

Regulation of the Pannexin-1 Promoter in the Rat Epididymis¹

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ABSTRACT

Pannexins (PANXs) are channel-forming proteins implicated in cellular communication through the secretion of biomolecules, such as ATP and glutamate. PANX1 and PANX3 are expressed in the male rat reproductive tract and their levels are regulated by androgens in the epididymis. There is currently no information on the regulation of the *Panx1* promoter. The objective of the present study was to characterize the *Panx1* promoter in order to understand its regulation in the epididymis. RNA ligase-mediated rapid amplification of cDNA ends identified three transcriptional start sites, at positions –443, –429, and –393. In silico analysis revealed that transcription was initiated downstream of binding sites for CREB and ETV4 transcription factors, in a CpG island context. To determine the importance of this region in gene transactivation, a 2-kb fragment of the promoter was cloned into a vector containing a luciferase reporter gene. Deletion constructs indicated that the highest transactivation levels were achieved with shorter constructs (–973 to –346 and –550 to –346). Electrophoretic mobility shift assay and supershifts indicated that both transcription factors were able to bind to the promoter region. Chromatin immunoprecipitation using rat caput epididymis cells confirmed the binding of ETV4 and CREB on the *Panx1* promoter. Site mutation of either the ETV4 or CREB binding site decreased the transactivation of the reporter gene. Previous studies indicated that orchidectomy increased epididymal PANX1 levels. Likewise, we observed an increase in both ETV4 and CREB in orchidectomized rats. These results indicate that ETV4 and cAMP response elements play a role in the transcriptional regulation of *Panx1* in the epididymis.

CREB, epididymal RCE cells, ETV4, gene expression, transcription factor

INTRODUCTION

Pannexins (PANXs) are transmembrane proteins that form hemichannels at the level of the plasma membrane. These proteins are structurally similar to innexins, the invertebrate gap junction proteins, but lack sequence homology with connexins (CXs), which form gap junctions in vertebrates [1–4]. While initial studies suggested that PANXs could form gap junctions, it is now generally

accepted that these proteins do not form gap junctions, but rather form large pores, or pannexons, at the level of the plasma membrane [5–7]. Upon activation, open pannexons are permeable to ions, small molecules, and metabolites up to 1 kDa [8, 9]. Unlike CXs, PANXs are glycoproteins [1–4]. Mutation studies have shown that PANX1 is glycosylated at the Asn⁸⁶ site [10, 11]. The degree of glycosylation is important for intracellular targeting, as the most highly glycosylated forms of PANXs are generally found at the level of the plasma membrane [12–14]. In addition to glycosylation, PANX1 can undergo nitrosylation, which has been shown to be important for the function of the pannexon pore [15]. Furthermore, PANX1 is also a target for caspases, which can cleave the intracellular C terminal of the protein, resulting in increased ATP secretion, which serves as the “find-me signal” for the recruitment of monocytes and phagocytosis of dying cells [16]. While there are numerous studies on the posttranslational regulation of PANXs, few studies have focused on either their endocrine or transcriptional regulation.

We have identified transcripts and proteins for *Panx1* and *Panx3* in the testis and epididymis of the rat [17]. In the testis, PANX1 is present in the Sertoli cells of the seminiferous epithelium at the level of the blood-testis barrier, and in peritubular myoid cells, while PANX3 localizes to Leydig cells. In the epididymis, PANX1 protein and mRNA levels are present in all segments of the epididymis at similar levels, while PANX3 protein levels are higher in the initial segment and caput epididymidis [17]. While both PANXs are expressed by principal cells, PANX1 localization is restricted to the basal region of the epithelium, whereas PANX3 is found at the apical region of the epithelium. PANX1 is also expressed in basal, halo, and myoid cells. Studies in the epididymis have indicated that orchidectomy results in an increased number of PANX1 and PANX3 immunoreactive bands, as well as increased total protein levels throughout the epididymis [17]. Testosterone maintenance with orchidectomy reduced the number of bands in the caput and corpus, suggesting that testosterone, as well as other testicular factors, are involved in both the transcriptional regulation and the posttranslational modification of these PANXs. Although the use of orchidectomized animals does have inherent limitations as an experimental model, the regulation of PANXs appears to be complex and involves multiple testicular factors [17].

Studies have shown that PANXs are implicated in ATP release, and can work in cooperation with purinergic receptors to regulate ATP-mediated intercellular signaling [18–20]. It has been reported that epididymal cells release ATP into the lumen [21–23] where there appears to be considerable crosstalk involving ATP between the epididymal cells and the maturing sperm. Several studies have shown that ATP secretion in the epididymis is regulated by the cystic fibrosis transmembrane conductance regulator (CFTR) [23]. *Cftr* has been shown to be essential for male fertility [24–28]. Recently, Ruan et al. [29] have shown that CFTR is

¹Supported by NSERC discovery grant 155065-06 and by the FRSQ-Réseau de Recherches en Santé Environnementale (D.G.C.). This work was presented in part at the Annual Meeting of the American Society for Cell Biology, December 14–18, 2013, New Orleans, LA.

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Received: 11 June 2014.

First decision: 8 July 2014.

Accepted: 15 October 2014.

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eISSN: 1529-7268 <http://www.biolreprod.org>

ISSN: 0006-3363

implicated in the secretion of ATP in the mouse epididymis. Whether or not CFTR operates on its own has been questioned. Recent studies have shown that, in erythrocytes, CFTR cannot directly secrete ATP, but instead regulates PANX1 channels, which are responsible for the release of ATP from the erythrocytes to the endothelium. ATP then stimulates the production of endothelial signaling molecules including nitrous oxide [30].

PANXs have also been implicated in other physiological processes. The release of ATP by Jurkat T cells entering apoptosis are thought to be responsible for the find-me signal, which activates the immune system to target apoptotic cells [16]. Recent studies have reported that, in CD4⁺ lymphocytes, PANX1 hemichannels are critical for human immunodeficiency virus (HIV) infection and replication [31]. The HIV virus interacts with chemokine receptors, which activate PANX1 channels to release ATP. ATP then binds to purinergic receptors and activates intracellular signaling pathways, depolarizing the plasma membrane and allowing the virus to fuse with the plasma membrane and enter its target cell [32, 33]. PANX1 in the hippocampus has also been associated with murine models of epilepsy. Studies have shown that PANX1 levels are increased in epilepsy, and that the N-methyl-D-aspartate receptor can activate the opening of PANX1 channels, thereby raising extracellular ATP [39] and arachidonic acid levels, and promoting epileptiform activity [19, 34–37].

There have been few studies to date on the regulation of *Panx* genes, and none on the regulation of the *Panx1* promoter in any tissue. In bone, the *Panx3* promoter is regulated by the runt-related transcription factor 2 (RUNX2) [38]. Given the role of PANX1 in the release of ATP [39] and its importance for male fertility, the objective of this study was to characterize the regulation of the *Panx1* promoter in the rat epididymis.

MATERIALS AND METHODS

Animals and Tissues Sampling

Animal studies were approved by the University of Quebec animal care committee. All tissues used in this study were sampled from adult male Sprague-Dawley rats of 350–400 g. Animals were purchased from Charles River Canada Inc. (St.-Constant, PQ, Canada), and received water and food ad libitum. They were maintained under a 12L:12D photoperiod.

The orchidectomy experiment was conducted as described previously [17]. Sham-operated rats ($n = 4$) were used as controls and compared to 7-day orchidectomized rats ($n = 4$). At the time of sampling, caput epididymides were paired, flash frozen in liquid nitrogen, and kept at -80°C until protein extraction.

Rapid Amplification of cDNA Ends

Total RNA was extracted from the caput epididymidis of an adult rat using a commercial kit (Illustra RNAspin Mini RNA Isolation Kit; GE Healthcare, Baie d'Urfé, PQ, Canada). The 5' untranslated region of the *Panx1* mRNA was amplified with the RNA Ligase-Mediated (RLM)-Rapid Amplification of cDNA Ends (RACE) Kit (Ambion, Austin, TX) and the Advantage GC 2 PCR Kit (Clontech Laboratories Inc., Mountain View, CA) according to the manufacturer's instructions. Gene-specific primer sequences are provided in Table 1. Following a first round of PCR amplification using the outerRACE primer, two different nested primers (innerRACE long and short) were used in separate reactions. These primers are located 227 bp away from each other on the *Panx1* promoter, and were designed to amplify nested PCR products with distinct molecular weights. PCR products were first analyzed on a 2% agarose gel and subsequently on a 6% nondenaturing polyacrylamide gel stained with ethidium bromide. The bands were excised from the gels, purified, and sequenced using specific innerRACE primers (Genome Quebec Innovation Centre, Montreal, PQ, Canada).

