression was usually lower than in the brain and showed no consistent pattern in different lines. One of the transgenic lines (line 29) showed no CAT activity in any tissue examined, probably due to insertion of the transgene at a chromosomal location that suppresses its activity.

Analysis of 5' deletions of the rat α 3 isoform gene in transgenic mice

To localize potential regulatory sequences directing specific expression of the gene in the brain, deletion constructs containing various lengths of the 5'-end (Fig. 1B) were made as described in Materials and Methods. Transgenic lines from these constructs were established and characterized as Δ AvaI described above. There were 2 lines analyzed for Δ XbaI, 6 for Δ KpnI, 4 for Δ ApaI, 3 for Δ BamHI, and 6 for Δ PvuII. Of the 21 transgenic

founders bred, three lines had double insertions (one for Δ ApaI and two for Δ PvuII) and one line (Δ ApaI) did not transmit the transgene. Analysis of CAT expression from the different transgenes in adult mouse brains revealed that sequences present within 210 bp of the transcription start site are sufficient to direct brain-specific expression of the α 3 isoform gene (Table I). All deletion constructs, including Δ BamHI (–210), express in the brain. However, the smallest deletion construct (Δ PvuII), containing only 118 bp of the 5'-flanking region relative to the transcription start site, exhibited virtually no expression in the brain of the eight lines examined.

To determine whether the deletions had any effect on expression in non-neuronal tissues, CAT activity was determined in other tissues in all constructs and these results are shown in Table II. There appears to be some level of transgenic expression in non-neuronal tissues in certain lines, however, as with the construct used in the initial studies, this expression is generally lower than in brain and not consistent in different lines containing the same construct. The only consistent expression is gonadal tissues, ovary and testis. Expression of the α 3 isoform gene in these tissues has not been reported and therefore analysis of the endogenous pattern of isoform expression in these tissues was carried out and is described below.

Expression of the endogenous $\alpha 3$ isoform gene in gonadal tissues in mice

Since several transgenic lines showed expression in the gonadal tissues, the expression of the endogenous $\alpha 3$ isoform gene in

these tissues was examined by Northern blot analysis. The results (Fig. 3) indicate that the ovaries express the $\alpha 3$ isoform gene while the testes do not, although it is possible that the level of $\alpha 3$ mRNA in the testes is too low to be detected or that the expression is restricted to a very small population of cells in the testis (26). The expression of the endogenous $\alpha 3$ isoform in the ovary has not been reported previously. These results indicate that CAT expression seen in the ovaries of some transgenic lines is in accordance with the expression of the endogenous gene.

Table I. CAT activity in adult brain in various lines of transgenic mice

Construct	Line No.	Copy No./ Haploid Genome	Expression in Brain
Δ AvaI (-1596)	30	8	97* (± 0.77)
	23	16	10 (± 0.07)
	22	18	8.5 (± 0.7)
	11	21	2.0 (± 0.21)
	7	18	0.2 (± 0.04)
	29	ND	0
Δ XbaI (-1027)	X18	75	23 (± 7.4)
	X7	18	4.9 (± 3.5)
Δ KpnI (-637)	K9	47	95* (± 7.1)
	K2	42	37.5 (± 3.5)
	K24	37	20 (± 0.4)
	K17	5	3 (± 0.28)
	K38	5-10	0
	K44	14	0
Δ ApaI (-375)	A9-1	90-100	48*
	A9-2	37	0.4 (± 0.28)
	A5	45	0
	A43	>100	0
Δ BamHI (-210)	B10	>100	28.5 (± 0.77)
	B9	>100	27 (± 1.6)
	B4	>100	12.8 (± 0.42)
Δ PvuII (-118)	P28	ND	0.3 (± 0.21)
	P8	ND	0
	P14	7.0	0
	P1	ND	0
	P3-1	1-10	0
	P3-2	1-10	0
	P21-1	1-10	0
	P21-2	35	0

The numbers indicate the percent conversion of ^{14}C chloramphenicol substrate to acetylated products per 50 μg of total protein. * indicates that these conversions are beyond the linear range of the assay. Results shown include analysis of at least two animals. Standard deviation is indicated in parenthesis. Nontransgenic animals showed no CAT activity under these assay conditions (data not shown). ND is 'not determined'.

