The expression of the human neuronal $\alpha 3$ Na^+, K^+ -ATPase subunit gene is regulated by the activity of the Sp1 and NF-Y transcription factors

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The Na^+, K^+ -ATPase is a ubiquitous protein found in virtually all animal cells which is involved in maintaining the electrochemical gradient across the plasma membrane. It is a multimeric enzyme consisting of α , β and γ subunits that may be present as different isoforms, each of which has a tissue-specific expression profile. The expression of the Na^+, K^+ -ATPase $\alpha 3$ subunit in humans is confined to developing and adult brain and heart, thus suggesting that its catalytic activity is strictly required in excitable tissues. In the present study, we used structural, biochemical and functional criteria to analyse the transcriptional mechanisms controlling the expression of the human gene in neurons, and identified a minimal promoter region of approx. 100 bp upstream of the major transcription start site which is capable of preferentially driving

the expression of a reporter gene in human neuronal cell lines. This region contains the cognate DNA sites for the transcription factors Sp1/3/4 (transcription factors 1/3/4 purified from Sephacryl and phosphocellulose columns), NF-Y (nuclear factor-Y) and a half CRE (cAMP-response element)-like element that binds a still unknown protein. Although the expression of these factors is not tissue-specific, co-operative functional interactions among them are required to direct the activity of the promoter predominantly in neuronal cells.

Key words: Na^+, K^+ -ATPase, neuroblastoma cell line, neuron, promoter, transcription factor.

INTRODUCTION

Na^+, K^+ -ATPase is a ubiquitous protein found in the plasma membrane of virtually all animal cells, in which it plays a pivotal role in maintaining the electrochemical gradient across the membrane. It consists of an α subunit [1–3], which is responsible for its catalytic and pharmacological properties, and β and γ subunits that may have regulatory functions [4–7]. Cloning techniques have made it possible to isolate four α subunit genes ($\alpha 1$ – $\alpha 4$) [8–11] encoding isoforms with different substrate affinities and kinetic properties, and discrete expression patterns [12]. In particular, the $\alpha 1$ subunit can be considered ubiquitous [10,13,14], the $\alpha 2$ isoform is mainly present in adipocytes, muscle, heart and brain [15–18], the $\alpha 3$ subunit predominates in neural tissues [19], and $\alpha 4$ is strictly restricted to testis [11].

The expression of Na^+, K^+ -ATPase is most complex in the nervous system where all of the isoforms, other than $\alpha 4$, can be expressed. This heterogeneity (which is made even greater by the fact that different β subunit isoforms can also be co-expressed) [10,13,14] is probably due to the need for a wide spectrum of ion pumping capability in order to counteract the dissipation of sodium and potassium gradients under the various metabolic and firing conditions of neuronal activity.

It has been suggested that the $\alpha 3$ isoform plays a key role in adult neurons during depolarization and the repeated firing of action potentials [12]: because of its low affinity for cations and its high affinity for ATP, this isoform may start operating when the

other Na^+, K^+ pumps are already working at saturation, thus significantly contributing to restoring resting membrane potential.

The spatial and temporal expression patterns of the $\alpha 3$ Na^+, K^+ -ATPase isoform in the brain, its cell-type-specificity (including neurons compared with glia), and the precise control of its amounts in a certain neuron are variables that are probably controlled at the transcriptional level.

Although the sequence of the 5'-regulatory regions of the rat and human $\alpha 3$ genes were published more than 10 years ago [20], the functional data currently available mainly concern the regulation of its gene expression in the heart [21,22]. Little is known about the molecular determinants that are responsible for the expression of $\alpha 3$ in neurons, and there is no information at all for $\alpha 3$ expression in human neurons.

In order to gain new insights into the genetic mechanisms governing the expression of the human $\alpha 3$ Na^+, K^+ -ATPase isoform in neuronal cells, we have defined the structural and functional properties of the basal promoter, and started identifying the transcription factors controlling its activity.

EXPERIMENTAL

Cell lines and cultures

The SY5Y human neuroblastoma cell line was grown in RPMI 1640 (Sigma–Aldrich, St. Louis, MO, U.S.A.), 10% foetal calf serum (Euroclone Life Science Division, Milano, Italy),

Abbreviations used: AP2, activator protein 2; ATF, activating transcription factor; CRE, cAMP-response element; CREB, CRE-binding protein; Egr1, early growth response 1; EMSA, electrophoretic mobility-shift assay; MHC II, major histocompatibility complex II; NF-Y, nuclear factor-Y; NF-YB, NF-Y B subunit; NRSE, neural restrictive silencer element; Sp1/3/4, transcription factors 1/3/4 purified from Sephacryl and phosphocellulose columns; UTR, untranslated region.

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100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine (Sigma–Aldrich). The HeLa cells were grown in Dulbecco's modified Eagle's medium (Sigma–Aldrich), 10% foetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 10 mM sodium pyruvate (Sigma–Aldrich).

RNA preparation and Northern blot analysis

The RNAs were prepared and the Northern blot analysis performed as described previously [23]. The RNAs were hybridized with a human $\alpha 3$ cDNA probe (10^6 c.p.m./ml) extending from nucleotide –31 to nucleotide +1143 with respect to ATG (GenBank® accession no. NM_152296). After stripping, the same blots were re-hybridized with a human 18 S cDNA probe (nucleotides 715–794; Ambion, Austin, TX, U.S.A.) in order to check the quality of the RNAs and normalize the signals obtained previously.

The human multiple tissue expression array (Clontech, Palo Alto, CA, U.S.A.) was hybridized with the same $\alpha 3$ cDNA probe as that used for the Northern blots ($\sim 2 \times 10^6$ c.p.m./ml) in accordance with the manufacturer's instructions, and washed at a final stringency of $0.1 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.5% (w/v) SDS at 50°C.