Sequence Analysis

Sequence comparison and alignments were done using the Basic Local Alignment Search Tool (BLAST; <http://blast.ncbi.nlm.nih.gov/>). Multiple sequence alignment of the rat, mouse, and human promoters was done with the Clustal 2.1 alignment tool (<http://www.ebi.ac.uk>). Response elements for specific transcription factors present in the *Panx1* promoter were predicted using the Transcription Element Search System (TESS) and the TRANSFAC database (<http://www.cbil.upenn.edu/tess>) as well as TFSEARCH (<http://www.cbrc.jp/research/db/TFSEARCH.html>). The CpGplot software (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpGplot/) was used to predict CpG islands.

Promoter Cloning, Plasmid Constructions, and Mutagenesis

All restriction enzymes used in the study were purchased from New England Biolabs (NEB; Whitby, ON, Canada). Rat genomic DNA was extracted from adult rat liver using the GenElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Oakville, ON, Canada). A fragment of 2583 bp of the *Panx1* promoter (from position -2508 to position $+75$ relative to the ATG) was amplified by touchdown PCR using the Advantage GC 2 PCR Kit and specific primers (Table 1). Amplicons were purified by agarose gel electrophoresis. The amplified promoter DNA was first cloned into a pCR2.1 TOPO cloning vector (Invitrogen, Carlsbad, CA) and its 5'–3' orientation was verified by enzyme restriction digestion. The *Panx1* promoter was then excised from the plasmid using a Kpn I-EcoR V double digestion and subcloned into the Kpn I-Sma I cloning sites of the pGL3-Basic reporter vector (Promega, Madison, WI) upstream of the *Luciferase* gene. After transformation of chemically competent bacteria (TOP10; Invitrogen), the construct was sent for sequencing to confirm its orientation and integrity (Genome Quebec Innovation Centre). The *Panx1* promoter construct was then modified to remove a 428-bp fragment containing the ATG start codon by digestion with Nhe I and Bgl II restriction enzymes, filled in using T4 DNA polymerase and ligated with T4 DNA ligase (NEB). This provided the final full-length (2163-bp) promoter construct starting from position -2508 to -346 relative to the ATG translational start site.

Deletion constructs were produced by restriction digests using the Kpn I restriction site located upstream of the promoter and other unique restriction sites (Xho I, BstE II, and Rsr II) present along the promoter sequence. After removal of 5' portions of the promoter, bands were purified by electrophoresis and plasmids were filled in and ligated as described above. Plasmids containing 1118, 628, and 205 bp of the *Panx1* promoter (positions -1463 , -973 , and -550 to -346 relative to the ATG, respectively) were generated in this fashion. A construct containing 140 bp (positions -550 to -406) of the promoter was also generated by the removal of a Sac I-Hind III fragment from the 205-bp promoter plasmid. All constructs used in the study were transfected into chemically competent TOP10 bacteria. Resulting colonies were grown and plasmid DNA extracted using a Mini-Prep Kit (Macherey-Nagel, Bethlehem, PA) and analyzed by restriction digest. Positive clones were sequenced (Genome Quebec Innovation Centre), amplified, and purified using a commercial midi-prep kit (Plasmid Midi kit; Qiagen, Toronto, ON, Canada). Constructs were stored at -20°C until being utilized in transfection experiments. Transcription factor binding sites from the -550 to -346 promoter construct were mutated using the QuickChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions, with PAGE-purified sense and antisense mutagenic oligonucleotides (IDT, Toronto, ON, Canada) listed in Table 1. Four nucleotides were mutated in either CREB or ETV4 consensus binding sequences (GCGT to TTAC and TTCC to CCAA, respectively). The resulting clones were grown, purified, and sequenced as described above and stored at -20°C .

Cell Culture, Reporter Assays, and Cell Treatment with 5-Azacytidine

Rat caput epididymis (RCE)-1 cells [40] were grown on mouse collagen IV-coated plates (BD Biosciences, Mississauga, ON, Canada) in Dulbecco modified Eagle medium (DMEM)/Ham F12 culture medium (Sigma-Aldrich) containing antibiotics and nutrients at 32°C in a humidified chamber with 5% CO_2 [40]. PC12 cells (kindly provided by Dr. Alain Fournier, INRS) were grown in DMEM/Ham F12 culture medium containing antibiotics and supplemented with 7% fetal bovine serum and 7% horse serum (Sigma-Aldrich). PC12 cells were grown at 37°C in a humidified chamber with 5% CO_2 .

RCE-1 cells were cultured in DMEM/Ham F12 medium containing the appropriate supplements in a humidified incubator at 32°C with 5% CO_2 as

TABLE 1. List and sequences of oligonucleotides used in the present study for characterization of the *Panx1* promoter.

| Assay | Name | Sequence 5' to 3'* | |
|------------------------------|---------------------------|--|--|
| 5' RACE | outerRACE | CAGAGGCAGACCACGGCAATG | |
| | innerRACE long | GAACCTGGGGCTCGGTGGGCTCCT | |
| | innerRACE short | GCCTACCTCGCAGGGACCGCACG | |
| Promoter cloning | -2508 pmt <i>Panx1</i> -F | CCACTCCCTGCTGCCCATTGACA | |
| | +75 pmt <i>Panx1</i> -R | GAACCTGGGGCTCGGTGGGCTCCT | |
| RT-PCR | <i>Nhlh1</i> forward | TCCTGCCTTTCTCACAACCC | |
| | <i>Nhlh1</i> reverse | CCAGCAGCTGGTTCAGGTAA | |
| | <i>Gapdh</i> forward | ACCACAGTCCATGCGCATCAC | |
| | <i>Gapdh</i> reverse | TCCACCACCCCTGTTGCT | |
| | <i>Panx1</i> forward | CCCACCGAGCCCAAGTTCAA | |
| | <i>Panx1</i> reverse | GCCCAGCAGTAAGAGTCCACGAAG | |
| | Mutagenesis | <i>Panx1</i> mCREB sense | CGCCCACTCCGG CGTTAC CGCGCTTCCGG |
| | | <i>Panx1</i> mCREB antisense | CCGGAAGCGCGCT GTGTAAC GGCGGAGTGGGGC |
| <i>Panx1</i> mETV4 sense | | CCGGCGTTCAGCGC GCCCAAG GAAACTGCTCGCCC | |
| <i>Panx1</i> mETV4 antisense | | GGGCGAGCAGTTT CCITGGG CGCGTACGCCCGG | |
| EMSA | CREB sense | CACTCCGCGCGCTCAGCGCGC | |
| | CREB antisense | GCGCGCTGACGCCGGCGGAGTG | |
| | mCREB sense | CACTCCGCG CGTTAC CAGCGCGC | |
| | mCREB antisense | GCGCGCT GTGTAAC GCGCGGAGTG | |
| | ETV4 sense | CAGCGCGCTTCCGGAAACTGCT | |
| | ETV4 antisense | AGCAGTTTCCGGAAGCGCGCTG | |
| | ETV4 sense | CAGCGC GCCCA GAAACTGCT | |
| | ETV4 antisense | AGCAGTTTCC TGGG CGCGCTG | |
| ChIP | <i>Panx1</i> ChIP-F | TAGTCTATCGCCGCAAGATT | |
| | <i>Panx1</i> ChIP-R | AGCTCTTCCGGAACCTACGC | |

*Binding sites for transcription factors are in bold, and mutated bases are underlined.

previously described [40, 41]. Cells were seeded at a density of 100 000 cells per well in collagen IV-coated 24-well plates (BD Biosciences). Cells were allowed to adhere overnight and the next day washed with PBS and the medium replaced with 500 µl of medium without antibiotics. The cells were transfected using 2 µl of Lipofectamine 2000 (Invitrogen) and 1 µg of each of the *Panx1*-pGL3 constructs combined with 100 ng of pRL-TK vector (Promega), in a total volume of 100 µl. The pRL-TK vector expresses the *Renilla* luciferase, which was used as an internal control for transfection efficiency. A promoterless pGL3-Basic empty vector was used as a negative control. A plasmid containing the *Luciferase* gene under the control of the Rous sarcoma virus long terminal repeat (pRSV-L) was used as a positive control (kindly provided by Dr. R.S. Viger, Laval University). All experiments were done in triplicate. The cells were placed in a CO₂ incubator for 24 h; medium was subsequently removed and the cells washed with PBS. Cells were lysed directly in the plate using 125 µl of Passive Lysis Buffer (Promega). Firefly and *Renilla* luciferase activities of a 20-µl aliquot were determined using the Dual-Luciferase Assay Kit (Promega) and an MLX microtiter plate luminometer (Dynex Technologies, Chantilly, VA). Relative luciferase activities were expressed as the ratio of firefly to *Renilla* luciferase activity.

