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# Expression Of Sodium Pump Subunit Genes During Mouse Preimplantation Development

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# EXPRESSION OF SODIUM PUMP SUBUNIT GENES
# DURING MOUSE PREIMPLANTATION DEVELOPMENT

by

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# ABSTRACT

The Na,K-ATPase or sodium pump is an enzyme that is found in most animal cells The enzyme pumps sodium and potassium ions against their concentration gradients and is hypothesized to be a mediator of fluid accumulation during mouse preimplantation development The purpose of the present study was to determine, using reverse transcriptase-polymerase chain re ction (RT-PCR), whether all $\alpha$ ($\alpha$1, $\alpha$2, $\alpha$3, $\alpha$4) and $\beta$ ($\beta$1 and $\beta$2) isoforms of the sodium pump were expressed during preimplantation development Previous work by Watson and Kidder (1988) had shown that the $\alpha$ subunit was localized to the basolateral domains of mural trophectoderm surrounding the blastocoel The spatial localization of $\alpha$1 transcripts in mouse morulae and blastocysts was examined with the development of a non-radioactive wholemount *in situ* hybridization procedure and the use of a laser scanning confocal microscope

$\alpha$3 and $\beta$2 isoform transcripts were detected throughout mouse preimplantation stages $\alpha$2 transcripts were only detected in oocytes while $\alpha$4 transcripts were not detected in any embryo stages None of the $\alpha$3, $\alpha$2, or $\beta$2 isoform subunits were detected, by immunocytochemistry, in membranes of preimplantation stages $\alpha$3 subunits were detected in morulae/blastocyst total protein on a western blot

$\alpha$1 subunits were found in membranes of all preimplantation stages while $\beta$1 subunits were only detectable from the morulae stage onwards $\alpha$1 transcripts were detected throughout the cytoplasm of mouse morulae and blastocysts correlating with the presence of $\alpha$1 subunits in membranes These results provide evidence that $\alpha$1 and $\beta$1 subunits are the most probable subunit isoforms of the sodium pump, to mediate fluid accumulation in mouse morulae and blastocysts

Using antisense oligodeoxynucleotides (ODNs), $\beta$1 subunit mRNA was targeted in the mouse preimplantation embryo to help determine the role of the $\beta$1 subunit during cavitation The number of cavitating embryos were significantly decreased ($p < 0.10$) compared to controls, but future experiments will require a better understanding of ODN

uptake characteristics in mouse embryos in order to increase the observed effects The results do suggest that the ß1 subunit could be a trigger for cavitation to occur

Sodium pump gene expression was also examined in another mammalian species, the horse. to provide further evidence that the enzyme is a mediator of mammalian blastocyst development $\alpha 1$ and ß1 transcripts were detected between day 12 and 28 of horse development, a period of maximal blastocoel expansion The two corresponding subunits were also detected, by immunocytochemistry, in trophoblast of day 16 conceptuses The presence of the two subunits corresponds to the enzyme's potential role of pumping sodium ions into the developing blastocoel, creating osmotic pressure, resulting in the movement of fluid into the blastocoel

Key Words Na,K-ATPase, mouse, preimplantation, development, horse, gene expression, subunits, mRNA

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# LIST OF ABBREVIATIONS

AMOG= adhesion molecule on glia

Amp= ampicillin

AMRE= adhesion molecule on glia regulatory element

ANOVA= analysis of variance

ARE= $\alpha 1$ regulatory element

ATP= adenosine triphosphate

bp= base pairs

BSA= bovine serum albumin

cAMP= cyclic adenosine monophosphate

cDNA= complementary deoxyribonucleic acid

CIAP= calf intestinal alkaline phosphatase

CTP= cytidine triphosphate

Da= daltons

DEPC= diethylpyrocarbonate

DGD= diethylene glycol distearate

DIG= digoxigenin

DTT= dithiothreitol

EDTA= ethylenediamine tetraacetic acid

EGF-R= epidermal growth factor-receptor

FCS= fetal calf serum

GCG= genetics computer group

GTP= guanosine triphosphate

ICM= inner cell mass

IGF-II= insulin-like growth factor- II

kb= kilobase

LB= Luria-Bertani

MDCK= Madin Darby Canine Kidney

MOPS= 4-morpholinepropanesulfonic acid

mRNA= messenger ribonucleic acid

nt(s)= nucleotide(s)