Analysis of mutation constructs in transgenic mice

The transgenic studies suggested that DNA sequences in the 92 bp region between -210 and -118, relative to the transcription initiation site, are important for directing expression of the $\alpha 3$ isoform gene in the brain. To further delineate regions in the 92 bp fragment that are necessary for brain-specific expression in transgenic mice, scanning mutagenesis of this region was performed as described in Materials and Methods. A total of six different mutations were made (Fig. 4A), each representing a 10 bp replacement at different positions in the 92 bp region. The mutated proximal region was fused to the Δ PvuII-CAT fusion gene construct (-118 bp) and used to create several transgenic mouse lines. Of the 28 transgene founders only two did not transmit the transgene and one line was sterile. Various tissues of the transgenic lines established were analyzed for expression. The results (Table III) indicate that all the mutant constructs, with the exception of mutation 6, express in the brain, indicating that this sequence is required for expression in this tissue. Analysis of the transgene in non-neuronal tissues shows that high levels of expression in non-neuronal tissues in all lines analyzed is observed for mutation 3. High levels of CAT activity were also consistently observed in the brain containing this construct. This effect is possibly due to higher levels of expression in non-neuronal cell types in the brain as well as in other tissues. Examination of this region of the rat $\alpha 3$ isoform gene indicates that it contains a DNA sequence element exhibiting a high degree of similarity to the known neural-restrictive silencer element

Table II. Summary of expression of CAT constructs in various tissues of transgenic mice

CONSTRUCT	LINE NO.	BRAIN	HEART	LIVER	LUNG	KIDNEY	SPLEEN	OVARY	TESTIS	ADIPOSE	COLON	SMALL INTESTINE
Δ AvaI -1596	30	97*	0	0	7.1 (± 3.3)	0	5 (± 3.1)	0	0	0	0	0
	11	2.0	0	0	0	7.5 (± 0.35)	0	0	0	0	0	0
Δ XbaI -1027	X17	23	0	0	0	0	0	0	0	0	0	0
	X7	4.9	0	0	0	0	0	0	0	0	0	0
Δ KpnI -637	K17	3	0	0	0	0	0	7.3	1	0	0	0
	K9	95*	0	0	0	0	0	9.5 (± 0.49)	0	0	0	0
	K2	37.5	0	0	0	0	0	0	9.5	0	0	0
Δ ApaI -375	A9-1	48*	2	0	0	0	0	ND	1.4	0	0	0
	A9-2	0.4	0	0	0	0	0	0.3	ND	0	0	0
Δ BamHI -210	B9	27	0	0	0	0	0	9.1	4.6	0	0	0
	B4	12.8	1.1	2.1	0	0	0	3.2	2.9	0	0	0
	B10	28.5	0	0	0	0	0	5.6 (± 3.3)	ND	0	0	0
Δ PvuII -118	P28	0.3	0	0	0	0	0	1.8	0	0.8 (± 0.72)	0	0
	P14	0	0	0	0	0	0	0.3	0	0	0	0.6
	P8	0	0	0	0	0	0	0	0	0	1.3 (± 0.14)	4.3 (± 0.15)
	P1	0	0	0	0	0	0	4.2	ND	0	0	0
	P3-1	0	0	0	0	0	0	0.1	0	0	0.4 (± 0.14)	0.3 (± 0.28)
	P3-2	0	0	0	0	0	0	0	0	0	0.3	0
	P21-2	0	0	0	0	0	0	0.04	0	0	0.4	0

The numbers indicate the percent conversion of ^{14}C chloramphenicol substrate to acetylated products per 50 μg of total protein. * indicates that these conversions are beyond the linear range of the assay. Standard deviations are shown when more than one animal was used for the assay. The listed tissues were examined for expression of the transgene in all transgenic lines. ND is 'not determined'.

(NRSE, Fig. 4B), the overall identity being 76% (16 out of 21 match), while identity to the core element is 85% (11 out of 13 match).

Figure 3. Northern blot analysis of α isoform expression in mouse tissues. RNA from brain, heart, kidney, skeletal muscle (5 μ g), ovary and testis (10 μ g) was used to prepare the blot. The top panel shows hybridization to the $\alpha 1$ isoform-specific probe and the middle panel shows hybridization to the $\alpha 3$ isoform-specific probe. The bottom panel shows hybridization to the 18S probe. The positions of the 28S and 18S RNAs are indicated.

DISCUSSION

The $\alpha 3$ isoform of the Na,K-ATPase α subunit is expressed primarily in the brain (5). Several studies have shown that in this tissue expression is localized to neurons in all major regions of the brain; cerebrum, cerebellum, corpus callosum, axolemma and optic nerve (27–32). It is, however, absent in glial cells (32). This study was therefore undertaken to identify the molecular determinants in the $\alpha 3$ isoform gene that direct its neuronal-specific expression in the brain using transgenic mice.