For treatment of RCE-1 with 5-azacytidine (Sigma-Aldrich), cells were plated in six-well collagen IV-coated plates (BD Biosciences) at a density of 80 000 cells per well. The next day, medium was replaced with fresh medium or fresh medium containing 1 or 10 µM 5-azacytidine prepared from a 500-µM stock solution of the chemical dissolved in the same medium. Cells were exposed for 24 h, total RNA was extracted, and *Panx1* was amplified by RT-PCR assays using specific primers described previously [17] (Table 1).

Reverse Transcriptase PCR

Total RNA was extracted from the epididymis of an adult rat and from the RCE-1 or the PC12 cells, as described above. The mRNA transcripts were reverse transcribed using oligo d (T)16 primers (R&D Systems, Minneapolis, MN) and MMLV-reverse transcriptase (Sigma-Aldrich). Primers used for PCR were designed using the Primer-BLAST tool (<http://www.ncbi.nlm.nih.gov/>) with available sequences for *Rattus norvegicus* factor nescient helix loop helix 1 (*Nhlh1*) and Glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNAs (GenBank accession nos. NM_001105970.1 and NM_017008.4, respectively). *Panx1* primers were used in a previous study [17] (Table 1). PCR amplifications were done using 35 cycles (*Nhlh1*), 28 cycles (*Panx1*), or 25 cycles (*Gapdh*) of denaturation (95°C for 30 sec), annealing (62°C, 60.8°C, or 60°C for *Nhlh1*, *Panx1*, and *Gapdh*, respectively, for 30 sec), and elongation (72°C for 30 sec). PCR reactions were also performed on RNA transcripts prior

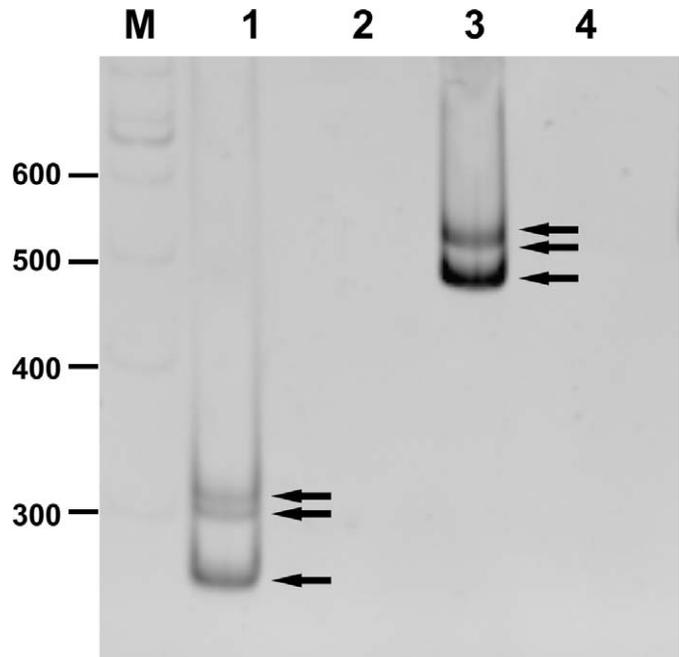


FIG. 1. 5' RACE experiments determine multiple transcriptional start sites for the *Panx1* gene. Amplifications were performed using a RLM-RACE kit and total epididymal RNA. After a first round of PCR using *Panx1* specific reverse primer, the 5' untranslated region (UTR) of *Panx1* mRNA was amplified with two different specific nested primers (lanes 1 and 3) located 227 bp away from each other on the *Panx1* promoter. Products were visualized on an ethidium bromide-stained nondenaturing 6% polyacrylamide gel. Three bands were obtained with each nested primer (arrows). The gel is representative of three separate experiments. Control reactions using TAP untreated capped mRNAs (lanes 2 and 4) are also shown. M, molecular weight marker.

PANNEGIN1 PROMOTER IN THE EPIDIDYMIS

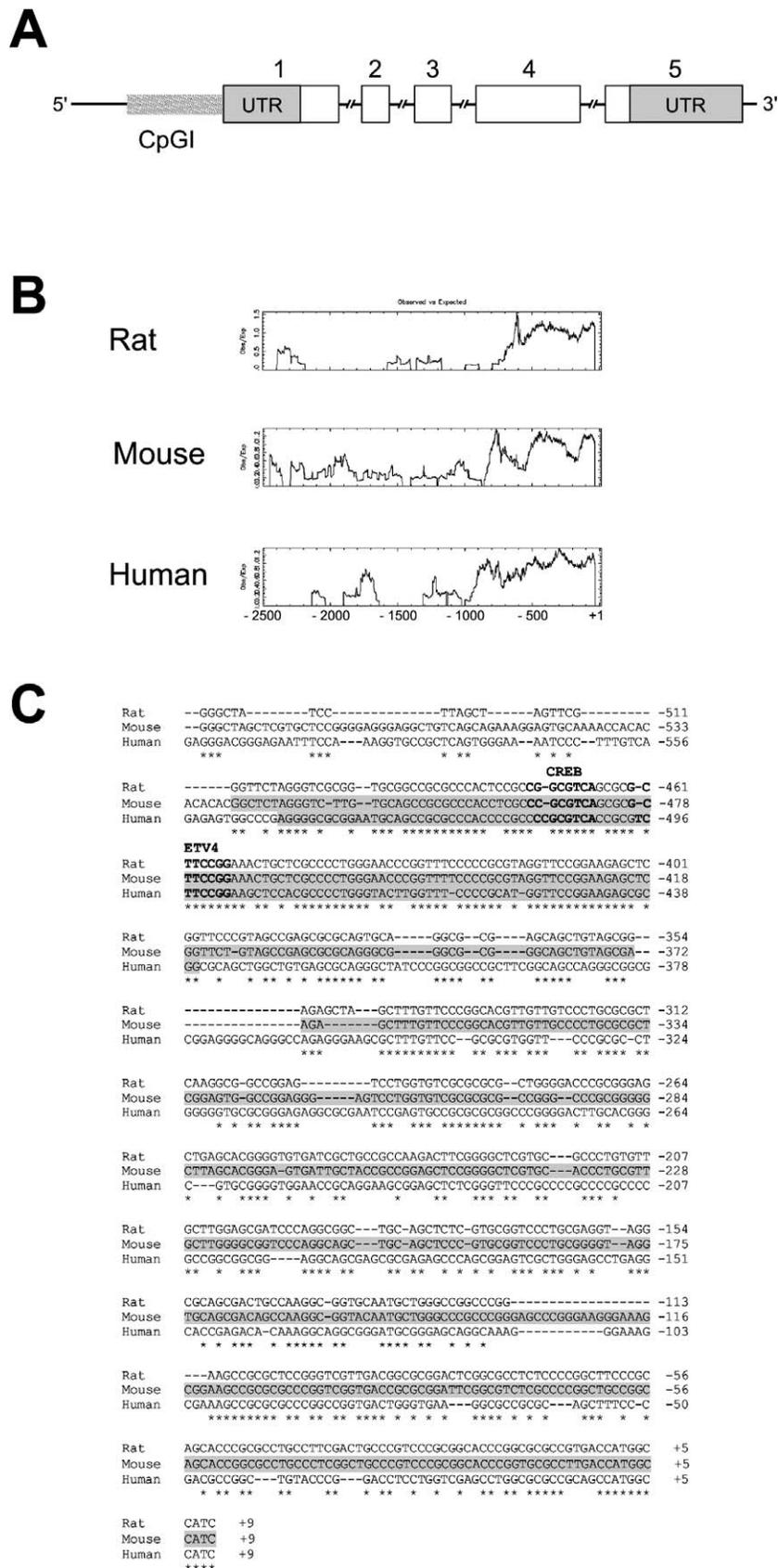


FIG. 3. The *Panx1* gene possesses a CpG island promoter, which shows conserved regions between species. A) Schematic representation of the rat *Panx1* gene showing the five exons (numbered 1–5), the 5' and 3' UTR and the location of the CpG island (CpGI). B) CpG islands located in the first 500 bp upstream of the ATG start codon were identified with the CpGPlot software in the *Panx1* promoter from different species (+1: translational start codon position). C) Alignment of the mouse and human *Panx1* promoters with the rat sequence revealed a highly conserved region in the 5' part of the CpGI. Homology to the rat sequence is highlighted in gray, and stars represent conserved nucleotides. Binding sites for ETV4 and CREB transcription factors are indicated in bold characters. Position numbers are given relative to the ATG translational start codon position.