ODNs= oligodeoxynucleotides

PAGE= polyacrylamide gel electrophoresis

PBS= phosphate buffered saline

PCR= polymerase chain reaction

PEG= polyethylene glycol

PKA= protein kinase A

PKC= protein kinase C

PMA= phorbol 12-myristate 13-acetate

post-hCG= post-human chorionic gonadotrophin

PVP= polyvinylpyrrolidone

RNaseA= ribonuclease A

SDS= sodium dodecyl sulphate

SSC= tri-sodium citrate

T3= triiodothyronine

TBS= tris buffered saline

TTP= thymidine triphosphate

UTP= uridine triphosphate

# CHAPTER 1

# GENERAL OVERVIEW

## 1.1 INTRODUCTION

Na,K-ATPase is an enzyme, found in membranes of most animal cells, that functions to pump sodium and potassium ions against their electrochemical gradients utilizing the energy from ATP hydrolysis The Na K-ATPase is electrogenic, pumping three Na ions out into the extracellular environment for every two K ions taken in intracellularly Turnover rate approximates $10^2$ ions/sec compared to channels which approximate $10^{6-8}$ ions/sec (Lauger, 1991)

The imbalance of transported charges leads to a pump generated current and forms the basis for electrophysiological characterization of the sodium pump Due to the enzyme's electrogenic nature, transport activity must depend on membrane potential In addition, each step in the reaction cycle that involves charge movements could depend on membrane potential and could contribute to the regulation of pump turnover (Vasilets and Schwarz, 1994)

The sodium pump plays a large role in a number of physiological processes regulation of cell volume, free calcium concentration, membrane potential, ion/solute uptake (Mercer, 1993, Rose and Valdes, 1994) In electrically active tissues or tissues that are involved in salt transport, up to 70% of the cell's total energy requirement may be used by the sodium pump (Mercer, 1993)

The reaction scheme for the Na,K-ATPase can be described by the Post-Albers cycle (below) (Albers, 1967, Post et al, 1972) The model assumes two conformations for the sodium pump during the reaction cycle, E1 and E2, with inward facing (E1) binding sites for Na and outward facing (E2) binding sites for K E1 binds preferably to Na and/or ATP while E2 binds K and/or inorganic phosphate (Pi) When ions bind to the appropriate conformation they become occluded

$(Na)_3 E_1\text{-}P$

$Na_3\text{-}E_1\text{-}ATP$          $P\text{-}E_2\text{-}Na_3$

$3\ Na^+\ cyt$          $3\ Na^+\ ext$

$E_1\text{-}ATP$          $P\text{-}E_2$

$2\ K^+\ cyt$          $2\ K^+\ ext$

$K_2\text{-}E_1\text{-}ATP$          $P\text{-}E_2\text{-}K_2$

$ATP\text{-}E_2(K_2)$     $E_2(K_2)$
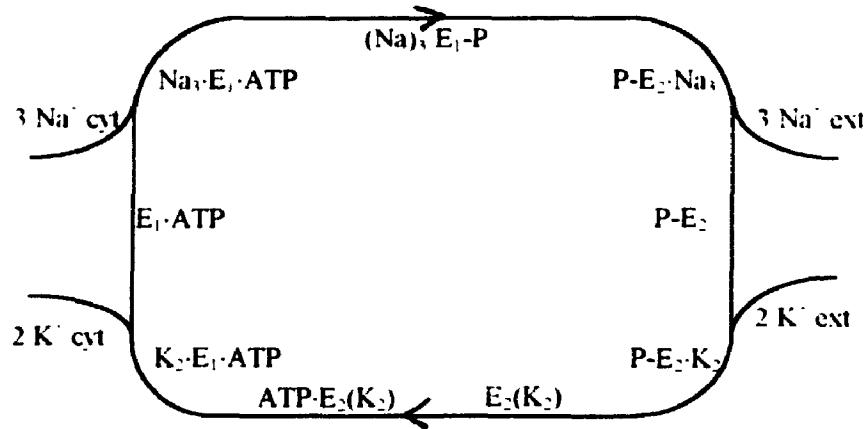
The Post-Albers model is a consecutive mechanism where one substrate (Na ) is translocated in one part of the cycle and the second substrate (K⁺) is translocated in the next part of the cycle This scheme can account for many, but not all observed properties of the Na,K-ATPase (Lauger, 1991) Transition between conformational states involves an intermediate state where ions are bound and occluded inside the protein A characteristic feature of the model is the change in binding affinities over the course of the reaction cycle The E1 conformation binding sites prefer sodium over potassium while in the E2 conformation, they prefer potassium over sodium The apparent affinities for sodium and potassium change 1000 fold and 50 fold, respectively, in the E1/E2 reaction scheme (Lauger, 1991)