Primer extension

Total RNA from the SY5Y cells and HeLa cells was used for the primer extension experiments, which were performed by using a 32 P-labelled oligonucleotide (5'-GGGCTCAGGCTCAGGCTTGGGCTGGGAGC-3') complementary to the human $\alpha 3$ sequence from nucleotide +102 to nucleotide +74 with respect to the major transcription start site (see Figure 2). Briefly, 5×10^5 c.p.m. of oligonucleotide was co-precipitated with 20 µg of total RNA and elongated with Superscript II (Invitrogen, Carlsbad, CA, U.S.A.) at 50°C for 90 min, with yeast RNA being elongated as a negative control. The reaction products were run on a 6% (w/v) polyacrylamide denaturing gel. The sequence of the same portion of DNA was run in parallel as a molecular marker.

Plasmid construction

All of the reporter constructs were obtained by subcloning fragments of the human Na^+, K^+ -ATPase $\alpha 3$ subunit 5'-flanking region into the pGL3basic plasmid (Promega, Madison, WI, U.S.A.) upstream of the firefly luciferase gene (details on plasmid construction are available in the Supplementary Experimental Procedures at <http://www.BiochemJ.org/bj/386/bj3860063add.htm>). All of the constructs were checked by means of restriction analysis and partial sequencing.

Transient transfection

Both cell lines were transiently transfected by means of lipofection using the DMRIE-C reagent (Invitrogen) as described previously [23]. An equimolar amount of pRL-TK plasmid (Promega) was used to normalize transfection efficiency. After 36 h, firefly and *Renilla* luciferase activities were measured using the Dual Luciferase Reporter Assay System (Promega) in accordance with the manufacturer's instructions. All of the transfections were performed in duplicate, and each construct was tested in at least three independent experiments using different batches of plasmid preparations. The transient transfection data were analysed as described previously [24].

In vitro DNase I footprinting assays

The –204/+70 region of the Na^+, K^+ -ATPase $\alpha 3$ promoter was amplified by PCR using *PvuII* up and *BssHII* low as primers,

and cloned in pGEM-T easy vector (the $\alpha 3$ wt construct). In order to prepare the 308 bp probe, the $\alpha 3$ wt construct was digested with *NcoI* (to label the top strand), filled-in with Klenow and [α - 32 P]dCTP (3000 Ci/mmol; Amersham Biosciences, Little Chalfont, Bucks., U.K.), and digested with *SpeI*. The probes were gel-purified, extracted with phenol/chloroform and precipitated with ethanol. In each footprinting reaction, 2 fmol of probes (corresponding to 20 000–30 000 c.p.m.) were incubated with 50 µg of nuclear extract, prepared as described previously [25]; the reactions were performed using a slightly modified version of a previously described procedure [25]. The binding was carried out in a volume of 50 µl; for competitive footprinting, before the addition of the probe, the extract was pre-incubated for 30 min on ice in the presence of competitor oligonucleotides. DNase I (DNase I RNase-free; Hoffmann-La Roche, Basel, Switzerland) was diluted in $1 \times$ binding buffer, 100 mM KCl and 20 mM MgCl_2 , and used at concentrations of 0.05–0.2 units/µg of DNA without extract and 1.5 units/µg of DNA in the presence of extract. The DNase I treatment was performed by adding 5 µl of DNase I to each sample, incubating the mixture for 150 s at 25°C, and stopping the reactions with 55 µl of DNase I $2 \times$ stop buffer (where $1 \times$ stop buffer is 100 mM NaCl, 10 mM Tris/HCl, pH 8, 10 mM EDTA, 0.5% SDS, 5 µg/ml proteinase K, 50 µg/ml glycogen). After 30 min of incubation at 50°C, the samples were processed as described previously [25].

EMSAs (electrophoretic mobility-shift assays)

The double-stranded oligonucleotides used in the EMSA were labelled by fill-in and purified on a G-25 Sephadex column (Hoffmann-La Roche). Reaction mixtures containing 2 µg of BSA, 10 µl of $2 \times$ binding buffer [$1 \times$ binding buffer is 20 mM Hepes, pH 7.9, 2 mM MgCl_2 , 4% Ficoll, 0.5 mM dithiothreitol], 2 µg of double-stranded poly(dI-dC) · (dI-dC) (Amersham Biosciences), 100 mM final salt concentration (NaCl + KCl)] were assembled in a final volume of 20 µl and pre-incubated for 15 min on ice; 10 000–20 000 c.p.m. of the labelled probes (1 fmol) were added to each reaction. After 15 min of incubation on ice, the reaction mixtures were loaded on to 0.5× Tris/borate/EDTA, 5% non-denaturing polyacrylamide gels (acrylamide/bisacrylamide, 29:1) and run at a constant voltage of 150 V. Competition experiments and supershifts were carried out by pre-incubating the reaction mixtures with the appropriate amounts of unlabelled oligonucleotides or antibodies. All the oligonucleotides used in the EMSA experiments are listed in Supplementary Table 1 (available at <http://www.BiochemJ.org/bj/386/bj3860063add.htm>). All of the antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), except for anti-NF-YB (nuclear factor-Y B subunit), which was kindly provided by Roberto Mantovani (Dipartimento di Biologia Animale, Università di Modena e Reggio Emilia, Modena, Italy).

RESULTS

Expression profile of the human $\alpha 3$ subunit in normal tissues and cell lines

In order to evaluate whether the expression of the human $\alpha 3$ subunit has the same tissue distribution as that described in rat, we analysed the RNA extracted from a number of human adult and foetal organs by means of dot-blot hybridization with an $\alpha 3$ cDNA probe (Figure 1A; see Figure 1B for a key).

