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# Differential Involvement of $Na^+,K^+$-ATPase Isozymes in Preimplantation Development of the Mouse[1]

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**$Na^+,K^+$-ATPase plays an essential role in mammalian blastocoel formation (cavitation) by driving trans-epithelial sodium transport. Previously, the $\alpha$1 and $\beta$1 subunit isoforms of this enzyme were identified in preimplantation mouse embryos and were assumed to be responsible for this function. Here we show that mRNAs encoding an additional $\alpha$ subunit isoform ($\alpha$3) and the remaining two $\beta$ subunit isoforms are also present in preimplantation embryos. Whereas $\alpha$3 mRNA accumulates between the four-cell and the blastocyst stages and thus results from embryonic transcription, the same could not be demonstrated for $\beta$2 and $\beta$3 mRNAs. Immunoblot analyses confirmed that these subunits are present in cavitating embryos. Using confocal immunofluorescence microscopy we found that $\alpha$1 and $\beta$1 subunits are concentrated in the basolateral membranes of the trophectoderm while being equally distributed in plasma membranes of the inner cell mass. In contrast, $\alpha$3, $\beta$2, and $\beta$3 subunits were not detected in plasma membranes. Our current assessment, therefore, is that as many as six isozymes of $Na^+,K^+$-ATPase could be involved in preimplantation development although it is primarily the $\alpha$1$\beta$1 isozyme that is responsible for blastocoel formation. Our findings imply that the regulation of sodium transport within the preimplantation mouse embryo is more complex than had been appreciated.** © 2000 Academic Press

***Key Words:*** **mouse; embryo; preimplantation; cavitation; $Na^+$ transport; sodium/potassium pump; $Na^+,K^+$-ATPase; isozymes; blastocoel.**

## INTRODUCTION

In mammals, successful embryogenesis depends on a complex interaction between the blastocyst and the receptive uterus into which it implants. This fact necessitates the setting aside, after relatively few divisions of the zygote, of an extraembryonic cell population that will mediate this interaction. Thus in the blastocyst, the outer cells constitute an epithelial monolayer, the trophectoderm. It is the ion and fluid transporting ability of the trophectoderm which enables blastocoel formation (cavitation), an event which prepares the blastocyst for implantation. The trophectoderm is also the part of the conceptus that initiates uterine contact and invasion. Trophectoderm arises *de novo* from previously unspecialized blastomeres, providing a unique experimental model for understanding how a polarized transporting epithelium develops (Watson *et al.,* 1990a; Wiley *et al.,* 1990).

There is general agreement that the plasma membrane sodium pump, $Na^+,K^+$-ATPase, plays a critical role in trophectoderm differentiation and cavitation (reviewed by Watson, 1992). This enzyme is present in all stages of preimplantation development in which it uses the energy of hydrolysis of ATP to transport $Na^+$ and $K^+$ across the membrane in opposite directions to maintain steep electrochemical gradients of both cations (Baltz *et al.,* 1997). Although total $Na^+,K^+$-ATPase activity increases only about twofold during preimplantation development, most of this increase occurs after the morula stage and is targeted to basolateral membranes (Van Winkle and Campione,

[1] This paper is dedicated to the memory of Clement L. Markert, who spent much of his career exploring the biochemical and developmental significance of isozymes.

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1991). Direct evidence for the involvement of $Na^+$,$K^+$-ATPase in the specific process of cavitation comes from experiments using ouabain, a specific inhibitor of the enzyme. Expansion of the blastocoel is due to transport of fluid across the trophectoderm, and this process is inhibited by ouabain provided the drug has access to the basolateral (juxtacoelic) side of the trophectoderm (DiZio and Tasca, 1977; Manejwala *et al.,* 1989; Betts *et al.,* 1997; MacPhee *et al.,* 1998). The involvement of $Na^+$,$K^+$-ATPase is also indicated by the fact that blastocoel expansion is significantly retarded in the absence of extracellular $Na^+$ (Manejwala *et al.,* 1989) or when the enzyme's function has been impaired by antisense treatment (Jones *et al.,* 1997; L. Barcroft and A. J. Watson, unpublished). As is the case in other transporting epithelia, $Na^+$,$K^+$-ATPase drives a trans-epithelial $Na^+$ flux (and corresponding osmotic flow of water) into the blastocoel because it is concentrated in the basolateral plasma membranes of the trophectoderm (Watson and Kidder, 1988; Kidder and Watson, 1990). Treatments that disrupt or prevent the development of the membrane–cytoskeletal complex in the blastocyst prevent $Na^+$,$K^+$-ATPase from assuming its basolateral localization, and fluid transport is blocked (Wiley *et al.,* 1990; Watson *et al.,* 1990a).

The enzymatic properties of $Na^+$,$K^+$-ATPase can be constituted by heterodimerization of two subunits, a catalytic $\alpha$ subunit and a noncatalytic, glycosylated $\beta$ subunit (reviewed by Blanco and Mercer, 1998). Although the $\beta$ subunit lacks catalytic activity, it is required for structural and functional maturation of the nascent $\alpha$ subunit (Geering *et al.,* 1996). Mammals have four isoforms of the $\alpha$ subunit ($\alpha1$, $\alpha2$, $\alpha3$, and $\alpha4$) and three of the $\beta$ subunit ($\beta1$, $\beta2$, and $\beta3$), all encoded by separate genes having specific patterns of expression among adult organs (Blanco and Mercer, 1998). Whereas the $\alpha$ subunit isoforms are very similar in amino acid sequence (76–86% identity; Shamraj and Lingrel, 1994) the $\beta$ subunit isoforms are less so (39–48% identity; Malik *et al.,* 1996). There is generally greater sequence identity between mammalian species for a given isoform than there is between isoforms within a species.

A variety of approaches have been used to explore the involvement of the various $Na^+$,$K^+$-ATPase subunit isoforms in blastocyst development. According to Northern blot analysis, both $\alpha1$ and $\beta1$ mRNAs accumulate during preimplantation development of the mouse and are thus the products of embryonic transcription (Watson *et al.,* 1990b; Gardiner *et al.,* 1990b). Transcripts encoding the $\alpha1$ isoform accumulate steadily from the time of genomic activation in the two-cell stage and reach a level of about 1000 copies/cell in early blastocysts (Kidder, 1993; Latham *et al.,* 1999). $\beta1$ mRNA, in contrast, has been undetectable until the late morula stage after which it becomes more abundant in early blastocysts (about 300 copies/cell; Kidder, 1993). More recently, a semiquantitative RT-PCR method was employed to show that there is a constant, low level of $\beta1$ mRNA (about 50 copies/cell), probably oogenetic in origin, that persists through the early cleavages to be superceded by new transcripts that accumulate rapidly in the late morula (MacPhee *et al.,* 1998). Thus it is expression of the $\beta1$ gene, not $\alpha1$, whose up-regulation coincides with the polarized deployment of sodium pumps in the trophectoderm and the onset of cavitation. Results from immunoblotting, showing a marked increase in $\beta$ subunits in late morulae, are consistent with these findings (Gardiner *et al.,* 1990b). A similar situation exists in bovine preattachment development in which, as in the mouse, late accumulation of $\beta1$ mRNA correlates with the onset of cavitation (Betts *et al.,* 1997). Both $\alpha1$ and $\beta1$ subunit isoforms have also been identified in rabbit and horse blastocysts (Gardiner *et al.,* 1990a; Waelchli *et al.,* 1997).