## 1.2 Na,K-ATPASE STRUCTURE

The Na,K-ATPase is an oligomeric protein generally understood to consist of two subunits $\alpha$ and $\beta$ (for reviews see Lingrel, 1992, Mercer, 1993) Both $\alpha$ and $\beta$ peptides are cotranslationally inserted into the endoplasmic reticulum where it is likely that posttranslational modifications occur before the subunits move to the membranes of the preimplantation embryo (Geering 1990, McDonough et l, 1990, Watson and Kidder, 1988, Kidder and Watson, 1990) Recently, Mercer et al (1993) found a putative γ subunit as well α and β subunits exist in multiple isozymic forms α1, α2, α 3, α4, β1, β2 (Kent et al., 1987, Malo et al., 1990, Shamraj and Lingrel, 1994) which are differentially distributed among adult rat tissues and expressed at different times during mouse development (Shull et al, 1986, Martin-Vasallo et al, 1989, Watson et

al, 1990a. Herrera et al 1994; Betts et al, 1996, in press) (see Chapter 3) reflecting the fact that both are encoded by families of related genes The Na,K-ATPase is a P-type ATPase because an unique characteristic of the enzyme is that a transient, phosphorylated aspartyl residue (Asp 372) is formed during the catalytic cycle (Vasilets and Schwarz, 1994) The $\alpha$-subunit is responsible for the physiological role of the enzyme (Jorgensen, 1986), while the $\beta$-subunit may facilitate the processing and insertion of the $\alpha$-subunit into the plasma membrane (Fambrough, 1988, Geering, 1991a,b, Ueno et al 1995), although increasing evidence suggests a role in K binding and translocation as well (Jaisser et al, 1992, Lutsenko and Kaplan, 1992)

The $\alpha$ subunit is a catalytic protein of about 1000 amino acids and approximately 100-115 kD, containing binding sites for ATP and cardiac glycosides In rodents the $\alpha$1 subunit is resistant to ouabain, a cardiac glycoside and specific inhibitor of the sodium pump, while $\alpha$2 and $\alpha$3 isoforms are ouabain sensitive (Vasilets and Schwarz, 1994) The $\alpha$-subunit may have six to ten transmembrane segments and a large cytoplasmic loop (Geering, 1990b, Herrera et al, 1987, Rossier et al, 1987, Shull et al 1986) The number of membrane spanning segments is still unclear

The sodium pump belongs to the E1E2 (see above) ATPase family including the $Ca^{2+}$- ATPase and H/K-ATPase When the enzyme is an E1 form, the amino terminus of the $\alpha$-subunit can be rapidly cleaved by trypsin, altering equilibrium between E1 and E2 conformations The tryptic site (K, 20) is within a conserved lysine rich region and Shull et al (1986) have proposed that this region may be involved in the conformational shift that occurs during cation occlusion and may function as a movable ion-selective gate

The E1E2 ATPase family shares sequence homology and a potentially common mechanism of cation transport (Fambrough et al, 1994) Hydropathy plots of the sodium pump and the calcium pump are quite similar and with the discovery of an epitope in a hydrophilic region of the $Ca^2$-ATPase SERCA1 on the lumenal side of the sarcoplasmic reticular membrane (Matthews et al, 1990, Clarke et al, 1990), there is a potential for restrictions on the enzymes' membrane topology Based on the localization of the epitope, ten membrane spanning regions may be more plausible for the ATPase family Further support for the ten membrane spanning rationale is the identification of a putative

cytoplasmic phosphorylation site for protein kinase A (PKA) in the α-subunit of the sodium pump, that could only be localized cytoplasmically if present in a ten membrane spanning subunit (Chibahn et al, 1992)

The cytoplasmic loop of the α-subunit contains a phosphorylation site and an ATP binding site The N-terminus of α has a cytoplasmic domain (Jorgensen and Collins, 1986) while the C-terminus' position is still unclear Ovchinnikov et al (1988) suggested that the C-terminus is extracelluar while similarities of the sodium pump α-subunit to the $Ca^2$ ATPase and epitope mapping suggest a cytoplasmic domain (Antolovic et al, 1991, Shull and Greeb, 1988)