There were strong hybridization signals throughout the nervous system (Figure 1A, columns 1–3) except for the corpus callosum (Figure 1A, dot C2), pituitary gland (Figure 1A, dot D3) and

Figure 1 Multiple tissue expression array and Northern blot analysis of the human $\alpha 3$ subunit gene expression in normal tissues and cell lines

(A) A multiple tissue expression array (Clontech) was hybridized with a probe corresponding to human Na⁺,K⁺-ATPase $\alpha 3$ cDNA. (B) Positions of polyadenylated RNA from the different

spinal cord (Figure 1A, dot E3), which seemed to express a lower amount of $\alpha 3$ transcript. The heart also showed consistent $\alpha 3$ mRNA expression, although at lower levels than those observed in the nervous system (Figure 1A, column 4). In line with the adult tissue results, the RNAs extracted from foetal brain (Figure 1A, dot A11) and foetal heart (Figure 1A, dot B11) were also positive for $\alpha 3$ expression, and a weak signal was also observed in testis (Figure 1A, dot F8). Lane 10 in Figure 1(A) contains the RNAs purified from different human cell lines, in which the absence of hybridization confirmed that $\alpha 3$ expression was restricted to neuronal and cardiac tissues.

In order to identify a cell model in which to study the expression of the gene encoding the $\alpha 3$ subunit of the Na⁺,K⁺-ATPase pump, total RNA, purified from three human neuroblastoma cell lines (SH-SY5Y, SK-N-BE and IMR32) and one human non-neuronal cell line (HeLa), was analysed by Northern blot analysis (Figure 1C). A unique transcript of approx. 4 kb was observed only in the neuronal SY5Y, SK-N-BE and IMR32 cell lines (Figure 1C, lanes 1, 2 and 4), whereas no signal was observed in HeLa cells (Figure 1C, lane 3). Hybridization of the total RNA extracted from human autonomic ganglia obtained from two different patients showed the expression of a transcript of the same length (Figure 1C, lane 5), thus suggesting that the expression of $\alpha 3$ in neuroblastoma cell lines parallels that observed in normal tissue. In order to check the quality and the quantity of the RNAs loaded onto the gel, the same blot was also hybridized with an 18 S probe (Figure 1D).

Structural and functional characterization of the human $\alpha 3$ 5'-flanking region

In order to examine the regulatory mechanisms controlling the expression of $\alpha 3$ in neuronal tissues, we isolated the 5'-flanking region of the human $\alpha 3$ gene. A phage clone obtained by screening a human genomic library was found to contain 3.2 kb of the upstream region of the human $\alpha 3$ gene (− 3266/+ 70 with respect to transcriptional start site; GenBank[®] accession number NT_011109, subsequence range 14769867–14766532). The missing region (+ 70/+ 155) was cloned by means of PCR using $\alpha 3$ cDNA as the template.

Primer extension experiments performed to map the start site of transcription revealed four bands in the SY5Y cells corresponding to positions − 157, − 155, − 154 and − 152 upstream of the start codon (Figure 2A, lane 1). The most intense band corresponded to site − 155, which was therefore considered to be the major transcription start site and was used as a reference (+ 1) for numbering the 5'-regulatory region. As expected, no signal was detected with either HeLa (Figure 2A, lane 2) or yeast RNAs (Figure 2A, lane 3).

In order to test whether the isolated sequence contained a functional promoter, a fragment of approx. 3200 bp (corresponding to the genomic region immediately upstream of the major transcription start site) and a fragment of 155 bp [corresponding to the genomic region specifying the 5'-UTR (untranslated region) of $\alpha 3$ mRNA], were fused upstream of the luciferase reporter gene, and the resulting plasmid was transiently transfected in a neuronal (SY5Y) and a non-neuronal cell line (HeLa). The construct was

tissues and cell lines (columns 1–11) and controls (column 12). (C) The cDNA probe encoding the Na⁺,K⁺-ATPase $\alpha 3$ subunit was hybridized to 10 μ g of total RNA purified from the indicated cell lines (lanes 1–4) and human lumbar ganglia (lane 5). The cDNA identified a unique transcript of approx. 4 kb in the neuroblastoma cell lines (lanes 1, 2 and 4) and human ganglia (lane 5); the RNA molecular-mass markers are indicated on the left. (D) Hybridization signals obtained using human cDNA for 18 S ribosomal RNA after stripping the $\alpha 3$ probe.

Figure 2 Primer extension mapping of the transcription start sites and functional characterization of the minimal promoter region of the Na^+ , K^+ -ATPase $\alpha 3$ subunit gene in neuronal and non-neuronal cell lines

(A) A ^{32}P -end-labelled oligonucleotide complementary to the -82/-54 region with respect to ATG was annealed to total RNA from the SY5Y (lane 1) and HeLa (lane 2) cell lines and then extended. Yeast RNA (lane 3) was used as a negative control. The arrows indicate the position of the identified transcription start sites with respect to the ATG codon. The sequence of the same portion of DNA was run in parallel as a molecular-mass marker. (B) Sequence of the -123/+155 region containing the $\alpha 3$ minimal promoter. The positions are numbered with the major transcription start site (-155 in Figure 3) set as +1. The putative binding sites for Sp1, CREB, NF-Y and AP2 are underlined. The CCAAT and TATA boxes are indicated in italics. (C) Left-hand panel: schematic representation of the chimaeric constructs used in the transient transfections. The putative binding sites for Sp1, the CRE-like element, and the CCAAT and TATA boxes are indicated. The numbers on the left limit the 5' end of the genomic region in each construct. Right-hand panel: the chimaeric plasmids were transiently transfected in SY5Y (open bars) and HeLa cell lines (striped bars). The bars represent the transcriptional activity of the constructs as fold increases over the activity of the -64/+155 plasmid (set as 1). Results are means $\pm$ S.E.M. for at least three independent experiments carried out in triplicate. *** indicates significantly different from the activity of the -64/+155 construct (Student's *t* test, $P < 0.001$).

active in both cell lines, with transcriptional activities that were respectively (177.5 ± 26) -fold and (62.2 ± 12.2) -fold that of a control plasmid, devoid of any promoter sequence (pGL3 basic), thus functionally confirming that the isolated fragment contained the 5'-regulatory region of the human $\alpha 3$ gene (results not shown).