From these studies, it is evident that the major determinant(s) in the expression of the $\alpha 3$ isoform gene lies within 210 bp upstream of the transcription initiation site. The results also suggest that there is at least one important element in the 92 bp region between –118 and –210 bp (relative to the transcription initiation site) contributing to expression of the $\alpha 3$ isoform gene in the brain. This can be seen by comparing the Δ BamHI construct (–210 bp), which appears to express in the brain in all lines examined, with the Δ PvuII construct (–118 bp), which is not expressed in the brain of seven out of eight lines analyzed. A detailed functional examination of this region using $\alpha 3$ –CAT gene fusion constructs containing mutagenized regions indicates that the neural-specific expression of the $\alpha 3$ isoform gene is in part governed by a silencer element. As described earlier, constructs containing mutation 3 show elevated expression of the $\alpha 3$ isoform gene in non-neuronal tissue. Moreover, this region of the rat $\alpha 3$ isoform gene has a high degree of similarity to the known NRSE (Fig. 4B). The wild-type rat $\alpha 3$ isoform gene has an overall 85% identity to the core consensus (11 out of 13 match), while mutation 3 reduces the homology to 69% (9 out of 13). In previous studies it has been demonstrated that the GG

A.

B.

Figure 4. (A) Mutational analysis of the proximal promoter region of the rat $\alpha 3$ isoform gene. The nucleotide sequences of the –210 to –118 region of the rat $\alpha 3$ isoform gene are shown above with potential transcription factor binding sites, as well as the NRSE core sequence, indicated. Also indicated are the six mutations that were made in this region. The dotted line represents nucleotide identity between the mutant and wild-type sequence and the bases listed indicate the substitutions. (B) Alignments of the NRSEs between the $\alpha 3$ isoform gene and other neuron-specific genes. The sequences are from the SCG10 gene (33), the NaII gene (38), the synapsin I gene (33,34) and the dopamine β -hydroxylase gene (36). The boxed sequence is the NRSE core. H and R represent human and rat respectively.

dinucleotide contained in the core sequence of the NRSE is particularly important for conferring the silencing effect in non-neuronal tissues on neuronally expressed genes. In the rat SCG10 gene, as well as the human synapsin I gene, conversion of GG to TT abolished silencing activity of the gene (33,34). In the case of the rat $\alpha 3$ isoform gene, mutation 3 causes an alteration in the core consensus sequence, and in particular in the GG dinucleotide, converting it to a GC. The mechanism by which the GG dinucleotide contributes to the silencing effect is not clearly understood, although reduced binding of a protein factor to the mutated sequence has been observed previously in other genes (33,34). Interestingly, this sequence is conserved in the human $\alpha 3$ isoform gene but not in $\alpha 2$.

The emerging theme in neural-specific gene expression appears to be one of selective repression in non-neuronal tissues mediated by the presence of a DNA element known as the neural-restrictive silencer element (NRSE). In addition to the SCG10 gene (33) and the synapsin I gene (34), this has been demonstrated for the Type II Na channel gene (35) the human β -hydroxylase gene (36) and the peripherin gene (37). In the case of both the synapsin I gene and the Type II Na channel NRSEs, repression is greater in the context of its own promoter as compared with a heterologous promoter (33–35,38,39). This suggests that the silencing effect of the neural-specific restrictive elements is dependent on promoter identity. Although the basis for such differential silencing is not understood, it has been postulated that interactions between proteins binding with the NRSE and other *cis* elements probably contribute to the effect (34). Whether

this is the case in the rat $\alpha 3$ isoform gene remains to be determined, although the close proximity of a functional silencer element to the basal promoter elements suggest that a specific interaction between the two could occur.

The transgenic results presented here suggest the presence of an additional *cis*-acting element, located between –140 and –118 bp relative to the transcription start site, that contributes to expression in the brain. Neither the $\Delta PvuII$ deletion construct nor mutation 6 express CAT activity in the brain, but both are transcriptionally competent as they express in the ovaries as well as certain other non-neuronal tissues. A detailed examination of this region reveals the presence of a region rich in G residues containing a 7 bp repeated sequence AAGGGGGAAGGGG located between –126 and –139 relative to the transcription initiation site. This may represent a potential neuronal-specific *cis*-acting element that is involved in governing expression of the $\alpha 3$ isoform gene in the brain.

In addition to the NRSE, the 92 bp proximal promoter region of the rat Na,K-ATPase $\alpha 3$ isoform gene contains consensus binding sites for transcription factors Oct-1, Oct-2 and AP-2, as well as a consensus binding site CACCC within the AP-2 sequence (Fig. 4A). It is possible that these may represent potential factors that play a role in governing tissue-specific expression of the gene. Although Oct-1 and Oct-2 bind the same octamer consensus sequence, they differ in their tissue distribution: Oct-1 is a ubiquitously expressed factor, while Oct-2 is more restricted in its expression, being found in B cells as well as neuronal cells (40,41). The two different cell types express