Recently, it became clear that the situation may be more complex than had been realized with respect to the involvement of specific subunit isoforms of $Na^+$,$K^+$-ATPase in blastocyst development. Using RT-PCR, it was discovered that bovine preattachment embryos express transcripts encoding the $\alpha1$, $\alpha2$, $\alpha3$, $\beta1$, and $\beta2$ isoforms (Betts *et al.,* 1997; $\beta3$ transcripts were not looked for). Furthermore, it was shown by confocal immunofluorescence microscopy that the $\alpha3$ isoform is concentrated in the apical surface of the bovine trophectoderm, suggesting a modulatory role in trans-epithelial $Na^+$ transport in opposition to basolaterally localized sodium pumps containing $\alpha1$ subunits (Betts *et al.,* 1998). In the present study, we have reexamined preimplantation mouse embryos to determine whether multiple subunit isoforms are expressed in that species as well and whether their distributions are similar to those in the cow.

## MATERIALS AND METHODS

### *Mouse Embryo Collection*

Random-bred CF1 female mice (Charles River Canada Ltd., St. Constant, Québec) were superovulated and mated as previously described (MacPhee *et al.,* 1994). The embryonic stages and times (hours post-hCG) of collection from the reproductive tract were as follows: oocytes, 18 h; two-cell, 48 h; four-cell, 60 h; eight-cell, 65 h; eight-cell compacting, 72 h; morula, 84 h; blastocysts, 90–92 h. Embryos were washed five times in calcium- and magnesium-free PBS containing 0.3% polyvinylpyrrolidone (PBS-PVP) and then either used immediately or stored frozen until use.

### *Reverse Transcriptase-Polymerase Chain Reaction*

Total RNA was extracted according to Valdimarsson *et al.* (1993). Pools of 100 murine embryos or 10 mg of mouse kidney or testis were lysed at room temperature in 100 $\mu$l of solution D (Chomczynski and Sacchi, 1987) in the presence of 20 $\mu$g of *Escherichia coli* rRNA. Embryo lysates were layered onto 100 $\mu$l of 5.7 M CsCl in 0.1 M EDTA, pH 7.5, and ultracentrifuged at 80,000 rpm (27,400$g$) for 4 h at 20°C utilizing a TLA-100 rotor and a Beckman TL-100 ultracentrifuge. RNA pellets were dissolved in 2.5 M ammonium acetate, precipitated in absolute ethanol, and then air-dried.

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**TABLE 1**
$Na^+,K^+$-ATPase Subunit Isoform PCR Primers

| | GenBank accession number | Primer sequences | Size of genomic amplicon (bp) | Size of cDNA amplicon (bp) | Amplified segment (codons of cDNA) | Diagnostic restriction enzyme | Sizes of restriction fragments (bp) |
|---|---|---|---|---|---|---|---|
| α2 | M14512 | (U) GGCTGCTTGGGAT-CCGCCTTGA<br>(L) AGGGGAAGGCACA-GAACCACCA | 2946 | 335 | 874–985 | *Ava*I | 267, 68 |
| α3[a] | M14513 | (U) ACCTGGTGGGCAT-CCGGCTCAA<br>(L) AGGGGAAGGCACA-GAACCACCA | 2946 | 336 | 874–983 | *Bcl*I | 179,157 |
| α3[b] | M14513 | (U) AGGAACCCACGCA-CAGA<br>(L) TTTCCTCAAACAGT-CCGAAGAT | 1600 | 376 | 827–952 | *Bgl*II | 346, 30 |
| α4 | U15176 | (U) GGAGTTGAAGAAGG-AAGTGG<br>(L) AGCCAGTGATGATG-ACCACG | 3478 | 338 | 39–150 | *Hae*III | 242, 96 |
| β1 | X16646 | (U) TTCAGCCCAGAAGG-ACGACATG<br>(L) AGGGAAGCCGTAGT-ATCCGCCCA | 6346 | 378 | 115–240 | *Hin*dIII | 266,112 |
| β2 | J04629 | (U) GCCTTACAACGACT-CATCCA<br>(L) CGGCATTCTACATTC-ACCTCC | 1664 | 441 | 116–262 | *Hae*III | 291,150 |
| β3 | U59761 | (U) TTGCCGTACAGGTC-AAAT<br>(L) CGGGAGCTCACTG-GTC | 384 | 384 | 231–280 (plus 234 bp of 5′-UTR) | *Eco*RI | 167,217 |

[a] Primer set used for studying the developmental expression profile.
[b] Primer set used for semiquantitative RT-PCR.

Purified RNA preparations were reverse transcribed according to De Sousa *et al.* (1993). Tissue RNA samples, for PCR positive controls, were extracted as described above except for a 2-μg sample of rat midbrain RNA that was a gift from Dr. Patricia Watson (Lawson Research Institute, London, Ontario). RNA pellets were resuspended in 11 μl of sterile water and incubated with 1 μg of oligo(dT)$_{12-18}$ for 10 min at 70°C. After cooling on ice, RNA was incubated in 1st Strand Buffer containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM deoxynucleoside triphosphates (dNTPs), and 200 units of Superscript reverse transcriptase (Life Technologies, Burlington, Ontario, Canada). Reverse transcription (RT) reactions were incubated at 42°C for 1.5 h. Reactions were terminated by heating at 94°C for 4 min and flash cooling on ice. Following the RT reactions, mouse embryo cDNA samples were at a final concentration of 5 embryo equivalents/μl.

PCR was carried out in a total volume of 25 μl. All assays were repeated at least twice on embryos from two different collections. One microliter of RT preparation, representing 2.5–5 embryo equivalents, was added to 0.4 units of *Taq* polymerase (Life Technologies) in a solution consisting of 20 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 1 mM $MgCl_2$, and 0.4 μM sequence-specific primers (4 μM for β2 primers). Reactions were overlaid with 25 μl of paraffin oil and amplified with a Perkin–Elmer Cetus DNA thermal cycler. Each cycle of PCR consisted of 1 min denaturation at 94°C, 30 s annealing at 53–60°C, and 1 min primer extension at 72°C. PCR products (11 μl) were resolved on 3% agarose gels (3:1 low-melting-point agarose and regular agarose) containing 0.75 mg/ml ethidium bromide.