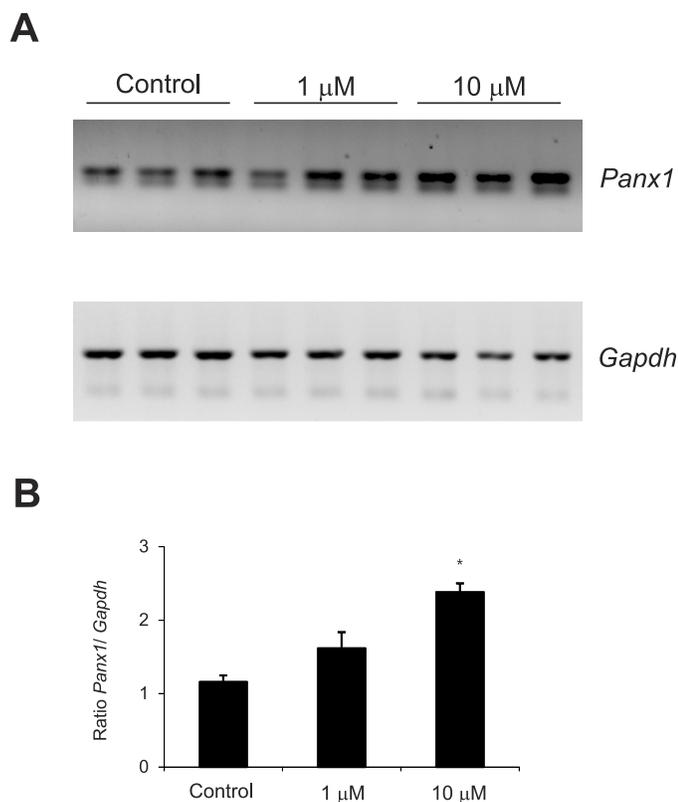


FIG. 4. Demethylating agent 5-azacytidine increases *Panx1* mRNA levels in RCE-1 cells. Epididymal cells ($n = 3$) were treated for 24 h with 1 μM or 10 μM of 5-azacytidine or with control normal culture medium, total RNA was extracted, and semiquantitative RT-PCR was performed. **A**) *Panx1* and *Gapdh* mRNAs were amplified and PCR products were visualized in ethidium bromide-stained agarose gel (200 bp and 450 bp, respectively). **B**) *Panx1* gene expression significantly increases at the 10- μM dose ($P < 0.05$). Data are expressed as the mean \pm SEM.

protein was mixed in Laemmli buffer, heated at 95°C for 5 min, and cooled on ice. Protein samples were separated on either a 10% (CREB and ETV4) or 15% (NHLH1) SDS-PAGE gel and transferred onto a polyvinylidene fluoride membrane using a Trans-Blot Turbo Transfer system (Bio-Rad). The membrane was blocked for 1 h at room temperature with 5% nonfat powdered milk dissolved in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20 (TBST). The membrane was then incubated overnight at 4°C with the rabbit polyclonal anti-CREB antibody (2 $\mu\text{g}/\text{ml}$) in blocking solution. After washing with TBST, the membrane was incubated with a horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (0.4 $\mu\text{g}/\text{ml}$; ab6721; Abcam, Toronto, ON, Canada). The signal was revealed using the Clarity Western ECL substrate (Bio-Rad) and analyzed using a ChemiDoc MP Imaging system (Bio-Rad). The membrane was stripped for 30 min at 60°C in stripping solution (62.5 mM Tris-HCl [pH 6.7], 2% SDS, 100 mM β -mercaptoethanol), rinsed with TBST, and then hybridized overnight at 4°C with the mouse monoclonal anti-ETV4 antibody (0.4 $\mu\text{g}/\text{ml}$) after blocking, as described above. The membrane was washed and incubated with an HRP-conjugated goat anti-mouse IgG (0.01 $\mu\text{g}/\text{ml}$; sc-2005; Santa Cruz Biotechnology), and proteins were revealed as described above. The membrane was stripped, blocked, and incubated for 1 h with anti- α -tubulin antibody (TUBA; 0.05 $\mu\text{g}/\text{ml}$; ab4074; Abcam), which was used to normalize protein levels. The membrane was then washed, revealed, and analyzed as for the other antibodies. A similar approach was also used with an anti-NHLH1 antibody (1 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology) and HRP-conjugated goat anti-rabbit secondary (0.04 $\mu\text{g}/\text{ml}$; Santa Cruz Biotechnology). Blots were normalized to TUBA, as described above.

Statistical Analysis

All statistical tests were performed using SigmaStat software (Jandel Scientific, San Rafael, CA). One-way ANOVA or Student *t*-tests were used followed by the Tukey post hoc test. *P* values for significant differences are indicated in the figure legends.

RESULTS

Panx1 Transcriptional Start Sites

Using rat total epididymal RNA as substrate, 5' RLM-RACE was performed to identify the transcription initiation site of the *Panx1* gene. Two nested primers were used (innerRACE short and long; Table 1), and amplified products showing a difference in molecular weight of 227 bp were identified (Fig. 1). Agarose gel electrophoresis (data not shown) and nondenaturing PAGE revealed the amplification of three separate bands obtained for each specific primer (Fig. 1, arrows), ranging in size from 274 to 326 bp for the shorter amplified product and 503 to 553 bp for the longer amplified product. DNA sequencing confirmed that all of the bands were *Panx1* transcripts. These data confirmed the presence of three distinct transcriptional start sites: a cytosine at position -443, a thymidine at position -429, and a guanine at position -393 relative to the ATG start codon.

The *Panx1* promoter is located in a GC-rich region containing putative transcription factor binding sites. Sequence analysis of the *Panx1* promoter using the TESS software with high-stringency parameters showed putative binding sites for CREB, ETV4, IL-6 RE-BP, and NHLH1 transcription factors in the vicinity of the identified transcription start sites (Fig. 2). High-stringency analysis with the TFSEARCH software, however, identified CREB, an ETS (including ETV4) response element, and an NFE2L2 (nuclear factor [erythroid-derived 2]-like 2, also known as NRF2) response element, which was not detected by TESS, in rat, mouse, and human. The IL-6 RE-BP putative binding site was identified by TFSEARCH as a response element for IKZF2, a lymphocyte-restricted transcription factor [42]. We therefore elected to focus on CREB, ETV4, and NHLH1 in the present study. Using TESS software with low-stringency parameters, multiple (seven) specificity protein 1 (SP1) putative binding sites were also present (data not shown), which is typical of GC-rich promoter regions. Analysis of the 2500-bp sequence upstream of the *Panx1* ATG start codon of the rat, mouse, and human sequences identified CpG islands in each species of 517, 506, and 673 bp, respectively (Fig. 3, A and B). These CpG islands start at position -47 and extended to -563, -552, and -719 of the 5' position in rat, mouse, and human, respectively. The 2.5-kb *Panx1* promoter sequence was compared to mouse and human homologous genomic sequences. This analysis revealed the presence of a highly conserved region in the three species of about 100 bp, located in the 5' portion of the CpG island (Fig. 3C). Mouse genomic DNA from position -526 to the ATG start codon of *Panx1* showed an 84% sequence homology with the rat, while the human genomic DNA from position -543 to -436 showed 83% sequence homology to the rat.

To determine if the methylation state of the CpG island promoter was a key regulator of *Panx1* transcription, semiquantitative RT-PCR was performed on total RNA from RCE-1 cells treated with the demethylating agent 5-azacytidine. *Panx1* mRNA levels were significantly increased relative to RCE-1 cells treated with vehicle alone (Fig. 4), suggesting that DNA methylation may regulate the *Panx1* promoter.