Table III. Expression of mutant constructs in transgenic mice

Mutation	Line No.	Copy No./ Haploid genome	Brain	Ovary	Liver	Kidney	Heart
-210 WT	B10	>100	28±0.77	9.1	0	0	0
	B9	>100	27±1.6	3.2	2.1	0	1.1
	B4	>100	12.8±0.42	5.4±3.3	0	0	0
1	M1-69	3	1.13±0.4	0	0	0	0
	M1-70	4	8.4±0.25	0	0	1.25±0.07	0
	M1-72	2	17.26±3.42	0	0	0	0
	M1-80	4	18.65±4.15	0	0	0	0
2	M2-2	2	2.8±1.1	5.95±0.15	0	0	0
	M2-96	9	1.5±0.44	ND	0	0	0
	M2-53	6	24±5.1	10.4±0.91	6.5±0.74	0	0
3	M3-84	5	89.1±4.8*(192)	30.5±4.3*(41)	39±1.1*(47.7)	27.4±0.56	6.2±0.35
	M3-87	19	97±1.1*(142)	6.2±1.6	7.4±2.4	25±2.4	8.7±0.35
	M3-88	8	95.8±4.9*(246)	8.27±2.2	4.7±0.7	6.36±0.1	26±1.49
	M3-19	7	79±4.4*(118)	5.3±0.84	1.7±0.2	23±1.75	22.5±2.1
4	M4-1	6	19.0±0.1	1.25±0.07	0	0	0
	M4-8	1	44.7±2.8*(57.2)	1.5±0.2	0	0	0
	M4-15	2	7.2±1.9	ND	0	0	0
	M4-17	2	4.1±0.41	1.0±0.16	0	0	0
	M4-22	3	5.9±0.34	1.52±0.03	0	0	0
	M4-25	1	7.8±1.1	1.65±0.07	0	0	0
5	M5-63	2	3.3±.42	0	0	0	0
	M5-39	11	6.62±1.0	1.9±0.02	0	0	11.2±0.32
	M4-45	2	13.8±1.4	0	0	0	0
6	M6-9	2	1.5±0.52	36±5.2*(42)	0	0	0
	M6-21	18	0	ND	0	0	0
	M6-29	2	0	32±2.5*(31)	0	0	0
	M6-30	2	0	40±0.7*(46)	0	0	0
	M6-32	2	1.5±0.11	1.9±0.7	0	0	0

The numbers indicate the percent conversion of ^{14}C chloramphenicol substrate to acetylated products per 50 μ g of total protein. * indicates that these conversions are beyond the linear range of the assay. Results shown include analysis of at least two animals. The numbers shown in parenthesis were obtained by using 5 μ g of protein to perform the assay so the conversions were in the linear range of the assay and subsequently multiplied by a factor of 10. ND is 'not determined'.

different forms of the Oct-2 protein that differ in binding affinity, the neuronal Oct-2 protein having a lower affinity for binding to the consensus sequence than the form found in B cells (42). Additionally, neuronal Oct-2 inhibits octamer-mediated expression, while the B cell protein activates an octamer-containing promoter. In the case of the AP-2 transcription factor, the pattern of expression in neuronal tissues is not consistent with expression of the rat Na,K-ATPase $\alpha 3$ isoform and therefore this transcription factor is unlikely to affect expression of the $\alpha 3$ isoform gene in brain (43). CACCC elements, on the other hand, have been shown to interact with a number of different proteins, such as Sp-1, TEF-2 and erythroid-specific factors (44–46). Until recently, proteins interacting with CACCC boxes were thought to be important as auxiliary factors aiding in the functioning of glucocorticoid response elements (47,48). Recently, a CACCC box binding factor was shown to interact with the erythroid-specific factor GATA-1 and this interaction is important in the functioning of the human γ -globin gene promoters (49). Additionally CACCC binding factors are important in the functioning of the human β -globin promoter (50). Whether these elements play a role in governing $\alpha 3$ isoform expression remains to be examined.

An unexpected finding of our studies is the expression of the $\alpha 3$ isoform in the ovaries. Ovarian expression of $\alpha 3$ appears to involve some DNA element(s) within –210 bp of the transcription initiation site. A number of the transgenic constructs express CAT activity in the testes, although endogenous expression of the $\alpha 3$ isoform gene was not seen. Transgenic experiments with the neuronally expressed synapsin I gene as well as the enolase gene have shown expression of the transgene in the testes, although neither is physiologically expressed in the testes (51,52). One possible explanation for this observation has been that some cell types in the testes provide most of the requirements for transcription of neuronal genes, such that transgene insertion then overcomes the last constraint for promoter activation (52).

In conclusion, the 92 bp region of the rat $\alpha 3$ isoform gene contains a functional NRSE that restricts expression in non-neuronal tissues. Our results suggest the presence of at least one additional *cis*-acting element that contributes to brain-specific expression. Further analysis of the promoter region of this gene should provide answers regarding the exact mechanisms by which $\alpha 3$ isoform expression is governed.

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