PCR primers were obtained from the Core Molecular Biology Facility, Department of Biochemistry, University of Western Ontario. $Na^+,K^+$-ATPase isoform primer pairs were derived from published rat and mouse cDNA sequences; accession numbers and the sizes of the expected amplicons are shown in Table 1. The primer pairs were designed to produce isoform-specific RT-PCR products and, with the exception of the β3 primers, to bracket introns for discrimination of products from genomic DNA and cDNA. As an initial confirmation of identity, PCR products were subjected to cleavage with appropriate diagnostic restriction enzymes. Following that, amplicons were cleaned with a QIAquick Spin PCR purification kit (Qiagen, Chatsworth, CA) and directly sequenced by the dye termination method with an ABI Prism 377

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DNA sequencer using the PCR primers as sequencing primers (Genalytic, Department of Zoology, University of Guelph, Guelph, Ontario).

A semiquantitative RT-PCR protocol was used to compare the relative amounts of subunit isoform mRNAs between different developmental stages. This protocol is described in Davies *et al.* (1996) and involves the addition of $\alpha$-globin mRNA (0.1 pg/embryo) before RNA purification to serve as an internal standard for monitoring RNA recovery and the efficiency of reverse transcription and PCR. For each PCR, 0.2–2.5 embryo equivalents of cDNA were used. After the PCR, the $\alpha$-globin and subunit isoform amplicons were separated and quantified by capillary electrophoresis using a Beckman P/ACE 2100 CE system equipped with laser-induced fluorescence detection.

### SDS–PAGE and Western Blot Analysis

Rat organ samples were pulverized under liquid nitrogen with a mortar and pestle and homogenized in RIPA lysis buffer [50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, 100 $\mu$M $Na_3VO_4$, and Complete Mini EDTA-free protease inhibitors (Roche Diagnostics, Laval, Québec)]. Samples were centrifuged at 15,000*g* at 4°C for 15 min and the supernatants collected.

Mouse organ samples, frozen and pulverized as above, and preimplantation embryos (mixtures of late morulae and blastocysts) were homogenized in equal volumes of PBS and 2× SDS loading buffer (100 mM Tris–HCl, 200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol). The last PBS-PVP wash from which the embryos had been collected was also utilized to provide a negative control. Samples were then left at room temperature for 10 min and centrifuged 10 min at 13,000*g* and the supernatants recovered. Protein concentrations for all tissue samples were determined by the Bradford assay using Bio-Rad protein assay dye reagent (Bio-Rad Laboratories, Mississauga, Ontario). Total protein from rat or mouse organs and from preimplantation embryos was separated by polyacrylamide gel electrophoresis in Tris–glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8.3, 0.1% SDS) using either 4% stacking and 9% resolving gels (30:1 acrylamide:bis) or 4–12% gradient gels (Helixx Technologies, Scarborough, Ontario).

Following electrophoresis, gels were soaked in transfer buffer (39 mM glycine, 48 mM Tris base, 0.037% SDS) for 20 min. Polyvinylidene difluoride membranes (0.45 $\mu$m; Millipore Canada, Nepean, Ontario) were rinsed in methanol for 10 s, rehydrated in distilled tap water for 3 min, and soaked in transfer buffer for 3 min prior to electroblotting for 2 h at 250–400 mA at 20°C. Membranes were then rinsed in PBS to remove residual acrylamide and air-dried.

All incubations for protein detection were done at room temperature (unless otherwise noted) and with constant agitation. Blots were blocked in 5% skim milk powder for 1 h, rinsed once for 15 min in Tris-buffered saline (20 mM Tris, 137 mM NaCl, pH 7.6) with 0.1% Tween 20 (TBST) and twice for 5 min each in TBST. Primary antibodies (see Table 2) were diluted in blocking solution and incubated with blots for 1 h or overnight at 4°C. Membranes were rinsed once for 15 min in TBST, followed by two more washes for 5 min each in TBST. Secondary antibodies were also diluted in blocking solution and incubated with membranes for 1 h. Membranes were washed once for 15 min in TBST and then four times for 5 min each in TBST. Proteins were detected using the Amersham Pharmacia Biotech ECL or Roche Diagnostics ECL detection system and multiple exposures of Kodak XAR X-ray film were generated to ensure the linearity of the film responses.

### Confocal Immunofluorescence Microscopy

Preimplantation embryos were fixed in methanol as recommended by Becker and Davies (1995). Embryos were fixed for 3 min each in 2:1 PBS:methanol, 1:1 PBS:methanol, 1:2 PBS:methanol, and then 100% methanol. They were then rehydrated through the same series in reverse. Prior to immunostaining, embryos were blocked in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM $MgCl_2$, pH 6.9) containing 0.01% Triton X-100, 0.1 M lysine, and 1% goat serum for 45 min and then washed in PBS-PVP for 5 min. Primary antibody (see Table 2), diluted in PBS, was added to the embryos for incubation overnight at 4°C. Embryos were then washed three times, for 10 min each, in PBS-PVP followed by addition of secondary antibody diluted in PBS. Embryos were incubated in secondary antibody for 1 h at 4°C then washed three times, for 5 min each, with PBS-PVP. Embryos were mounted on glass slides in FITC-Guard (Testog, Inc., Chicago, Illinois) and viewed with a Bio-Rad MRC-600 laser scanning confocal microscope.

### Antibodies

Details about the primary antibodies used in this study are summarized in Table 2. Secondary antibodies for immunofluorescence experiments were fluorescein-conjugated rabbit anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG (ICN ImmunoChemicals, Montreal, Québec), both used at the recommended dilution of 1:50. For Western blots, donkey anti-rabbit IgG and sheep anti-mouse IgG, both conjugated to horseradish peroxidase (Roche Diagnostics, Laval, Québec; Amersham Pharmacia Biotech, Baie d'Urfe, Québec), were used at dilutions of 1:1000 and 1:2000, respectively.

## RESULTS

### Detection of $Na^+,K^+$-ATPase Subunit Isoform Transcripts by RT-PCR

This study was begun with the knowledge that mRNAs encoding the $\alpha$1 and $\beta$1 subunit isoforms accumulate during preimplantation development of the mouse (Gardiner *et al.,* 1990b; Watson *et al.,* 1990b; Kidder, 1993; MacPhee *et al.,* 1994, 1998). RT-PCR was used to search for mRNAs encoding the other subunit isoforms; results are shown in Fig. 1. Transcripts encoding the $\alpha$2 isoform could be detected only in oocytes, and this was an inconsistent finding both within and between cDNA preparations, indicating very low mRNA abundance. Transcripts encoding the $\alpha$3 isoform were detected throughout preimplantation development. There was an apparent increase in abundance of this mRNA from the four-cell stage onward, suggesting its derivation from both maternal and embryonic gene transcription. This conclusion was confirmed by semiquantitative RT-PCR, which revealed that the relative amount of $\alpha$3 mRNA per embryo increases approximately threefold between the four-cell and the blastocyst stages (Table 3). The