Panx1 Promoter Transactivation Activity

In order to map the minimal promoter region of the *Panx1* gene, deletion constructs coupled to a *Luciferase* reporter

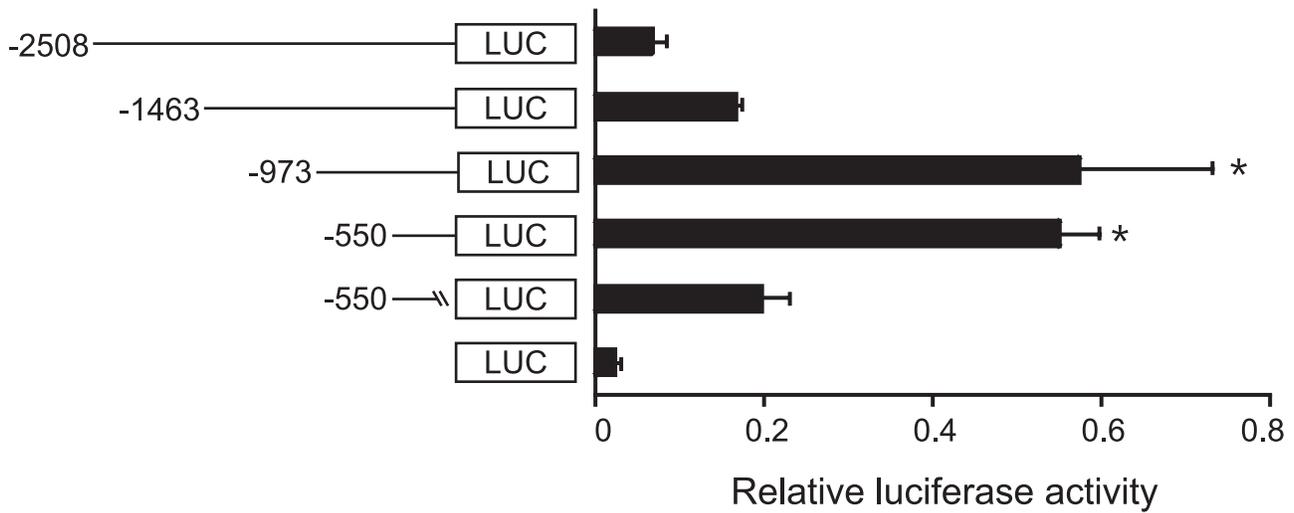


FIG. 5. *Panx1* promoter activity in RCE-1 cells. The rat *Panx1* promoter (–2508 to –346 relative to the ATG) was PCR amplified and cloned into the pGL3-Basic luciferase reporter vector. Deletion constructs were produced by restriction digests (–1463 to –346, –973 to –346, –550 to –346, and –550 to –406). RCE-1 cells were transfected with each construct and with the pRL-TK vector to normalize for transfection efficiency. The pGL3-Basic empty vector was used as a negative control. Cells were incubated for 24 h and the firefly and *Renilla* luciferase activities were measured. Data are expressed as a ratio of firefly to *Renilla* activity (mean of triplicate ± SEM; n = 3 separate experiments). Asterisks indicate a significant difference ($P < 0.05$).

gene were produced and tested in RCE-1 cells. The full-length construct containing 2163 bp of the promoter (–2508 to –346) showed low transactivation activity, as well as the first 5' truncated vector containing the –1463 to –346 region (Fig. 5). Further 5' deletion of the promoter resulted in an increase of the luciferase activity, which was highest when cells were transfected with the –973 and –550 to –346 constructs. Removal of a 3' portion of the promoter (–550 to –406 construct) also resulted in a loss of the transactivation activity.

While the deletion of transcription factor NHLH1 response element was associated with decreased *Panx1* transcriptional activity of the 3' truncated construct (–550 to –406), mRNA and protein (data not shown) levels for the transcription factor were below detection levels in the adult epididymal tissue, and were therefore not examined in subsequent promoter experiments (Fig. 6).

Electrophoretic Mobility Shift Assays

EMSA were done to determine if epididymal CREB and ETV4 bound to their specific response elements on the *Panx1* promoter in vitro. Nuclear extracts from RCE-1 cells, adult rat initial segment, or caput epididymidis were used for EMSA. Nuclear proteins specifically bound to a double-stranded oligonucleotide corresponding to the CREB response element on the *Panx1* promoter (Fig. 7A; Table 1). High-molecular weight complexes were particularly intense in the RCE-1 nuclear extracts. The protein-DNA complexes were out-competed by an excess of cold competitor oligonucleotides, but not by an excess of the same competitor mutated in the CREB binding site. Preincubation of the nuclear extracts with an anti-CREB antibody resulted in a supershift of the complexes in RCE-1 cells and also in the initial segment or caput epididymidis (Fig. 7B; upper arrow). Using a second double-stranded oligonucleotide containing the ETV4 consensus response element (Table 1), high molecular weight complexes were detected when incubated with nuclear proteins from RCE-1 cells and both adult rat initial segment and caput epididymidis (Fig. 8A). Again, complexes were detected at higher levels in the nuclear extracts from RCE-1 cells. An anti-

ETV4 antibody failed to supershift the complexes when preincubated with the nuclear extracts, but instead prevented protein-DNA complex formation (Fig 8B).

Binding of CREB and ETV4 transcription factors to the promoter region of *Panx1* in vivo was verified by ChIP assay. Chromatin of RCE-1 cells was immunoprecipitated with anti-CREB or anti-ETV4 antibody and was enriched for the *Panx1* promoter region containing binding sites for each transcription factor when compared to normal IgG negative control (Fig 9).

Role of CREB and ETV4 Transcription Factors in RCE-1 Cells

To identify the transcription factors implicated in the high transcriptional activity of the 205 bp region of the *Panx1* promoter, site-directed mutagenesis of the ETV4 and CREB putative response elements was done. Mutation of the CREB response element significantly reduced the transactivation of the reporter gene when compared to the parental construct. Furthermore, mutation of the ETV4 binding site completely abolished the luciferase activity (Fig. 10).

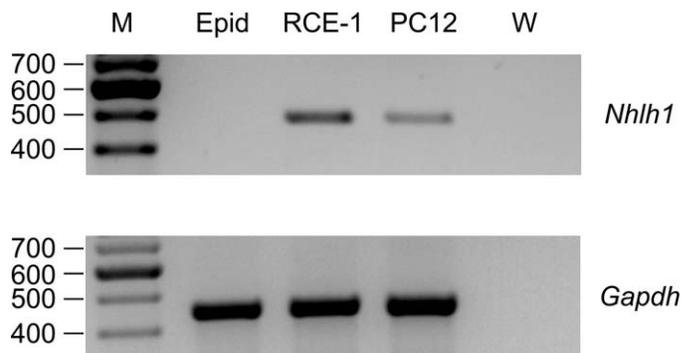


FIG. 6. The NHLH1 transcription factor is expressed at very low levels in the rat epididymis. A 481-bp fragment of the mRNA coding for NHLH1 was amplified by RT-PCR in a control cell line (PC12 cells) and in RCE-1 cells, but was below detection in rat epididymis (Epid). A 450-bp fragment of the *Gadph* mRNA was amplified in each cDNA sample as a control. M, molecular weight marker; W, water negative control.