Functional characterization of the minimal promoter

A preliminary deletion analysis of the $\alpha 3$ 5'-flanking region showed that the region -123/-64 could still sustain a consistent level of transcription in both neuronal and non-neuronal cells (results not shown). As computer-assisted analysis of this region showed the presence of putative binding sites for the Sp1 (transcription factor 1 purified from Sephacryl and phosphocellulose columns), CREB (cAMP-response-element-binding protein)/ATF (activating transcription factor), NF-Y (nuclear factor-Y)/C-EBP (CCAAT-enhancer-binding protein) and AP2 (activator protein 2) transcription factors (Figure 2B), we carried out a deletion analysis in order to establish the contribution of these *cis*-acting elements to $\alpha 3$ promoter activity.

We first assessed the transcriptional activity of the core promoter, which included the TATA box, the transcription initiation sites and the genomic region specifying the 5'-UTR of the mRNA. A construct containing this region (-30/+155) did not show any transcriptional activity in SY5Y cells, but it still retained some activity in HeLa cells (results not shown). Basal activity in neuronal cells was obtained when a few nucleotides upstream of the core promoter were included in a novel construct (-64/+55), including two Sp1 and one AP2 putative binding sites. As expected, these sites were also functional in HeLa cells (Figure 2C).

As the -64/+155 region defined the minimal region capable of driving transcription in neuronal cells, we expressed the activities of all of the other constructs as a ratio in relation to that of the -64/+155 plasmid. The presence of the CAAT box (construct -80/+155) significantly increased the activity of the promoter (approx. 3-fold) in both cell lines (Figure 2C). However, when 20 and 43 bp were added at the 5' end (constructs -100/+155 and -123/+155 respectively), a substantial increase in promoter activity (4-fold and 9-fold respectively) was only observed in SY5Y cells (Figure 2C), thus suggesting that the two regions may function as neuro-specific *cis*-acting elements.

Figure 3 DNase I footprinting analysis of the $-204/+70$ region of the $\alpha 3$ promoter

Each footprinting reaction used 2 fmol of the 308 bp probe, spanning the region $-204/+70$ of the $\alpha 3$ gene promoter, labelled on the top strand, in the presence of 50 μ g of SY5Y (lanes 5–10 in **A**) and HeLa nuclear extract (lanes 4–9 in **B**). (**A**) Lanes 1–4, no nuclear extract. In lanes 2–4, 0.02, 0.1 and 0.2 units/ μ g DNA of DNase I respectively were added to the reaction mixture. A 5000-fold molar excess of unlabelled oligonucleotide containing the MHC II CCAAT box (lane 6), the CRE (lane 7), the AP2 (lane 8), the Sp1 (lane 9) or the Egr1 canonical binding site (lane 10) was added to the reaction mixtures. The protected regions F1, F2 and F3 are indicated on the right of the autoradiogram. The numbers on the left indicate the reference nucleotides on the probe; -128 , on the right, indicates the hypersensitive site in lane 9. (**B**) Lanes 1–3, no nuclear extract. In lanes 2 and 3, 0.1 and 0.5 units/ μ g DNA of DNase I respectively were added to the reaction mixtures. A 5000-fold molar excess of unlabelled oligonucleotide containing the MHC II CCAAT box (lane 5), the CRE (lane 6), the AP2 (lane 7), the Sp1 (lane 8) or the Egr1 canonical binding site (lane 9) was added to the reaction mixtures. The protected regions F1, F2a, F2b and F3 are indicated on the right of the autoradiogram. The numbers on the left indicate the reference nucleotides on the probe. The asterisks in both lanes 1 indicate an artifactual band.

Footprinting analysis of the $\alpha 3$ minimal promoter region

In order to map the DNA elements of the $\alpha 3$ minimal promoter that are involved in the binding of specific transcription factors, we carried out *in vitro* footprinting experiments using nuclear extracts of SY5Y and HeLa cells. DNase I treatment of a probe labelled on the top strand and spanning the $-204/+70$ region defined slightly different patterns of protected areas depending on the source of the nuclear extract (Figure 3A, lane 5, and Figure 3B, lane 4) in comparison with the controls with no added nuclear proteins (Figure 3A, lanes 2–4, and Figure 3B, lanes 2–3). In particular, the protected $-121/-92$ region (F1), which includes the Sp1 site located at $-110/-100$ (Figure 2B), was observed with both extracts (Figure 3A, lane 5, and Figure 3B, lane 4). A second footprinted area (F2) spanned a wider region in SY5Y cells ($-85/-48$), including part of a putative CRE (cAMP-response element)-like element ($-83/-87$), the CCAAT box ($-61/-64$) and an Sp1 site located at $-47/-59$ (Figure 2B). In HeLa cells, F2 could be actually divided into two sub-regions (F2a, $-86/-78$ and F2b, $-69/-53$), because the sequence between the CRE-like element and the CAAT box ($-77/-69$ in Figure 2B) was not protected. A third protected area (F3) extended from -43 to -33 in SY5Y cells, and from -51 to -38 in HeLa cells, and encompassed the second Sp1 site ($-38/-51$) and the putative AP2 site ($-45/-36$) (Figure 2B).

Competitive footprinting characterization of the protected region

In order to confirm the involvement of Sp1, AP2, CREB and CAAT-binding proteins in the protection of the $-159/+70$ region of the $\alpha 3$ minimal promoter, competitive footprinting experiments

were carried out using unlabelled oligonucleotides carrying canonical binding sites for the different transcription factors. In the case of SY5Y nuclear extract, an oligonucleotide containing the CAAT box of the MHC II (major histocompatibility complex II) gene promoter, which has been shown previously to bind NF-Y [26,27], affected protein interactions in the $-85/-63$ region (Figure 3A, compare lanes 5 and 6). Moreover, in the presence of the CAAT box competitor, the digestion patterns also changed near the F1 and F2 protected regions which respectively seemed to be more extended at their 3' and 5' ends (Figure 3A, lane 6 compared with lane 5, and lanes 2–4). This suggests that other transcription factors may be recruited to this region in the absence of the specific CAAT-box-binding factors or, simply, that the factors bound to F1 and F2 can contact the DNA more easily, and produce sharper and wider protected areas. In HeLa cells, the impact of the CAAT box competitor on the NF-Y protection pattern was slightly different: the oligonucleotide did not seem to modify the binding of NF-Y to DNA, but rather promoted the formation of more defined F1 and F3 protection patterns (Figure 3B, lanes 4 and 5).