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**TABLE 2**
Primary Antibodies Used for Immunoblotting and Immunofluorescence Localization

| Antibody | Type | Raised against | Dilution for immunoblot | Dilution for immunofluorescence | Reference for specificity | Source |
|---|---|---|---|---|---|---|
| mAb 6H | Mouse monoclonal | $\alpha$1 in dog kidney microsomes | Not used | 1:100 | Pietrini *et al.,* 1992; Gottardi and Caplan, 1993 | M. Caplan, Yale Univ. |
| HERED | Rabbit polyclonal | aa 489–500 of rat $\alpha$2 | 1:100 | 1:100 | Pressley, 1992 | T. Pressley, Texas Tech. Univ. |
| McB2 | Mouse monoclonal | rat $\alpha$2 (N-terminal half) | Not used | 1:4 | Urayama *et al.,* 1989 | K. Sweadner, Mass. Gen. Hospital |
| MA3 915 | Mouse monoclonal | $\alpha$3 in dog heart microsomes | 1:1000 | 1:300 | Zahler *et al.,* 1996; Mobasheri *et al.,* 1997 | Affinity BioReagents, Inc. (Golden, CO) |
| Caplan $\alpha$3 | Rabbit polyclonal | aa 2–14 of rat $\alpha$3 | 1:500 | 1:100 | Pietrini *et al.,* 1992 | M. Caplan, Yale Univ. |
| TED | Rabbit polyclonal | aa 489–500 of rat $\alpha$3 | Not used | 1:100 | Pressley, 1992 | T. Pressley, Texas Tech. Univ. |
| mAb 8a | Mouse monoclonal | $\beta$1 in dog kidney microsomes | Not used | 1:50 | Gottardi and Caplan, 1993 | M. Caplan, Yale Univ. |
| SM-GP50 | Mouse monoclonal | $\beta$2 in rat brain synaptic membranes | 1:5 | 1:1 | Arystarkhova and Sweadner, 1997 | J. Gurd, Univ. of Toronto |
| 06171 | Rabbit polyclonal | aa 63–285 of rat $\beta$2 | 1:500 | 1:200 | Shyjan *et al.,* 1990b | Upstate Biotechnology, Inc. (Lake Placid, NY) |
| Levenson $\beta$3 | Rabbit polyclonal | aa 70–279 of rat $\beta$3 | 1:1000 | 1:250 | Unpublished, but see www.upstatebiotech.com/antibody.asp?ProductID=06-817 | R. Levenson, Penn State U. |
| RNT$\beta$3 | Rabbit polyclonal | N-terminal 13 aa of rat $\beta$3 | Not used | 1:500 | Arystarkhova and Sweadner, 1997 | K. Sweadner, Mass. Gen. Hospital |

identity of the $\alpha$3 amplicon was confirmed initially by restriction endonuclease digestion (Table 1) and then by direct sequencing (Fig. 2). Transcripts of the $\alpha$4 gene were not detected in oocytes nor in any of the preimplantation stages examined, although an amplicon of the predicted size was obtained from a mouse testis cDNA preparation (Fig. 1). The identity of the amplicon was confirmed by restriction enzyme digestion (Table 1). The predicted $\beta$2 and $\beta$3 amplicons were also generated from cDNA preparations from all stages of preimplantation development. Again, the hypothesis that these mRNAs accumulate through preimplantation development was tested by semiquantitative RT-PCR and found to be incorrect: neither mRNA increases in abundance between the four-cell and the early blastocyst stages (Table 3). Indeed, $\beta$2 mRNA may actually decline, although the difference was not significant. Thus it cannot be concluded that these two genes are embryonically transcribed. $\beta$2 and $\beta$3 amplicon identities were confirmed by restriction enzyme digestion (Table 1) followed by direct sequencing (Fig. 2).

### *Detection of $Na^+$,$K^+$-ATPase Subunit Isoforms by Immunoblotting*

Although $\alpha$2 mRNA was not detected in any preimplantation stage, its presence in ovulated oocytes (Fig. 1) raised the possibility that oogenetic $\alpha$2 subunits might contribute to overall sodium pump activity during blastocyst development. However, while we were able to detect an $\alpha$2-immunoreactive protein on immunoblots of

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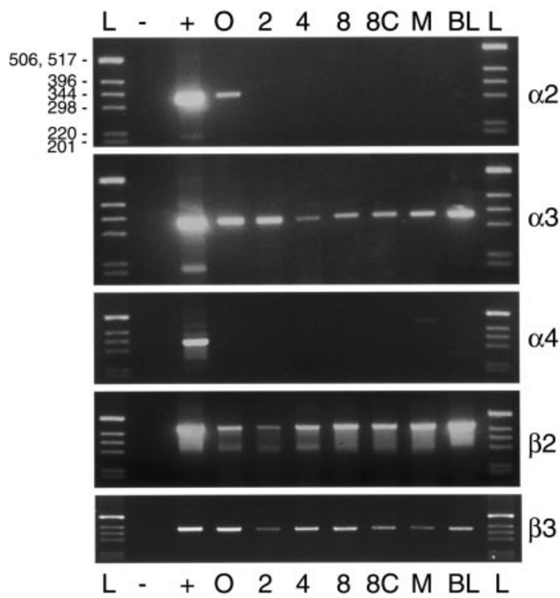


**FIG. 1.** Detection of $Na^+$,$K^+$-ATPase subunit isoform mRNAs in preimplantation embryo stages by RT-PCR. Five embryo equivalents of cDNA were used for each reaction except for the $\beta3$ series, which used 2.5 embryo equivalents. Amplification for $\alpha2$, $\alpha3$, $\alpha4$, $\beta2$, and $\beta3$ isoforms was for 40, 34, 40, 38, and 25 cycles, respectively. L, DNA ladder (sizes in base pairs are given at the top); −, negative control (no template); +, positive control (rat midbrain cDNA as template for $\alpha2$, $\alpha3$, and $\beta2$; mouse testis cDNA for $\alpha4$; and mouse kidney cDNA for $\beta3$); O, ovulated unfertilized oocytes; 2, 2-cell; 4, 4-cell; 8, 8-cell; 8C, compacted 8-cell; M, morula; BL, blastocyst.

mouse brain homogenates, no such band was detected in blastocyst lysates (not shown). The $\alpha3$ isoform was detected in both brain and blastocysts (Fig. 3). Immunoreactive bands corresponding to $\beta2$ and $\beta3$ isoforms were also detected in blastocyst lysates along with the respective positive control samples (brain for $\beta2$, liver for $\beta3$; Fig. 3). Several more rapidly migrating bands reactive with the SM-GP50 antibody were also evident, probably representing incompletely glycosylated forms of the $\beta2$ subunit (Shyjan *et al.,* 1990b). In all cases the last wash samples were negative, showing that the positive immunoreactivity came from the blastocysts and not the female reproductive tract.