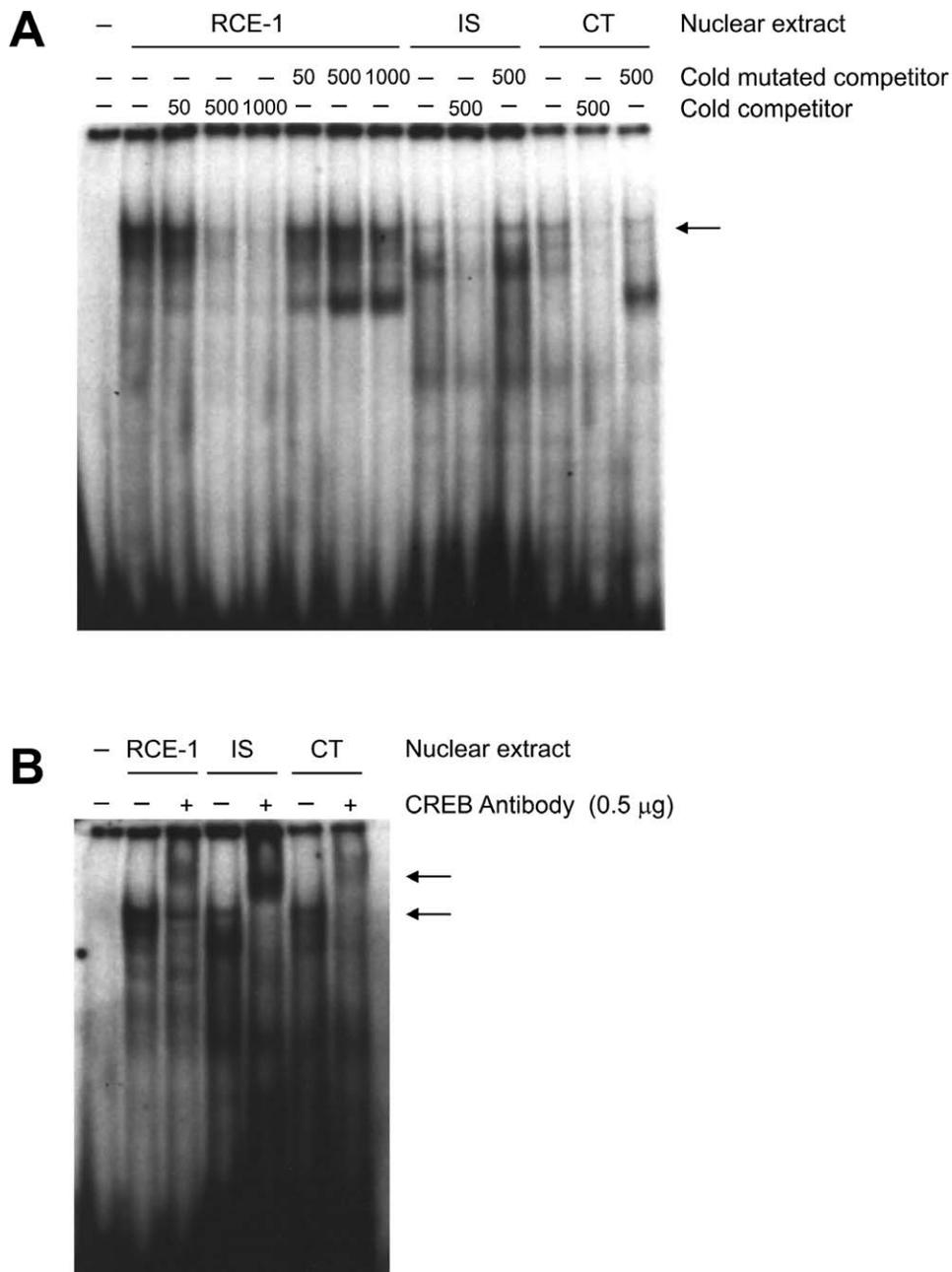


FIG. 7. Epididymal CREB binds to the *Panx1* promoter in vitro: EMSA on the promoter region of *Panx1* containing the CREB binding site. Nuclear extracts from RCE-1 cells, adult rat initial segment, or caput epididymidis were incubated with radiolabeled oligonucleotide containing the CREB binding site. **A)** For competition studies, proteins were preincubated with 50, 500, or 1000 molar excess of unlabeled (cold) competitor, unmutated or mutated in the CREB binding site. **B)** For supershift studies, proteins were preincubated with 0.5 µg of CREB-1 antibody. Protein-DNA complexes and supershifts are indicated by arrows. Gels are representative of three experiments.

Epididymal CREB and ETV4 Protein Levels in Orchidectomized Rats

We have previously shown that PANX1 protein levels were increased 7 days following orchidectomy [17]. In order to determine if levels of CREB and ETV4 were also altered by orchidectomy, Western blot analyses were performed on total protein extracted from caput epididymidis of control-sham operated and orchidectomized rats 7 days after surgery (Fig. 11A). Western blots of ETV4 yielded a single band of 62 kDa, while two bands were observed for CREB, corresponding to nonphosphorylated CREB (39 kDa) and the phosphorylated form of CREB (43 kDa). Quantification of Western blots using

tubulin as a loading control revealed that ETV4 levels were increased significantly in orchidectomized rats when compared to control (Fig. 11B). There were no significant differences in total CREB protein levels between control and operated rats (Fig. 11C). However, levels of phosphorylated CREB, which binds to DNA and activates transcription, were significantly increased (Fig. 11D). NHLH1 was also verified to determine whether or not levels increased with orchidectomy. NHLH1 protein levels were measured and, while the levels were barely detectable, these did not increase in orchidectomized rats (data not shown).

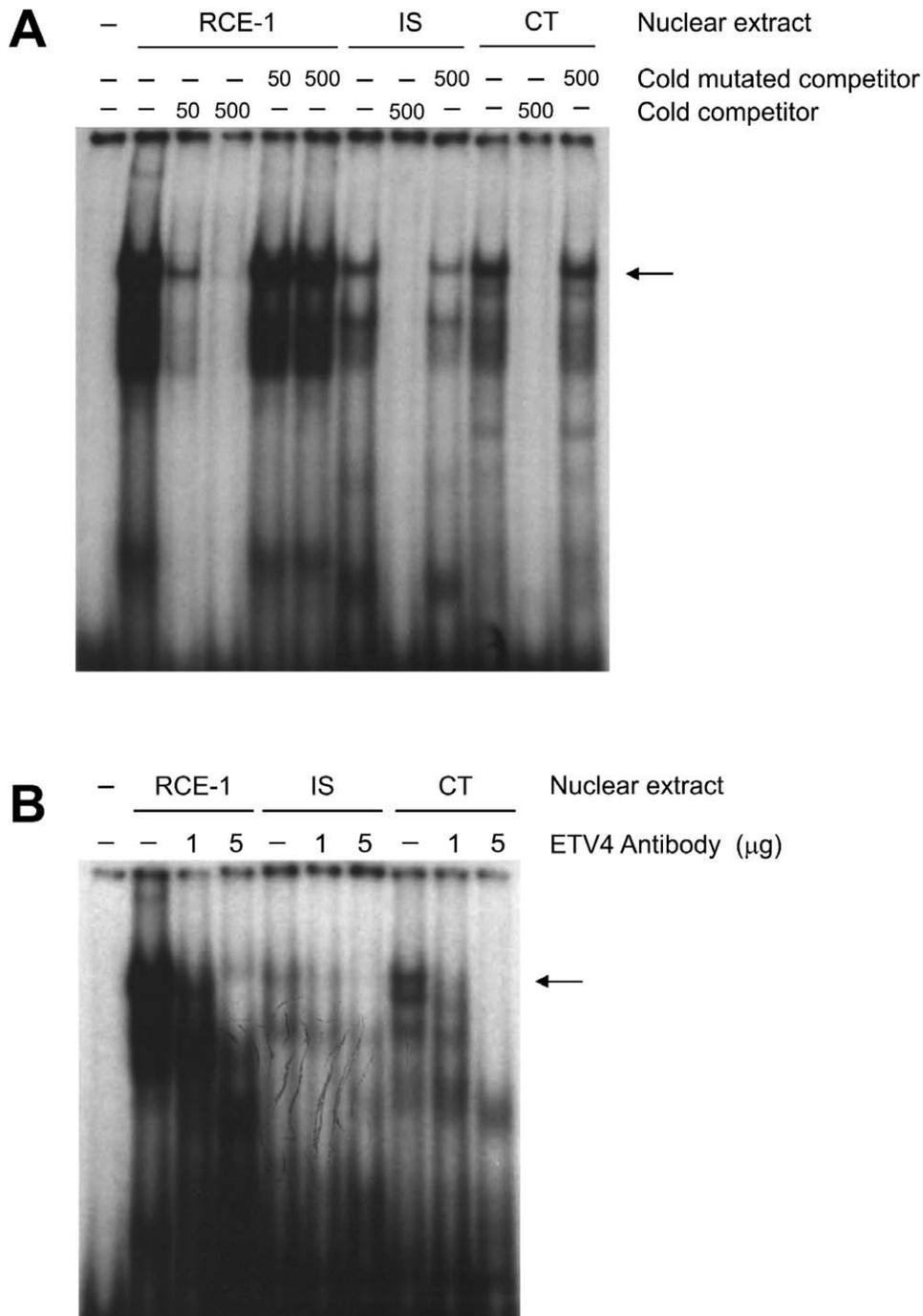


FIG. 8. Epididymal ETV4 binds to the *Panx1* promoter in vitro: EMSA on the promoter region of *Panx1* containing the ETV4 binding site. Nuclear extracts from RCE-1 cells and from adult rat initial segment or caput epididymidis were incubated with radiolabeled oligonucleotide containing the ETV4 binding site. **A**) For competition studies, proteins were preincubated with 50 or 500 molar excess of unlabeled (cold) competitor, unmutated or mutated in the ETV4 binding site. **B**) For supershift studies, proteins were preincubated with 1 or 0.5 µg of ETV4 antibody. Protein-DNA complexes are indicated by arrows. Gels are representative of three experiments.