A canonical sequence for CREB did not perturb the digestion pattern with either nuclear extracts, thus suggesting that CREB is not involved in binding its putative element in the $\alpha 3$ minimal promoter (Figure 3A, lane 7, and Figure 3B, lane 6; also see Figure 6B). When using an excess of unlabelled oligonucleotide bearing the canonical sequence for Sp1, the formation of the F1 region was abolished with both nuclear extracts (Figure 3A, lane 9, and Figure 3B, lane 7). Furthermore, a hypersensitive site formed at position -128 with the SY5Y extract, whereas a hypersensitive site at -122 disappeared with the HeLa extracts. Although never

resembling that of the control, the digestion pattern at the 3' end of F2 was also affected under these experimental conditions, with the formation of another hypersensitive site at position -45 (Figure 3A, compare lanes 9 and 5). Finally, the formation of F3, which contained the second Sp1 and the AP2 sequences, was also severely affected in the presence of the canonical Sp1 oligonucleotide, with the additional appearance of a hypersensitive site at the 3' end of F3 (Figure 3A, lane 9). In contrast, an oligonucleotide containing the canonical AP2 binding site did not affect the formation of F3 (Figures 3A and 3B, lanes 8).

An oligonucleotide bearing the consensus sequence for Egr1 (early growth response 1) partially interfered with the formation of F1 in the presence of SY5Y nuclear extract (Figure 3A, lane 10), but the competition was much less than that observed with the Sp1 canonical oligonucleotide and did not lead to the formation of the hypersensitive site at -128.

Molecular characterization of the transcription factors binding the $\alpha 3$ minimal promoter

EMSA were used to identify the transcription factors that bind to the minimal promoter of the human Na⁺,K⁺-ATPase $\alpha 3$ gene. When an oligonucleotide spanning the -118/-94 region (Figure 4A) was used in the presence of SY5Y nuclear extract, four major complexes were detected (Figure 4B, lane 2) and competed out by an excess of the same unlabelled oligonucleotide (Figure 4B, lanes 3 and 4). In order to confirm that this retardation pattern was related to the presence of the Sp1 transcription factor in the complex, we carried out competition experiments. As shown in Figure 4(B) (lanes 7 and 8), an oligonucleotide bearing the canonical consensus sequence for Sp1 interfered greatly with the formation of the four retarded complexes, whereas an oligonucleotide bearing two point mutations in the Sp1-binding site (Figure 4A) did not lead to any competition (Figure 4B, lanes 5 and 6). As Egr1 and AP2 transcription factors (like Sp1) can bind to GC-rich sequences, we carried out competition experiments using oligonucleotides bearing AP2 or Egr1 consensus binding sites. Figure 4(B) (lanes 9–12) shows that these oligonucleotides did not modify the retardation pattern, thus leading to the conclusion that they did not participate in the formation of the nuclear complexes assembling on the -118/-94 region. In order to identify which member of the Sp1 family was actually involved in the binding to this region, we performed supershift experiments using specific antibodies. Both anti-Sp1 and anti-Sp4 antibodies supershifted complex I (Figure 4B, lanes 14 and 16), whereas the anti-Sp3 antibody supershifted complex II specifically (Figure 4B, lane 15). As expected, AP2 and Egr1 antisera (Figure 4B, lanes 17 and 18) failed to supershift any of the complexes.

A similar analysis was made using an oligonucleotide spanning the -63/-33 region (Figure 4A), which contained two overlapping sites for Sp1 (Figure 2B, -46/-58 and -39/-50) and one putative site for AP2 (Figure 2B, -45/-36). When this oligonucleotide was incubated with the SY5Y nuclear extract the five detected complexes (Figure 4C, lane 1) were specifically competed by the same unlabelled oligonucleotide (Figure 4C, lanes 2 and 3). A shorter oligonucleotide (spanning the -63/-44 region) that excluded the putative binding site for AP2 and approximately half of the downstream Sp1-binding site (Figure 4A), was equally efficient in interfering with the formation of the retarded complexes (Figure 4C, lane 4 and 5), thus indicating that Sp1, but not AP2, is involved in the formation of the retarded bands. For purposes of further confirmation, we carried out competition experiments with oligonucleotides bearing canonical AP2- or Sp1-binding sites and demonstrated that only the Sp1 oligonucleotide was an efficient competitor (Figure 4C, lanes 10–13). By the

Figure 4 EMSA identification of the Sp1-binding sites in the human $\alpha 3$ minimal promoter

EMSA were performed using probes corresponding to different parts of the -123/-29 $\alpha 3$ minimal promoter in the presence of SY5Y nuclear extract. (A) The sequence of the -118/-94 probe spanning the most 5' Sp1 site and the -63/-33 probe spanning the 3' composite Sp1 binding sites. The letters above each sequence indicate the mutated nucleotides. (B) The -118/-94 probe. The competitions were carried out by adding a 100- or 500-fold molar excess of the indicated unlabelled oligonucleotides [wt (wild-type), lanes 3 and 4; mut (mutant), lanes 5 and 6; Sp1, lanes 7 and 8; AP2, lanes 9 and 10; Egr1, lanes 11 and 12]; lane 1 had no nuclear extract and lane 2 had no competitor. Supershift analyses were performed by adding the specific antibodies as follows: α Sp1 (lane 14), α Sp3 (lane 15), α Sp4 (lane 16), α AP2 (lane 17) or α Egr1 (lane 18), where α indicates anti-. There were no antibodies in lane 13. I–IV indicate the four retarded complexes. * indicates the supershifted complexes. (C) The -63/-33 probe. Competition was performed in the presence of a 100-fold (lanes 2, 4, 6, 8, 10, 12, 14 and 16) or 500-fold molar excess (lanes 3, 5, 7, 9, 11, 13, 15 and 17) of the indicated unlabelled oligonucleotides. Lanes 18–25, supershift analysis in the presence of the indicated antibodies (the prefix α indicates anti-); lane 1, no competitor or antibodies. I–IV indicate the retarded complexes; * indicates the supershifted complexes.

same approach, we also ruled out the participation of members of the Egr1 family in the binding to the –63/–33 region (Figure 4C, lanes 14 and 15).