### Detection of $Na^+$,$K^+$-ATPase Subunit Isoforms by Immunofluorescence

Previously, we investigated the distribution of $\alpha1$ and $\beta1$ subunits in preimplantation mouse embryos by immunofluorescence using sections cut from wax-embedded embryos (Watson and Kidder, 1988; MacPhee *et al.,* 1998). In the present study, confocal immunofluorescence microscopy was applied to whole-mount preparations, hence we repeated the analysis of $\alpha1$ and $\beta1$ subunit distribution along with the analysis of the other isoforms. The $\alpha1$ subunit was detected from the four-cell stage to the blastocyst stage (Fig. 4). In four-cell embryos ($n = 61$ embryos examined), fluorescence was localized to blastomere membranes that were in contact or close apposition to one another. In eight-cell embryos ($n = 31$), $\alpha1$ subunits were detected throughout blastomere membranes and as embryos compacted ($n = 10$) and became morulae ($n = 25$), this distribution was maintained but became more intense. In blastocysts ($n = 43$), fluorescence was localized in basolateral plasma membrane domains of mural and polar trophectoderm and in membranes of the inner cell mass (ICM). Little or no $\alpha1$ immunofluorescence was detected in apical membranes of mural or polar trophectoderm. In contrast to $\alpha1$, $\beta1$ subunits were not detected in four-cell, eight-cell, or eight-cell compacting mouse embryos ($n$ = 25, 17, 17, respectively) but became detectable in apposed membranes and, to a more limited extent, in the cytoplasm of late morulae ($n = 35$) (Fig. 5). In blastocysts ($n = 26$), the $\beta1$ isoform was localized in the basolateral plasma membranes of the mural and polar trophectoderm and in membranes of the inner cell mass, matching the distribution of the $\alpha1$ isoform.

At least two different antibodies were used to study the subcellular localization of each of the other isoforms,

**TABLE 3**

Changes in Relative Abundance of $Na^+$,$K^+$-ATPase Subunit Isoform mRNAs during Preimplantation Mouse Development as Determined by Semiquantitative RT-PCR

| | $\alpha1$ | $\alpha3$ | $\beta1$ | $\beta2$ | $\beta3$ |
|---|---|---|---|---|---|
| Four-cell[a] | n.d.[b] | 0.22 ± 0.04 | 0.02 ± 0.01 | 2.75 ± 0.81 | 0.50 ± 0.03 |
| Blastocyst[a] | n.d.[b] | 0.70 ± 0.08[c] | 3.45 ± 0.05[c] | 1.58 ± 0.41 | 0.60 ± 0.04 |
| Blastocyst/four-cell | 5.6[b] | 3.2 | 172.5 | 0.6 | 1.2 |

[a] Target amplicon/$\alpha$-globin amplicon ratios (mean ± SD) were determined from triplicate PCRs.
[b] Relative $\alpha1$ mRNA levels were not determined in this study, but had been compared previously by Northern blotting (Kidder, 1993).
[c] Significantly different from the four-cell value ($P < 0.01$).

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α3

```
2784  GATGACCGCACTGTCAACGACCTGGAAGACAGCTACGGGCAGCAGTGGACTTATGAGCAG 2843
         ||      |||||| |||  |  | |||| || ||||||||||||||||||||||||
1     TGGGAGACGCATGTCAATGACTAG..AAACAGTTATGGGCAGCAGTGGACTTATGAGCAG 58

2844  AGGAAGGTGGTTGAGTTCACGTTCCACACAGCCTTCTTCGTGAGCATAGTGGTGGTCTCA 2903
      ||||| | ||| |||||||| | ||||||||||||||| ||||| || ||||||||||||
59    AGGAA.G.GGTAGAGTTCACATGCCACACAGCCTTCTTTGTGAGTATCGTGGTGGTCTCA 116

2904  GTGGGCTGACCTGATCATTTGCAAGACCAGGAGGAATTCCGTCTTCCAGCAGGGCATGAA 2963
      |||||||||||||||||| ||||||||||||||||| |||||||||||||||||||||||
117   GTGGGCTGACCTGATCATCTGCAAGACCAGGAGGAACTCCGTCTTCCAGCAGGGCATGAA 176

2964  GAATAAGATCTTGATCTTCGGACTGTTTGAGGAAACGGCCCTCGCTGCCTTCCTGTCCTA 3023
      |||||||||||||||||||||  |||||||||| ||||||||||||||||||||||||||
165   GAATAAGATCTTGATCTTCGGCTTGTTTGAGGAGACGGCCCTCGCTGCCTTCCTGTCCTA 236

3024  CTGCCCAGGCATGGATGTGGCCCTTCGCATGTACCCGCTCAAGCCCAGCTGGTGGTTCTG 3083
      |||||||||||||||||||||||||||||||||||| |||||||||||||||||||||||
237   CTGCCCAGGCATGGATGTGGCCCTTCGCATGTACCCTCTCAAGCCCAGCTGGTGGTTCTG 296

3084  TGCCTT 3089
      | ||
297   TTCCCC 302
```

β1

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423   CCCAGTGAACCCAAGGAACGGGGCGACATCAATCACGAACGAGGAGAGAGGAAGGTGTGC 482
      |||||| |||||||||||||||||| ||||||| ||||||||||||||||||||||||||
1     CCCAGTNAACCCAAGGAACGGGGCG.CATCAAT.ACGAACGAGGAGAGAGGAAGGTGTGC 58

483   AGGTTCAAGCTTGACTGGCTGGGGAACTGCTCCGGTCTCAATGATGACTCTTACGGCTAC 542
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
59    AGGTTCAAGCTTGACTGGCTGGGGAACTGCTCCGGTCTCAATGATGACTCTTACGGCTAC 118

543   AGAGAGGGGAAGCCCTGCATCATTATCAAGCTCAACCGAGTGTTGGGCTTCAAACCGAAG 602
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
119   AGAGAGGGGAAGCCCTGCATCATTATCAAGCTCAACCGAGTGTTGGGCTTCAAACCGAAG 178

603   CCTCCCAAGAATGAATCCTTGGAGACTTACCCACTGATGATGAAGTATAATCCAAATGTC 662
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
179   CCTCCCAAGAATGAATCCTTGGAGACTTACCCACTGATGATGAAGTATAATCCAAATGTC 238

663   CTGCCTGTTCAGTGCACTGGCAAGAGAGATGAAGATAAGGATAAAGTCGGGAACATAGAG 722
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
239   CTGCCTGTTCAGTGCACTGGCAAGAGAGATGAAGATAAGGATAAAGTCGGGAACATAGAG 298