The various α-isoforms show sequence similarity Each rat α isoform's amino acid sequence differs from the others by approximately 14%, while each α-subunit compared between human and other mammalian species has about 98% identity (Shull et al, 1986, Mercer, 1993) The degree of amino acid identity of each α-subunit isoform across species is greater than 92% Most amino acid divergence among isoforms occurs in the amino terminus, the loop between the first and second membrane spanning domains and the cytoplasmic region from amino acids 403-503 The greatest similarities occur in the phosphorylation site, major hydrophobic regions, and in the cytoplasmic region amino acids 589-785 (Shull et al, 1986, Mercer, 1993) Pairwise nucleotide sequence comparisons of the protein coding regions of the α1, α2, and α3 isoform cDNAs found 77%-79% identity and no extended regions of similarity in the untranslated regions (Shull et al., 1986)

The β1-subunit is an approximately 305 amino acid, 35-55 kD glycosylated protein (McDonough et al, 1990) It, and possibly all P-type β-subunits, has one transmembrane segment (from amino acids 35-62), a short cytoplasmic tail, and a large extracellular domain In fact, most of the subunit appears to be extramembranous and extracellular (McDonough et al, 1990) The β-subunit's N-terminus is in the cytoplasmic domain while the C-terminus is in the extracellular domain The C-terminal domain of β1 contains three or four glycosylation sites and three disulphide bridges at amino acids 125-148, 158-172, and 212-275 (Geering, 1990, Mercer, 1993) Mammalian β1-subunits have about 95% amino acid sequence identity while in non-mammalian species, amino acid

sequences are approximately 60% identical  Hydropathy profiles and predicted secondary structure analysis indicate that any amino acid substitutions that have occurred in the different ß1 subunits, do not greatly change the structure of the protein (Mercer, 1993)

The ß2 subunit of the mouse has 42% amino acid sequence identity with mouse ß1 (Schmalzing and Gloor, 1994)  The subunit is made up of 290 amino acid residues and has seven potential glycosylation sites, three of which may be conserved relative to the three N-linked glycosylation sites in ß1 (Martin-Vasallo *et al*, 1989)  These sites can be found at amino acid positions 159, 193 and 250 of ß2 and at the corresponding positions of 158, 193 and 266 of the rat ß1  Hydropathy profile suggests that the ß2-subunit contains a charged cytoplasmic amino terminus, a single hydrophobic domain located between residues 40 and 67 and a large extracellular carboxy terminus (Martin-Vasallo *et al*, 1989)  The predicted secondary structures of the rat ß1 and ß2 subunits appear to be virtually identical  A ß3 subunit is also known to exist but it appears to be restricted to the nervous system of *Xenopus laevis* (Good *et al* 1990)

The ß2 isoform of mouse brain was originally identified as adhesion molecule on glia (AMOG), involved in astrocyte-neuron adhesion (Antonicek *et al*, 1987)  Expression of the AMOG in *Xenopus* oocytes showed that it could associate with α1-subunits to form catalytically active sodium pumps (Antonicek *et al*, 1987)  Cloning and sequence analysis indicated it was an isoform of the ß subunit of the sodium pump (Gloor *et al*, 1990)

A recently characterized, putative γ subunit (Mercer *et al*, 1993) is a poorly understood component of the enzyme  It is a hydrophobic peptide of about 10 kD (Mercer et al, 1993) and peptide sequencing has established that it is not a breakdown product of α or ß (Collins and Lesznk, 1987)  There are no sites for N-linked glycosylation and hydropathy plots suggest a cytoplasmic N-terminus, a single membrane spanning region and a highly charged extracellular domain  Its function or importance is yet to be determined

## 1.3 GENE AND SUBUNIT EXPRESSION

Rat α1, α2, and α3 subunit cDNAs were isolated by Shull *et al* (1986) and by Herrera *et al* (1987) by screening rat brain, kidney and liver cDNA libraries with a sheep

kidney Na,K-ATPase α-subunit hybridization probe (Shull *et al.*, 1985) or a previously characterized rat brain sodium pump cDNA (Schneider *et al*, 1985) The kidney cDNAs exhibited a single restriction pattern (α1) whereas the brain cDNAs had three distinct patterns representing α1, α2, and α3isoforms The various cDNAs were sequenced and their amino acid sequence deduced