Given that two overlapping sites may be responsible for the Sp1 binding, we investigated which was involved by introducing two point mutations in the upstream Sp1 site contained in the –63/–44 oligonucleotide (Figure 4A), which completely prevented it from working as a band-shift competitor (Figure 4C, lanes 6 and 7, compare with lanes 4 and 5). We also carried out competition experiments with two different unlabelled oligonucleotides: the first corresponded to the –52/–29 genomic region, which included the entire downstream Sp1 and AP2 sites, but only the half at the 3' end of the upstream Sp1 site (Figures 2B and 4A), and the second corresponded to the –79/–48 genomic region, which included all of the NF-Y site and most of the upstream Sp1 site, with the exception of the last two nucleotides at the 3' end (Figure 2B). Neither was capable of competing (Figure 4C, lanes 8, 9, 16 and 17), thus confirming that the upstream Sp1 site is mainly responsible for the binding of nuclear transcription factors in the –63/–33 genomic region. Supershift experiments showed that Sp1, Sp3 and Sp4 were all involved in the formation of the retarded complexes (Figure 4C, lanes 18–20) and various controls confirmed the specificity of the supershifted complexes (Figure 4C, lanes 21–25).

We then studied the molecular interactions that take place in the genomic region that includes and surrounds the CAAT box (Figures 2B and 5). An oligonucleotide spanning this region (–79/–48; Figure 5A) detected two major complexes in SY5Y nuclear extract (Figure 5B, lane 1) that were competed specifically by an excess of unlabelled oligonucleotide (Figure 5B, lanes 2 and 3). Mutations in the CAAT box (Figure 5A) abolished the prevention of the formation of the two complexes (Figure 5B, lanes 4 and 5), whereas an oligonucleotide bearing the sequence of the CAAT box of the MHC II gene promoter worked very efficiently as a competitor (Figure 5B, lanes 6 and 7). As the CAAT box in the MHC II promoter has been shown to bind NF-Y, we performed a supershift analysis by using an antiserum against NF-YB. As shown in Figure 5(B), lanes 9–11, the two complexes were supershifted by an increasing amount of NF-YB antiserum, thus demonstrating that the CAAT box of the $\alpha 3$ gene promoter is also bound by this transcription factor.

The –100/–80 region contains a sequence resembling a hemipalindromic CRE-like element, and, when a probe spanning this region was used in EMSA (Figure 6B), two major complexes were detected in the presence of SY5Y nuclear extract (Figure 6B, lane 1) and specifically competed by an excess of unlabelled oligonucleotide (Figure 6B, lanes 2 and 3). However, mutations in the residues required for CREB binding [28] (Figure 6A) prevented the formation of the nuclear complexes (Figure 6B, lanes 5 and 6), whereas a consensus sequence for CREB derived from the somatostatin promoter [29] did not affect the retardation pattern (Figure 6B, lanes 7 and 8). CREB and ATF-2 antisera (Figure 6B, lanes 10 and 11) also failed to supershift the complexes. All of these data ruled out the possibility that factors belonging to the CREB family of transcription factors can bind to the CRE-like element of the Na⁺,K⁺-ATPase $\alpha 3$ gene promoter, and suggest that, in order to contact DNA, the transcription factor binding to the CRE-like sequence requires nucleotides that are different from, or additional to, those predicted for a member of the CREB family.

In order to test this hypothesis, we generated a series of mutated oligonucleotides bearing nucleotide substitutions in the core of the putative CRE-like sequence or in the regions surrounding it (Figure 6A, probe –98/–66). An oligonucleotide bearing four nucleotide substitutions in the core sequence of the putative CRE-like element (mut 1, Figure 6A) was unable to prevent the

Figure 5 Molecular characterization of the CCAAT-box-binding protein

(A) The CCAAT-box-spanning oligonucleotide (–79/–48). The letters above the sequence indicate the mutated nucleotides. (B) Competitor oligonucleotides were added in a 100-fold (lanes 2, 4 and 6) or 1000-fold molar excess (lanes 3, 5 and 7); lanes 9–11, supershift in the presence of 0.1, 0.3 and 1 μ g of anti-NF-YB (α NF-YB) antibody; lanes 1 and 8, no competitor or antibodies. **a** and **b** indicate the two retarded complexes, * indicates the supershifted complex. The MHC oligonucleotide contains the CCAAT box sequence of the MHC II gene promoter; NE, nuclear extract; wt, wild-type; mut, mutant.

formation of the retarded bands (Figure 6C, lanes 4 and 5); however, when mutations were introduced into the sequence at the 3' end of the CRE-like core sequence, the resulting oligonucleotides (mut 2, mut 3 and mut 4; Figure 6A) were still capable of interfering with the formation of the retarded complexes (Figure 6C, lanes 6–11), thus indicating that this region was not involved in the binding of the transcription factor of interest. On the contrary, point mutations in the sequence at the 5' end of the CRE-like core sequence (mut 5; Figure 6A) prevented the oligonucleotide from working as a competitor (Figure 6C, lanes 12 and 13). The data shown in Figure 6 led to the conclusion that the unknown transcription factor(s) binding to the putative CRE-like sequence, recognized this motif with a nucleotide specificity that was different from that of a member of the CREB family of transcription factors and needed to contact additional nucleotides at the 5' end. When these experiments were repeated in HeLa cells, no substantial difference was observed in the retardation patterns or competitive experiments (results not shown), thus indicating that the unknown factor binding the CRE-like sequence is not neuro-specific.