723   TACTTTGGGATGGGCGGATACTACGGCTTCCCT 755
      |||||||||||||||||||||||||||||||||
299   TACTTTGGGATGGGCGGATACTACGGCTTCCCT 331
```

**FIG. 2.** Verification of the identity of subunit isoform RT-PCR amplicons by direct sequencing (the amplicon sequence is the lower line of each pair). The sequences were compared using the BLAST-N program from NCBI. The $\alpha$3 amplicon was compared with the rat $\alpha$3 sequence (nt 2784–3089; GenBank Accession No. M14513), revealing 95% sequence identity. The $\beta$1 amplicon was compared with the mouse $\beta$1 sequence (nt 423–755; GenBank Accession No. X16646), revealing 99% sequence identity. The $\beta$2 amplicon was compared with the mouse $\beta$2 sequence (nt 515–851; GenBank Accession No. X16645), also revealing 99% sequence identity. The $\beta$3 amplicon was compared with the mouse $\beta$3 sequence (nt 846–1203; GenBank Accession No. U59761), revealing 97% sequence identity.

with congruent results being obtained in each case. As expected, no immunoreactivity corresponding to the $\alpha$2 isoform was detected in any preimplantation stage (73 embryos examined in total; results not shown). When embryos ($n = 112$) were immunostained with $\alpha$3 antibodies, cytoplasmic and nuclear immunoreactivity was

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```
ß2

515   AGAAGAATGATGTCTGCCGTCCAGGGCGATATTATGAGCAACCTGATAATGGGGTTCTGA 574
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1     AGAAGAATGATGTCTGCCGTCCAGGGCGATATTATGAGCAACCTGATAATGGGGTTCTGA 60

575   ACTACCCAAAACGTGCCTGCCAGTTCAACCGGACCCAACTGGGCGATTGCTCTGGCATTG 634
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
50    ACTACCCAAAACGTGCCTGCCAGTTCAACCGGACCCAACTGGGCGATTGCTCTGGCATTG 120

635   GGGACCCTACCCACTATGGTTACAGCACCGGGCAGCCCTGTGTCTTCATCAAAATGAATC 694
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
121   GGGACCCTACCCACTATGGTTACAGCACCGGGCAGCCCTGTGTCTTCATCAAAATGAATC 180

695   GGGTCATCAACTTCTATGCAGGGGCAAACCAGAGCATGAATGTCACTTGTGTTGGCAAG. 753
      ||||||||||||||  |||||||||||||||||||||||||||||||||||||||||||
181   GGGTCATCAACTTCCTTGCAGGGGCAAACCAGAGCATGAATGTCACTTGTGTTGGCAAGA 240

754   GAGATGAAGATGCTGAGAACCTTGGCCACTTTGTCATGTTCCCTGCTAATGGCAGCATTG 813
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241   GAGATGAAGATGCTGAGAACCTTGGCCACTTTGTCATGTTCCCTGCTAATGGCAGCATTG 300

814   ATCTGATGTACTTTCCCTACTATGGCAAAAAGTTCCAT 851
      ||||||||||||||||||||||||||||||||||||||
301   ATCTGATGTACTTTCCCTACTATGGCAAAAAGTTCCAT 338


ß3

846   GGTCAAATTTGACTCTGGTCTTAACAAGAAAGAAGTAACAGTTGAGTGCCATATTGCTGG 905
      |||||||||||||||||||||||||||||||||||||||||||||||||||||||| |||
1     GGTCAAATTTGACTCTGGTCTTAACAAGAAAGAAGTAACAGTTGAGTGCCATATTGNTGG 60

906   AACCAGGAACCTAAAAAACAAGAATGAGCGTGACAAGTTCTTGGGACGTGTTTCGTTCAA 965
      ||||||||||||||||||| || |||||| ||||||||| ||||||||||||||||||||
61    AACCAGGAACCTAAAAAACNAGNATGAGCGNGACAAGTTCNTGGGACGTGTTTCGTTCAA 120

966   AGTCACAGCACGAGCCTAGGAATAGGATGTCTCCACAGAATTCATGTTGTGTTGTCGCCA 1025
      |||||||||||||||||||||||||| |||||||||||||||||||||||||||||||||
121   AGTCACAGCACGAGCCTAGGAATAGGANGTCTCCACAGAATTCATGTTGTGTTGTCGCCA 180

1026  TTTTGTATCAGCTGGACCTGCCATTCTAGGATTATGAGGCCACCTTGGAGGAGGAAGTGG 1085
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
181   TTTTGTATCAGCTGGACCTGCCATTCTAGGATTATGAGGCCACCTTGGAGGAGGAAGTGG 240

1086  TGTGGTACACACTTGGGTGACATCATAACATGCTTCCAGATCATAGTGTTCAGTGTCCTC 1145
      ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
241   TGTGGTACACACTTGGGTGACATCATAACATGCTTCCAGATCATAGTGTTCAGTGTCCTC 300

1146  TGAAGTAACTGCCTGCTGCCT.CTGCTGCCCTTGAACCCATGTACGGTCGCCAGACAGG  1203
      |||||||||||||||||||||  |||||||||||||||||||||| |||||||||||||
301   TGAAGTAACTGCCTGCTGCCTGGTGCTGCCCTTGAACCCATGTACAGTCGCCAGACAGG  359
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**FIG. 2**—*Contined*

seen in all preimplantation stages (Fig. 6) but $\alpha 3$ subunits were not detected in plasma membranes, including those of the trophectoderm. This is in contrast to cow blastocysts, in which the Caplan $\alpha 3$ antibody under the same fixation conditions generated apical membrane staining in trophectoderm (Betts *et al.,* 1998). Likewise, no membrane staining was detected with either of the $\beta 2$ or $\beta 3$ antibodies used, although cytoplasmic immunoreactivity was apparent in each case ($n = 90$ for $\beta 2$, 55 for $\beta 3$; Fig. 6). As also shown in Fig. 6, neither of the two secondary antibodies used in these latter experiments caused noticeable background fluorescence.

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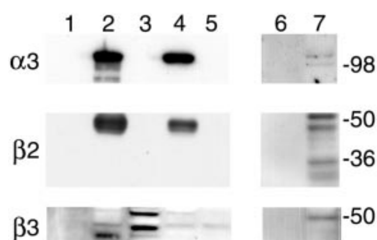