Shull *et al* (1986) characterized the various forms from brain and kidney The brain and kidney α1 forms are nearly identical except for several nucleotide differences, however, the differences do not cause amino acid subsititutions The α1 forms had a 237 nucleotide (nt) 5'-untranslated sequence 1023 codon open reading frame and 328 nts of 3' untranslated sequence It was deduced that the mature protein consists of 1018 amino acids with a molecular weight of 112, 573 Daltons The brain α2 cDNA isolate contained a 96 nt 5'-untranslated region, a 1020 codon open reading frame and 1851 nts of 3'-untranslated sequence There are 3 polyadenylation sites at 3293, 4853, and 5085 nts The mature α2 protein consists of 1015 amino acids and has a molecular weight of 111, 736 Daltons The α3 cDNA isolate has a 141 nt 5'-untranslated sequence, a 1013 codon open reading frame and a 378 nt 3'-untranslated sequence The primary translation product has 1013 amino acids and a deduced molecular weight of 111,727 Daltons

Shull *et al* (1986) found that the general organization of the three rat isoforms is identical with that of the sheep (Shull *et al.*, 1985), pig (Ovchinnikov *et al*, 1986), and electric ray (*Torpedo californica*) enzymes (Kawakami *et al.*, 1985) The location of hydrophobic domains, phosphorylation site, and a potential phosphorylation site for a cAMP-dependent protein kinase are very similar or identical among isoforms

Herrera *et al* (1987) used the cDNA clones to make probes and investigate the presence of the various α-isoform mRNAs in a variety tissues The α1-isoform mRNA (~4 5 kb) was detected in adult and fetal brain, liver, heart, skeletal muscle and uterus The α2-isoform mRNA (~4 5 kb) was detected in brain and fetal heart The α3 probe detected two mRNA species (~4 5 kb and ~6 kb) with the larger species being detected in fetal and adult brain, heart, and skeletal muscle The smaller species was detected in fetal and adult kidney, brain, heart and in adult skeletal muscle and uterus The authors, however, could

not completely rule out the possibility that the probes did not hybridize specifically to the appropriate isoforms

Gick *et al* (1993) used the same α-isoform cDNAs, described above, to probe northern blots of total rat RNA Their results were similar with the exception that α2 mRNA was detected in skeletal muscle, as well as heart and brain Furthermore, two mRNA species for α2 and one for α3 were detected with the cDNA probes, unlike the Northern blot analysis of Herrera *et al* (1987) The authors speculated that differences might be attributable to different hybridization and wash conditions

The rat α4 cDNA isoform was isolated (Shamraj and Lingrel, 1994) by screening a rat testis cDNA library with the human α4 279 bp cDNA fragment The cDNA was found to consist of 3446 nts which contains a 3087 bp open reading frame and a primary translation product of 1028 amino acids and a calculated molecular weight of 113,873 Daltons The open reading frame was found to be preceded by 89 nts of 5'-untranslated sequence and 270 nts of 3'untranslated sequence followed by a poly(A) stretch The rat α4 isoform had 78%, 78%, 76% amino acid identity with the rat α1, α2, α3 isoforms The proposed transmembrane regions of the four isoforms varied between 79%-100% in amino acid identity and the α4 N-terminus exhibited considerable dissimilarity from the other isoforms Using a rat specific α4 probe, Shamraj and Lingrel (1994) probed a rat multiple tissue northern blot of poly (A) RNA The α4 isoform 3 9 kb mRNA was detected in testis and at a 250 fold lower level in skeletal muscle

A β1 cDNA was originally isolated from a λgt11 cDNA library constructed from neonatal rat brain (Mercer *et al*, 1986) An initiation codon was assigned to the first ATG downstream of an in-frame termination codon and an open reading frame commencing with this codon continued to position 912 followed by 231 base pairs of 3'-untranslated sequence including a polyadenylation signal (AATAAA) The deduced amino acid sequence was virtually identical with a human Na,K-ATPase β-subunit (Kawakami *et al*, 1986) The cDNA was used to probe total RNA from rat tissues and several mRNA species were discovered (Mercer *et al* 1986) a 3 0, 2 8, 2 6 and 2 4 kb were found in rat brain, bladder, kidney, lung, testis and heart It was not indicated whether the different mRNAs were the result of alternative splicing of a single mRNA species, different

isoforms or differential polyadenylation  The 3 0 kb species predominates in kidney, whereas the 2 4 kb species predominates in brain  Martin-Vasallo *et al* (1989) found similar results and also extended their analysis to include 2-day old and 2-week old rats as well as adults