Figure 6 EMSA analysis of the CRE-like element spanning region

(A) Two oligonucleotides were used to analyse the CRE-like element spanning region: the -98/-74 probe and the -98/-66 probe. The mutated (mut) oligonucleotides are also indicated. (B) The -98/-74 probe. Competitor oligonucleotides were added in a 100-fold (lanes 3, 5 and 7) or 1000-fold molar excess (lanes 4, 6 and 8). CRE cs indicates a CRE canonical-binding-site-containing oligonucleotide. Lanes 10–12, supershift analysis in the presence of the indicated antibodies; lane 1, no nuclear extract; lanes 2 and 9, no competitor or antibodies. a and b indicate the two retarded complexes; α indicates anti-. (C) The -98/-66 probe. Competitor oligonucleotides were added in a 100-fold (lanes 2, 4, 6, 8, 10 and 12) or 500-fold molar excess (lanes 3, 5, 7, 9, 11 and 13); lane 1, no competitor; NE, nuclear extract. I and II indicate the retarded complexes. The asterisk indicates an unspecific band.

Functional characterization of the CCAAT box, Sp1 and half CRE-like elements

Deletion analysis and DNA–protein interaction studies provided compelling evidence that Sp1 (at two different DNA sites) and

Figure 7 Functional characterization of the Sp1, CRE and CAAT box binding sites in the $\alpha 3$ minimal promoter

(A) Schematic representation of the constructs transfected in SY5Y cells. The black boxes represent the putative binding sites for Sp1, the CRE-like element spanning region (mut 1) and the CCAAT box in which point mutations were inserted. The empty box is the mut 5 spanning region. Sp1 I indicates the Sp1 site in the -123/-100 region, Sp1 II is the site located in the -46/-58 region. Mut 6 (M6) contains mutations 1 (M1) and 5 (M5) in the CRE-like spanning region (see text and Figure 6A). Apa I indicates the restriction site used to generate the -444/+155 construct. Luc, luciferase. (B) Results are means $\pm$ S.E.M. of the transcriptional activity of the constructs as percentages of the activity of the -444/+155 construct for at least three independent experiments performed in triplicate. *, ** and *** indicate significant differences from the activity of the -444/+155 plasmid (Student's *t* test, $P < 0.05$, $P < 0.01$ and $P < 0.001$ respectively). Double mutants are indicated by the name of the mutated sites. The 3x mut construct contains the triple mutation in the Sp1 I, mut 6 region and CCAAT box elements.

NF-Y play an essential role in the activity of the promoter of the $\alpha 3$ isoform of Na⁺,K⁺-ATPase, whereas the identity of the factor acting at the half CRE-like site remained undefined. In order to gain further insights into the specific contribution of these factors to the general mechanisms regulating $\alpha 3$ transcription, we separately mutated their DNA-binding sites. Point mutations were introduced into the -444/+155 construct which was the most active construct derived from the -3266/+155 region, with 80 times the transcriptional activity of the -64/+155 construct (results not shown).

Mutations of the Sp1-binding site in the -123/-100 region (Sp1 I in Figure 7A) and the CCAAT box significantly reduced the activity of the -444/+155 construct, by approx. 35% and 40% respectively (Figure 7B), whereas the mutations of the Sp1 site in the region -46/-58 (Sp1 II in Figure 7A) did not affect promoter activity (Figure 7B). The double mutation in the Sp1 I and CCAAT box elements reduced the activity of the -444/+155 construct by approx. 80%, thus suggesting their additive contribution to the activity of the $\alpha 3$ promoter.

The mut 6 construct, which bears mutations both in the core consensus sequence and the 5' residues of the putative CRE-like element (see Figure 6A), did not show any decrease in the transcriptional activity. However, when these mutations were combined with those in the Sp1 I site (the m Sp1 I/mut 6 construct in

Figure 7B), the activity of the $-444/+155$ construct fell to 30 %, thus indicating that the two sites co-operate. This co-operative activity was not observed in the case of the double mutant mut 6/mCCAAT. When mutated Sp1 I and CCAAT box sites were present together with mut 6 (the 3 $\times$ mut construct in Figure 7B), the activity of the promoter was reduced to a residual 6 % in a manner that is compatible with synergistic interactions among factors. When these experiments were repeated in non-neuronal HeLa cells, none of the individual mutated constructs showed reduced transcriptional activity, whereas the triple mutant was less active than the wild-type, but still 26 times more active than pGL3 basic (results not shown).

Comparison of the structural features of rat and human $\alpha 3$ minimal promoters

In order to verify whether the DNA elements found to be relevant to the activity of the $\alpha 3$ minimal promoter in human neurons had been conserved during evolution, we compared human and rat DNA sequences in the regions immediately upstream of the start codon (see Supplementary Figure 1 at <http://www.BiochemJ.org/bj/386/bj3860063add.htm>) and found that they had an overall identity of 82 %. The region spanning the transcription start site is conserved in rats and humans [20], as is the TATA box. Moreover, the regions spanning the CCAAT box and the Sp1 II site are identical in the two species in terms of the core consensus sequences for NF-Y and Sp1 respectively. In contrast, the Sp1 I site has a nucleotide substitution in the consensus sequence of the rat gene which, in principle, may affect the binding of the cognate transcription factor. However, the greatest differences were in the region between the Sp1 I site and the CCAAT box; in particular, three nucleotide substitutions in the rat gene correspond to the mutations introduced in mut 1, mut 5 and (together) mut 6 constructs, and probably interfere with the binding properties of the CRE-like sequence.