**FIG. 3.** Detection of $Na^+$,$K^+$-ATPase $\alpha 3$, $\beta 2$, and $\beta 3$ subunits in preimplantation embryos by immunoblotting. Thirty micrograms of total protein from mouse kidney (lane 1), mouse brain (lane 2), rat liver (lane 3), rat brain (lane 4), and rat heart (lane 5) and total protein from a mixture of 1260 morulae and blastocysts (lane 7) was separated by polyacrylamide gel electrophoresis in 4–12% gradient gels. Lane 6 contains the last wash from which the embryos were taken. Positions of molecular size markers (kDa) are shown along the right margins. The blots were probed with the MA3 915, SM-GP50, and Levenson $\beta 3$ antibodies.

## DISCUSSION

Our results demonstrate that multiple subunit isoforms and hence, potentially, multiple isozymes of $Na^+$,$K^+$-ATPase are present in preimplantation mouse embryos. The genes encoding the $\alpha 1$, $\alpha 3$, and $\beta 1$ isoforms are transcribed embryonically as indicated by the fact that their mRNAs accumulate during blastocyst development. Transcripts encoding the $\beta 2$ and $\beta 3$ isoforms are also present in preimplantation embryos, but do not accumulate, suggesting either that they are strictly oogenetic in origin (as is the $\alpha 2$ transcript in oocytes) or that embryonic mRNA accumulation is balanced by mRNA turnover. Previous immunoblot analyses had identified both $\alpha 1$ and $\beta 1$ isoforms in preimplantation mouse embryos and demonstrated that both increase in abundance as cavitation proceeds (Gardiner *et al.,* 1990b). Here we show that $\alpha 3$, $\beta 2$, and $\beta 3$ isoforms are present as well. Whereas $\alpha 1$ and $\beta 1$ isoforms reside in the trophectodermal cell surfaces facing the blastocoel, where the $\alpha 1\beta 1$ isozyme would drive trans-epithelial $Na^+$ and fluid transport, the other three isoforms were not detected in plasma membranes. Although it remains possible that low levels of plasma membrane immunoreactivity for $\alpha 3$, $\beta 2$, and $\beta 3$ subunits escaped our detection, these would be quantitatively insignificant in relation to $\alpha 1$ and $\beta 1$ subunits. Thus it is most likely that, in the mouse, the $\alpha 1\beta 1$ isozyme plays the major role in the process of cavitation. This conclusion is supported by the fact that the mouse $\alpha 1$ isoform, unlike the other $\alpha$ isoforms, has a low affinity for ouabain (Blanco and Mercer, 1998) and our earlier observation that cavitation in the mouse is relatively insensitive to this drug (Betts *et al.,* 1997). The absence of an essential role for embryonically expressed $\alpha 2$ subunits in preimplantation

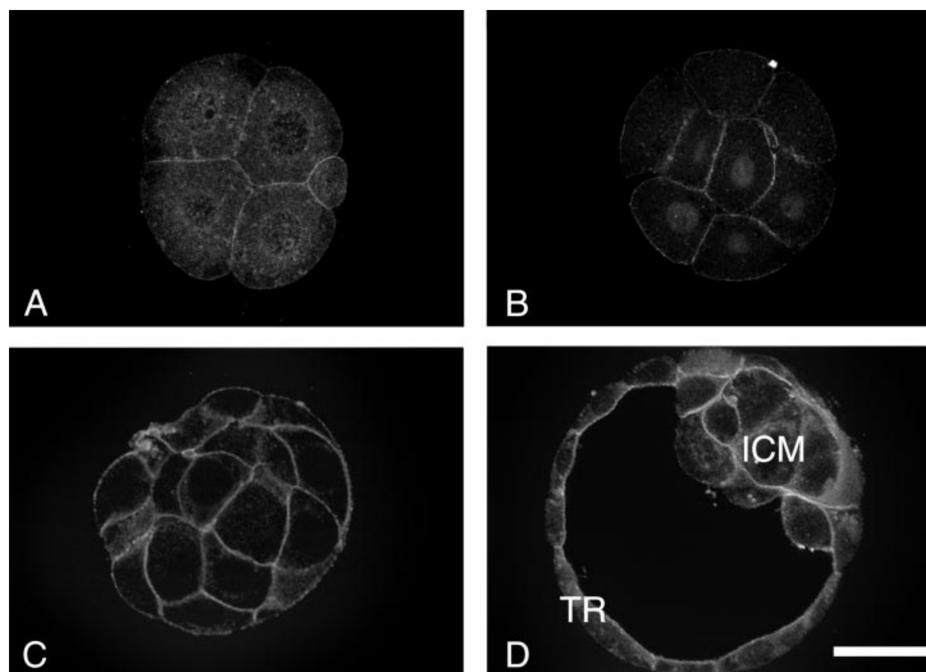


**FIG. 4.** Localization of $Na^+$,$K^+$-ATPase $\alpha 1$ subunit isoform in preimplantation embryos by confocal immunofluorescence microscopy. Stages shown are (A) 4-cell, (B) 8-cell, (C) morula, and (D) blastocyst. The embryos were immunostained with the mAb 6H antibody. Nuclear staining was an artifact of the secondary antibody that was especially prominent in 8-cell embryos. The scale bar indicates 25 $\mu$m.

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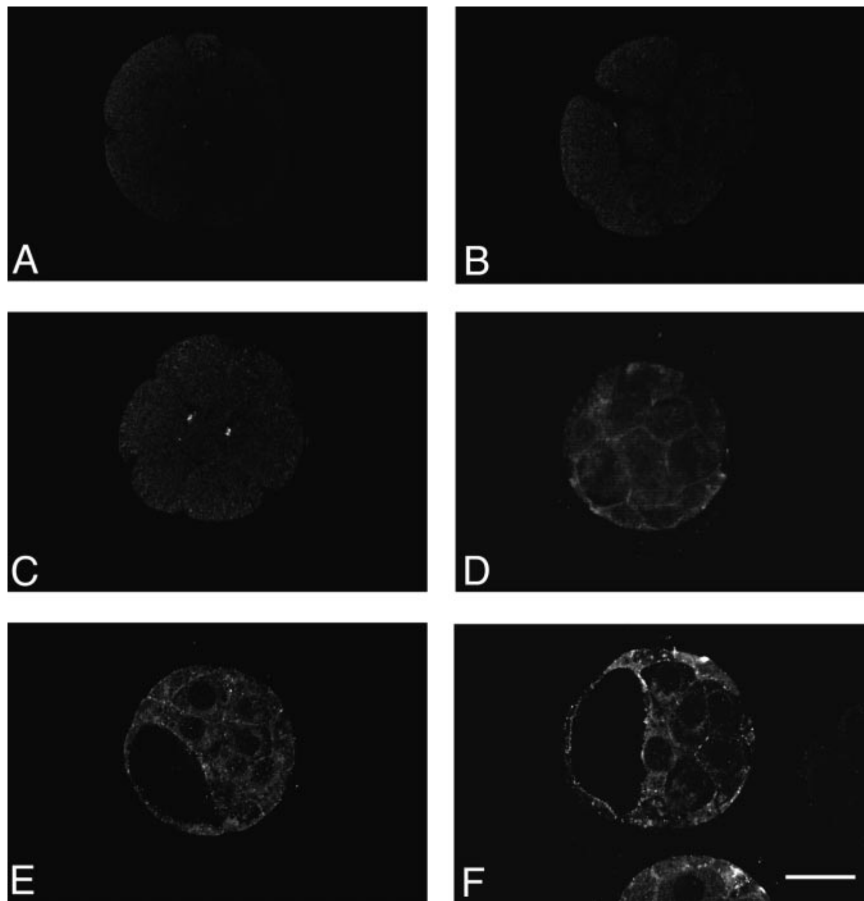


**FIG. 5.** Localization of $Na^+$,$K^+$-ATPase $\beta1$ subunit isoform in preimplantation embryos by confocal immunofluorescence microscopy. Stages shown are (A) 4-cell, (B) 6- to 8-cell, (C) compacting 8-cell, (D) morula, and (E and F) blastocyst. The embryos were immunostained with the mAb 8a antibody. The scale bar indicates 30 $\mu$m.

development was confirmed recently with the generation of offspring lacking this subunit; the homozygous mutant embryos established pregnancies and survived to term (James *et al.,* 1999). In the same report, it was explained that offspring homozygous for a null mutation in the gene encoding the $\alpha1$ subunit did not survive to term, but the time of embryo loss was not determined.

The results presented here for the $\alpha1$ and $\beta1$ isoform distributions differ from what we had published previously (Watson and Kidder, 1988; MacPhee *et al.,* 1998). In the earlier studies, sections were cut from wax-embedded embryos and immunostained for $\alpha1$ and $\beta1$ isoforms. Immunoreactivity for both isoforms in blastocysts was localized to the juxtacoelic surface of the trophectoderm including extensions covering the ICM and was not noted between ICM cells or between ICM and trophectoderm cells. In the present study, $\alpha1$ and $\beta1$ isoform immunoreactivity was confirmed in basolateral membranes of mural trophectoderm, but in addition, it was detected in ICM membranes and basolateral membranes of polar trophectoderm (including the interface between ICM and trophectoderm). We believe this difference arises from the greater sensitivity of immunodetection in whole-mount preparations and reflects the lower abundance of $Na^+$,$K^+$-ATPase in membranes of the ICM. Our present finding with respect to the distribution of the $\alpha1$ subunit is in agreement with that published by Becker and Davies (1995) and with the mRNA distribution as revealed by *in situ* hybridization (MacPhee *et al.,* 1994).

Our failure to detect the $\alpha3$ isoform in plasma membranes differs from our previous study of cow blastocysts in which the $\alpha3$ isoform was localized to the apical surfaces of trophectoderm cells (Betts *et al.,* 1998). We can offer two possible explanations for this difference, neither of which can be ruled out by available evidence. On the one hand it could be that the difference reflects differing physiological requirements of cow and mouse blastocysts, the former being a maximally expanding blastocyst and the latter expanding minimally. For example, apically localized $\alpha3\beta1$ isozymes in the cow might serve to moderate the rate of