DISCUSSION

PANX1 channels are involved in the movement of ions and small molecules, such as ATP and glutamate, and are implicated in a number of pathologies and physiological processes, including inflammation, stroke, epilepsy, and cancer [43]. In the epididymis, PANX1 is located in principal cells, basal cells, and halo cells, while PANX3 is localized at the apical plasma membrane of principal cells [17]. Numerous studies have shown that PANX1 is implicated in

the secretion of ATP and works in conjunction with purinergic receptors to regulate ATP secretion and intercellular communication through the propagation of ATP signaling [19, 35, 44–46]. Hence, the regulation of the *Panx1* gene provides an insight into its role and function in epididymal physiology.

Our results show that the rat *Panx1* promoter possesses multiple transcriptional start sites and lacks core promoter elements, such as a TATA box. The promoter is located in a CpG island, which spans 517 bp upstream from the ATG start

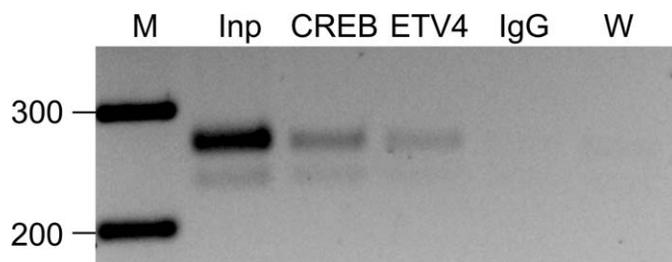


FIG. 9. CREB and ETV4 transcription factors interact with the *Panx1* promoter in vivo. Chromatin extracted from RCE-1 cells was immunoprecipitated with anti-CREB or anti-ETV4 antibodies. PCR amplifications were positive for a 267-bp fragment of the *Panx1* promoter and enriched for these antibodies when compared to the negative control (IgG). The gel is a representative of three separate experiments. Inp, 0.5% input; M, molecular weight marker; W, control water.

site. While CpG island promoters represent the majority of mammalian promoters [47, 48], they generally lack core promoter elements, and have dispersed transcription initiation sites as compared to TATA box-containing promoters, which usually have focused transcription initiation site [47–50]. It has been suggested that, in CpG island promoters, transcription factors can interact with the basal transcription machinery to activate transcription without core promoter elements defining the precise binding of the transcription apparatus, leading to dispersed transcription initiation sites [50, 51]. Zhang et al. [50] recently reported that, while transcription factors, such as SP1, have little transcriptional initiation capacity, members of the E-twenty six (ETS) family, nuclear respiratory factor 1 (NRF1), and the basic helix-loop-helix/leucine zipper (bHLH/ZIP) family of proteins showed a strong transcriptional initiation activity. Transcription of the reporter gene initiated by these transcription factors was also initiated at multiple sites [50]. The present observations suggest that the *Panx1* gene is likely to be initiated by transcription factors, which would explain the multiple start sites observed with RACE experiments. Transcription factors, such as ETV4, a member of the ETS family, and CREB, a member of the basic leucine zipper family of transcription factors, possess the ability to initiate transcription [50]. These transcription factors could act together with other regulators of transcription in binding to GC-rich regions, such as SP1, since multiple putative binding sites for this protein were

detected in the *Panx1* promoter, to regulate the *Panx1* transcription.

Sequence alignment of the *Panx1* promoter in the rat, mouse, and human indicate a strong degree of sequence homology, suggesting that the mechanism by which the *Panx1* gene is regulated is conserved between species. The up-regulation of *Panx1* mRNA levels observed in RCE cells treated with 5-azacytidine suggests that some of the regulation of the *Panx1* promoter occurs by DNA methylation. Few studies have examined DNA methylation in epididymal tissue. Reyes et al. [52] reported that the 5-alpha-reductase 2 gene was hypermethylated in the epididymis. Sato et al. [53] reported that neonatal exposure of mice to diethylstilbestrol resulted in changes in the expression of DNA methyltransferases and differences in DNA methylation. The significance of these in relation to gene expression was not investigated. Darwanto et al. [54], however, showed an increase in the methylation of the *CyclinD1* promoter during epididymal development, which appeared to be associated with a decreased expression of the gene. Nevertheless, the fact that DNA methylation may regulate the transcription of *Panx1* may be important not only for epididymal function, but also in other tissues.

Experiments in which 2.2 kb of the *Panx1* promoter was inserted into a pGL3 promoterless construct revealed that there was sufficient information in the promoter to drive the expression of the reporter gene. Deletion of the promoter indicated that the highest basal activity for the transactivation of the reporter gene occurred in the first 550 bp of the *Panx1* promoter sequence. Deletion of the –346 to –406 region of the promoter (140 bp construct) resulted in significantly lower ability to transactivate the reporter gene as compared to the intact –550 to –346 (205 bp) construct. Furthermore, constructs composed of the –973 to –346 region of the *Panx1* promoter sequence did not have significantly different activity from that of the 205-bp construct. However, larger constructs of 1.1 and 2.2kb (–1463 and –2508 to –346, respectively) yielded significantly lower activity than either the –550 or –973 constructs. These results indicate the presence of proximal binding sites for transcription factors implicated in the activation of the *Panx1* promoter, while inhibitory elements occur further upstream of the –973 position relative to the ATG. Analyses of these sequences indicated the presence of an NHLH1 response element in the first 406 bp 5' to the

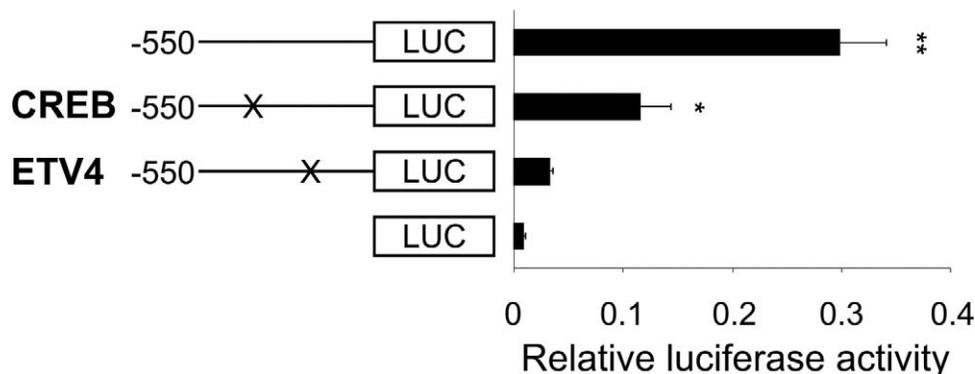


FIG. 10. CREB and ETV4 are crucial for transcriptional activity of the *Panx1* promoter. Point mutation constructs of CREB and ETV4 transcription factor binding sites were generated from the –550 to –346 *Panx1* reporter vector. RCE-1 cells were transfected with each construct and with the pHRL-TK vector to normalize for transfection efficiency. The pGL3-Basic empty vector was used as a negative control. Cells were incubated for 24 h and the firefly and *Renilla* luciferase activities were measured. Data are expressed as a ratio of firefly to *Renilla* activity (mean of triplicate \pm SEM) and are representative of three experiments. *Significant difference ($P < 0.05$) with both the empty construct and ETV4 mutated construct; **significant difference ($P < 0.05$) with constructs containing the CREB mutation, ETV4 mutation, or empty construct.