DISCUSSION

In the present study, we have characterized the promoter driving the expression of the human gene encoding the Na⁺,K⁺-ATPase $\alpha 3$ isoform. Dot-blot analyses showed that this gene is thoroughly and abundantly expressed in the nervous system and, in line with this pan-neuronal distribution, its transcript was found by Northern blot analysis in the ganglionic neurons of the autonomic nervous system. In agreement with Zahler et al. [30], we also found that the $\alpha 3$ gene is expressed in the adult heart, which marks a difference from the rat orthologue whose cardiac expression undergoes a developmental switch: it is present during the embryonic development, but is replaced by the $\alpha 2$ isoform in adulthood [17]. In addition to the brain and heart, we only detected the $\alpha 3$ transcript in testes, whereas the rat $\alpha 3$ isoform is expressed in ovaries [31]. However, a testis-specific isoform ($\alpha 4$) that is highly homologous with $\alpha 3$ has been described in rat [11], and so we cannot rule out that a cross-hybridization occurred in our experiments between the $\alpha 3$ probe and the human testis-specific $\alpha 4$ isoform.

Computer-assisted analysis suggested that the expression of the human $\alpha 3$ Na⁺,K⁺-ATPase gene is driven by a canonical promoter containing a TATA and a CAAT box, two Sp1 sites, and a half CRE-like element clustered in approx. 100 bp upstream of the major transcription start site. Although none of these *cis*-acting elements is known to confer tissue-specificity to gene expression, deletion analysis of the region showed clearly that the putative CRE-like and upstream Sp1 site are much more active in neuronal

than in non-neuronal cells, whereas the CAAT box was equally effective in both cell lines.

Given the possibility that tissue-specific transcription factors may bind these putative *cis*-acting elements, we investigated the DNA-protein interactions occurring in the entire region by means of DNase I and competitive footprinting experiments using both neuronal and non-neuronal cells. These assays showed that the sub-regions encompassing the Sp1 I, CRE-like, CCAAT box and Sp1 II elements are protected by both SY5Y and HeLa extracts, thus suggesting that no tissue-specific transcription factors were involved, although the extent of the protected areas and the appearance or disappearance of the hypersensitive sites in competition experiments were slightly different in the two cell types. EMSA and supershift experiments with specific antibodies allowed us to demonstrate that both the Sp1 I and Sp1 II sites can bind Sp1, Sp3 and Sp4, and that the putative CAAT box is actually recognized by the transcription factor NF-Y. In contrast, DNA-protein interaction studies ruled out that the half-CRE site could actually bind some members of the CREB transcription factor family, and showed that additional nucleotides at the 5' end of this site are needed for the binding of an unknown factor.

In order to obtain a more precise picture of the functional role of Sp1/3/4, NF-Y and the unknown factor binding the half-CRE-like site, constructs bearing single or combined point mutations in each site were generated and transfected in neuroblastoma cells. This analysis allows us to make a few general statements concerning the molecular basis underlying the activity of the Na⁺,K⁺-ATPase $\alpha 3$ promoter: (i) the presence of simultaneous mutations in the three *cis*-acting elements severely affects the activity of the promoter, thus establishing clearly that $\alpha 3$ gene expression depends on these sites and their cognate transcription factors; (ii) the mutation of the downstream Sp1 site (Sp1 II) does not modify the expression of the reporter gene, which suggests that Sp1 and the other members of its family operate on the human $\alpha 3$ promoter through the upstream site; and (iii) the CRE-like element does not seem to have an independent function in regulating the $\alpha 3$ promoter, but co-operates with the upstream Sp1 site. All of these data are consistent with the results of the deletion analysis, and, taken together, lead to the paradoxical conclusion that the expression of the $\alpha 3$ Na⁺,K⁺-ATPase isoform in the nervous system relies on a promoter that binds transcription factors whose expression is not restricted to neurons or, as in the case of Sp1 and NF-Y, may even be ubiquitous. One hypothesis that may reconcile this discrepancy is that, by means of protein-protein interactions, these transcription factors co-operate in recruiting one or more neuro-specific co-activators, that subsequently activate transcription in a tissue-specific fashion.

Although these data have been obtained in a cell culture model, neuroblastomas, which arise from migratory cells of the embryonal neural crest, conserve most of the features of the differentiating neurons of the autonomic nervous system, with particular regard to the transcription factors and the neurotrophic factor receptors that govern their specification *in vivo*. In particular, SY5Y cells, if treated appropriately with physiological differentiating agents, show many of the characteristics of human primary culture neurons [31]. Thus we are confident that these data are representative of mechanisms that also occur in normal neural cells.

The only available published information concerning the transcriptional control of the $\alpha 3$ Na⁺,K⁺-ATPase isoform in neurons refers to the rat promoter, and was obtained using transgenic mice [32]. It was found that a 210 bp fragment upstream of the transcription start site is sufficient to promote the transcription of the $\alpha 3$ gene in the brain and prevent its expression in non-neuronal tissue, other than the gonads. According to the authors, an

NRSE (neural restrictive silencer element) [33] is responsible for restricting the expression of the gene in brain, as its removal caused the appearance of an ectopic expression of CAT (chloramphenicol acetyltransferase) in the intestine [32]. However, most of the other non-neuronal tissues remained negative, and a biochemical analysis of this putative NRSE failed to demonstrate that it binds the factor (NRSF) that is responsible for switching off the expression of neuronal genes in non-neuronal tissues [34]. This putative NRSE is not well conserved in the human gene, and its deletion does not substantially change the expression of the reporter gene in non-neuronal cells (results not shown), thus suggesting that it is not relevant for the neuro-specific expression of the $\alpha 3$ Na⁺,K⁺-ATPase subunit in humans.

The transcriptional mechanisms underlying the expression of this Na⁺,K⁺-ATPase isoform have also been investigated in neonatal rat cardiocytes [21]. However, although it was demonstrated that NF-Y and Sp1/Sp3 play a role in the promoter activity, they found that it involved the downstream Sp1 *cis*-acting element (Sp1 II in our nomenclature), whereas the removal of the upstream Sp1 I site and the half-CRE-like element had no effect [21]. This is in line with the observation that these two sites are degenerated in the rat gene and may be devoid of any function.

In conclusion, very few papers have so far concentrated on the transcriptional mechanisms governing the expression of the $\alpha 3$ Na⁺,K⁺-ATPase subunit gene despite its fundamental functional role in excitable tissues. Our results offer the first contribution towards improving our understanding of how the transcription of this gene is regulated in human neurons.

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