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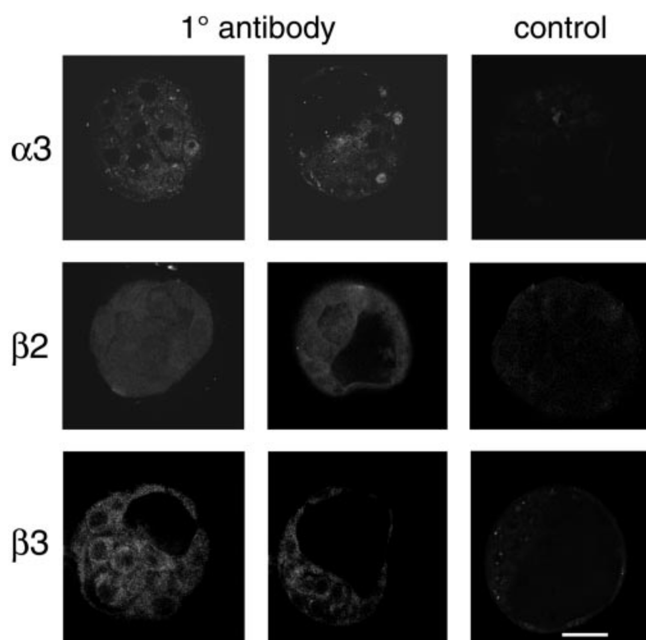


**FIG. 6.** Localization of $Na^+$,$K^+$-ATPase α3, β2, and β3 subunit isoforms in morulae and blastocysts by confocal immunofluorescence microscopy. The embryos were immunostained with the Caplan α3, SM-GP50, and RNT β3 antibodies, respectively. The third image in each row shows a negative control: secondary antibody alone for α3 and β2, preimmune serum for β3. The scale bar indicates 30 μm.

blastocoel expansion, working against basolaterally localized α1β1 isozymes when the intracellular $Na^+$ concentration reaches a certain threshold (Betts *et al.,* 1998); the α3 isoform's lower $K_m$ for $Na^+$ is consistent with this idea (Shyjan *et al.,* 1990a). On the other hand, there is the possibility that deployment of α3 subunits to apical membranes in the cow is part of a physiological response to suboptimal culture conditions, since sodium pump subunit isoform expression has not been studied in freshly isolated cow blastocysts.

Although we can be fairly certain that α1β1 isozymes drive cavitation in the mouse, what of the other isozymes that presumably assemble from the α3, β2, and β3 subunits that are present? It has become clear recently that the different isozymes of $Na^+$,$K^+$-ATPase have unique properties in addition to being differentially distributed (reviewed by Blanco and Mercer, 1998). Expression of different isozymes in HeLa or insect cells has allowed investigators to characterize their individual properties and to compare them directly in the same cellular milieu (Jewell and Lingrel, 1991; Blanco *et al.,* 1995a,b). These studies have demonstrated that the choice of β subunit does not greatly influence the kinetic properties of the enzyme, but can have an effect on $Na^+$ affinity. Changing the α subunit, however, can significantly alter the affinity for $Na^+$, $K^+$, and ATP as well as the enzyme's sensitivity to ouabain. The susceptibility of different sodium pump isozymes to regulation by protein kinases also depends on the α subunit. Modulation of sodium pump activity by activators of protein kinase A or protein kinase C (PKC) has been demonstrated in a variety of tissues and cell culture systems, and at least one amino acid residue phosphorylated by each kinase *in vivo* has been identified in the α subunits (Béguin *et al.,* 1994; Feschenko and Sweadner, 1995). The presence of these individual phosphorylation sites differs among the α subunit isoforms, suggesting that they might be differentially susceptible to kinase regulation. Indeed, the α1 but not the α2 or α3 isoform has been shown to be a good substrate for PKC (Béguin *et al.,* 1996). This fact is of interest in the present context because cavitation during mouse preimplantation development can be accelerated by a rise in intracellular free $Ca^{2+}$, possibly acting through stimulation of phospholipase C, the generation of diacylglycerol, and activation of PKC (Stachecki and Armant, 1996). These considerations cast further doubt on a role for α3 subunits in mouse cavitation. The recent demonstration of α3 subunits in mitochondria (but not plasma membranes) of rat renal tubules (Chang *et al.,* 1998) hints of a novel function for this isoform that would be consistent with its cytoplasmic distribution in preimplantation embryos. The function of α3 subunits in mitochondria is yet to be elucidated. Other investigators have proposed a role for cytoplasmically localized α subunits in protein export (Florkiewicz *et al.,* 1998).

Despite the fact that they do not appear to exert a major influence on the catalytic properties of $Na^+$,$K^+$-ATPase isozymes, β subunit isoforms do differ functionally. For example, the β2 subunit also serves as a neural recognition molecule mediating interactions between CNS neurons and glial cells, and this function cannot be substituted by β1 subunits (Müller-Husmann *et al.,* 1993; Weber *et al.,* 1998). However, an essential role for the β2 isoform in preimplantation development, either as a subunit of the sodium pump or as a mediator of cell recognition, can be ruled out because β2 knockout mice suffer no ill effects until after birth (they die as juveniles due to neurological defects; Magyar *et al.,* 1994). If β2 subunits do have a role in blastocyst development it is a redundant one and does not require deployment in plasma membranes. Likewise, a role for the β3 isoform in blastocysts, if indeed there is an essential role, must be sought in the cytoplasm. Further characterization of the functional, as opposed to enzymatic, roles of these subunit isoforms must be carried out before their significance for preimplantation development can be appreciated.

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