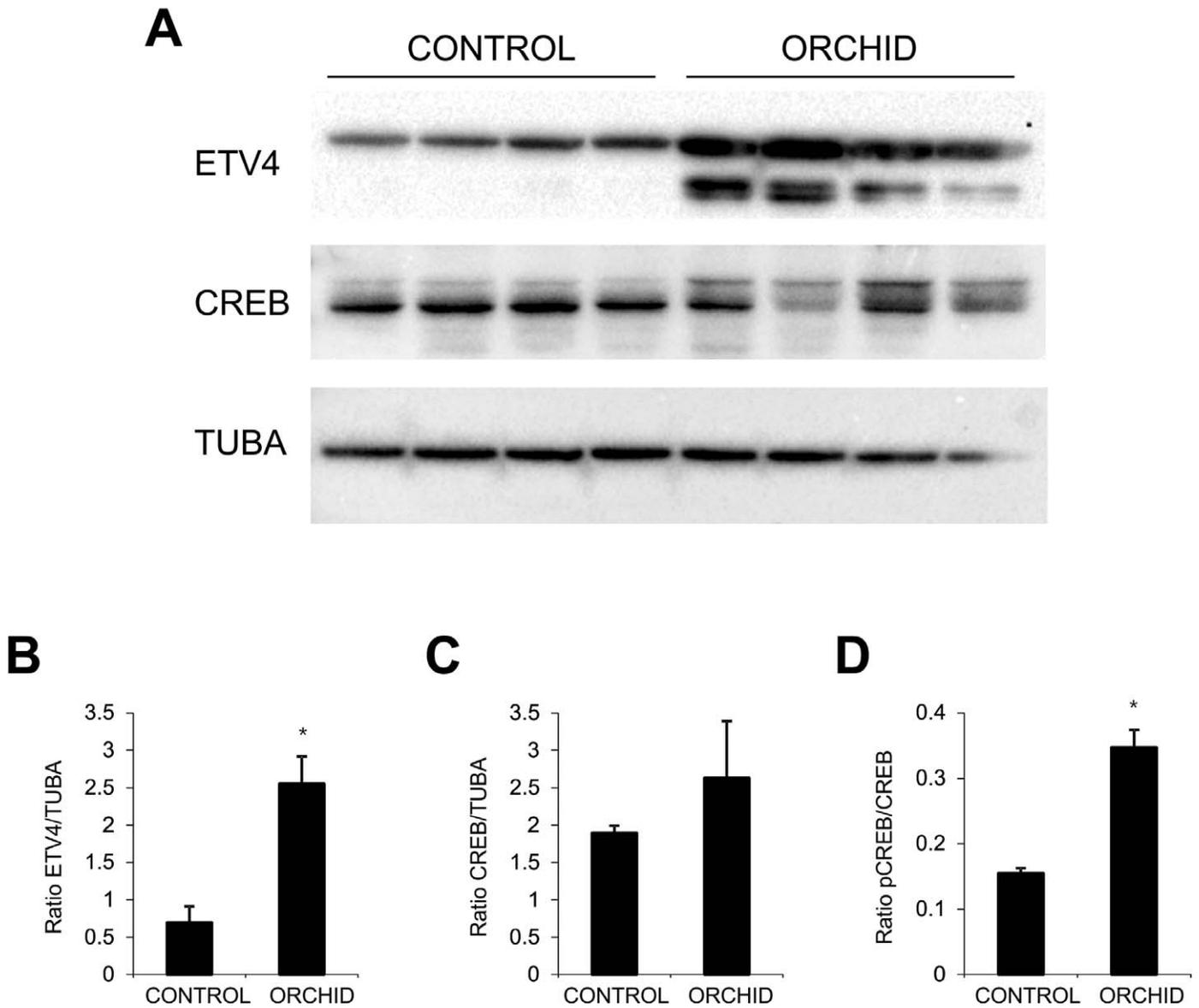


FIG. 11. ETV4 and CREB transcription factors levels increase in the caput epididymis in absence of testicular factors. Western blot analysis of ETV4 and CREB in sham-operated (control, n = 4) and orchidectomized (orchid, n = 4) rats (A). ETV4 protein levels (62 kDa) increase in the caput epididymis of orchidectomized rats (B). *Significant difference ($P = 0.005$). The increase of total CREB protein levels (39 and 43 kDa; in treated rats was not significant (C), but phosphoCREB (pCREB/CREB ratio) increased significantly (D). *Significant difference ($P < 0.001$). Total protein levels were normalized to TUBA levels. Data are expressed as the mean \pm SEM.

ATG (position -360 to -365), an ETV4 site at position -455 to -462, and a CREB site at position -467 to -474, in addition to multiple SP1 response elements.

NHLH1 is a transcription factor that has been identified in the ventricular zone of the brain, and has been shown to be an important regulator of neurogenesis and neuronal differentiation [55, 56]. While *Nhlh1* is expressed in RCE cells, it was detectable at very low levels in the adult rat epididymis in this study. In silico analyses of the transcriptome of embryonic murine epididymis indicated that *Nhlh1* is expressed during embryonic epididymal development, but that the mRNA levels are much lower in postnatal epididymis (National Center for Biotechnology Information, Geo Profile IDs 27723664 and 70598964). However, it is possible that other transcription factors may also bind to this site and contribute to the regulation of *Panx1*.

Both ChIP and EMSA studies using mutated response elements and specific antibodies confirmed that ETV4 and CREB bind to response elements on the *Panx1* promoter. Furthermore, transfection of RCE cells with *Panx1* promoter constructs in which either ETV4 or CREB was mutated resulted in a significant decrease in *Panx1* promoter activity. ETV4 and ETV5 are part of the PEA3 subfamily of the ETS transcription factors family [57] and expressed in the principal cells of the rat epididymal epithelium [58]. In the mouse, RNase protection assays have suggested that *Etv4* is expressed mainly in the epididymis and the brain [59]. In the rat epididymis, ETV4 protein expression was restricted to the caput epididymis [60], while mRNA transcripts were detected in all segments of the rat epididymis, although levels were higher in the initial segment [61].

Previous studies have shown that ETV4 regulates the transcription of *Gamma-glutamyl transpeptidase* mRNA IV

in the rat [62] and *Glutathione peroxidase 5* in the mouse [63]. Both are implicated in glutathione metabolism, and have high levels of enzymatic activity in the epididymis. In both rat and mouse epididymis, ETV4 is regulated by testicular factors, as shown by the significantly lower mRNA and protein levels observed following efferent duct ligation and orchidectomy [61]. Interestingly, androgens were shown to decrease *Etv4* mRNA levels in immortalized mouse caput epididymal cell lines following the overexpression of the androgen receptor [60]. Furthermore, since other ETV members can interact with the same response element on the promoter, it is possible that, in tissues other than the epididymis, other members of the ETV family contribute to the regulation of the *Panx1* gene.

CREB is a member of the ATF/CREB family of transcription factors [64]. It modulates the expression of genes, the promoter of which contains cAMP response elements. It is a bHLH/ZIP transcription factor and is converged upon by a large number of intracellular signaling pathways. Three major signaling pathways regulate CREB: cAMP and PKA pathways; Ca²⁺ signaling pathways; and numerous growth factor-mediated pathways. In the mouse epididymis, CREB regulates the expression of ER-alpha through the binding of its promoter [65], and was shown to be implicated in androgen regulation of gene expression in a mouse epididymal cell line as the result of kinase signaling pathways [66].

We previously reported that PANX1 levels were significantly increased following orchidectomy in the rat [17]. Western blot analyses of ETV4, CREB, and phosphorylated-CREB indicate that both ETV4 and phosphorylated-CREB levels were significantly increased in orchidectomized rats. Previous studies have reported a decrease in *Pea3* mRNA levels following orchidectomy [63]. However, in the case of study by Drevet et al. [63], the animals had been orchidectomized for 30 days, while the current study examined rats 7 days postsurgery. Thus, the differences in duration of time that the epididymis was deprived of androgens and other testicular factors may explain the discrepancy between their study and the present observations.

Total levels of CREB in the present study were unaltered by orchidectomy. It should be noted, however, that CREB is a phosphoprotein, which requires the phosphorylation of ser133 in order to activate transcription [64]. Our results indicated a significant increase in pCREB in the caput epididymis of rats that had been orchidectomized for 7 days. Whether or not the increases in ETV4 and pCREB are responsible for the increased PANX1 levels will require further studies. However, the data do suggest that there may be an association between the increase in both ETV4 and pCREB and the higher levels of epididymal PANX1 in orchidectomized rats.

The present article is the first report on the regulation of the *Panx1* promoter. Previous studies have shown that the transcription of the *Panx3* promoter in osteoblasts is regulated by the RUNX2 transcription factor [38]. The data from the present study clearly support the notion that *Panx1* and *Panx3*, both expressed in the epididymis, are regulated via different mechanisms. This may explain, at least in part, the cell-specific expression of the different PANXs in the rat epididymis.

ACKNOWLEDGMENT

Mary Gregory and Patrick Turmel (INRS) are thanked for their helpful suggestions.

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