The ß2 cDNA was originally isolated from a human fetal liver λgt11 cDNA library (Martin-Vasallo *et al.*, 1989) by screening with a ß1 1 2 kb *EcoRI* cut cDNA fragment originally isolated by Mercer *et al* (1986)  The cDNA of one of the clones isolated was used to screen for the rat ß2 cDNA in a neonatal rat brain λgt11 library  Clones were isolated, purified and sequenced  The rat cDNA initiation codon was assigned to the first ATG triplet downstream of the in-frame termination codon (TGA)  The cDNA had a 461 nucleotide 5'-untranslated region, an 870 nucleotide open reading frame and a 432 nucleotide 3'-untranslated sequence  The open reading frame encoded a 290 amino acid polypeptide with a deduced molecular weight of 33,412 Da (Martin-Vasallo *et al*, 1989) The nucleotide sequence of the coding region of rat ß2 cDNA exhibited 53% and 55% homology with the corresponding rat and *Torpedo californica* ß1 cDNA (Mercer *et al*, 1986, Young *et al*, 1987, Martin-Vasallo *et al*, 1989)  On the other hand, the 3'-untranslated region of rat ß2 showed virtually no sequence similarity with the 3'-untranslated region of rat or *Torpedo* ß1  The protein encoded by the human ß2 clones exhibited 98% identity with the rat ß2 amino acid sequence indicating the two cDNAs isolated encoded almost identical proteins (Martin-Vasallo *et al*, 1989)

Using the rat ß2 cDNA as a probe, northern blots of rat tissue RNA were analyzed to determine tissue specific expression of ß2 mRNA (Martin-Vasallo *et al*, 1989)  A single ß2 mRNA species of ~3 4 kb was detected in neonatal rat brain, and to a lesser extent in kidney, liver, and heart  In adult rats, the ß2 mRNA was extremely abundant in brain and moderate levels were detected in heart and spleen while fewer transcripts were found in kidney, lung, thymus and mammary gland (Martin-Vasallo *et al.*, 1989)

## 1.4 REGULATION OF Na,K-ATPASE ACTIVITY

Hormones such as aldosterone and triiodothyronine (T$_3$) are documented regulators of the sodium pump in a variety of mammalian organs (Verrey *et al*, 1989,

Corthesy-Theulaz *et al*, 1991, Ewart and Klip, 1995) Aldosterone is a mineralcorticoid which promotes sodium reabsorption and potassium release across tight epithelia of kidney and salivary glands (Ewart and Klip, 1995) Short term effects appear to be initiated by an increase in intracellular sodium ion concentration, with stimulation independent of protein synthesis (Shahedi *et al*, 1993), followed by a rapid stimulation of sodium pump activity The classical effect of aldosterone on the sodium pump is to increase activity by inducing synthesis of new $\alpha$ and $\beta$ subunits This response presumably involves interaction of aldosterone-receptor complexes with specific hormone responsive elements in the promoters of sodium pump $\alpha$ and $\beta$ subunit genes (Ewart and Klip, 1995) $T_3$ is a major determinant of steady state sodium pump activity in heart, skeletal muscle, liver and kidney (Ismail-Beigi, 1993) $T_3$ stimulates pump activity by increasing the number of pump molecules

$T_3$ was found to cause a dose dependent effect on $\alpha 1$, $\alpha 2$, $\beta 1$, and $\beta 2$ isoform mRNA levels in rat telencephalon cell cultures, increasing some isoforms levels from as low as 1 5 fold to as much as 7 fold (Corthesy-Theulaz *et al*, 1991) The effects on basal transcription rates of each isoform were insignificant although the rate of transcription differed for each isoform These results were contrary to work done by Gick *et al* (1988) who found that there were small but significant increases, following T. administration, in $\alpha$ and $\beta$-subunit transcription rate in rat renal cortex and rat liver nuclei, as assayed by nuclear run on

Phosphorylation and *cis* and *trans*-acting factors are potential regulatory mechanisms Analysis of the rat sodium pump $\alpha 1$ promoter has found an $\alpha 1$ positive regulatory element (ARE) at positions -102 to -61 (transcriptional start site + 1) (Watanabe, *et al*, 1993, Kawakami *et al*, 1994) Using gel retardation analysis and nuclear extracts from MDCK, HeLa and L6 cell lines, at least six different binding factors were found to interact with the ARE and designated C1, C2, C3, ATF1, HEB, and AREB6 (Watanabe *et al*, 1993) Using similar techniques and 5' sequential deletion mutations, Ikeda *et al* (1994) and Kawakami *et al* (1994) analyzed the $\alpha 2$ promoter The region between -175 and -108 was found to be a positive regulatory element In this region, two E-box (CANNTG) and one Sp1 binding consensus sequence was found The

distal E-box sequence acted as a negative regulatory element, while the Sp1 consensus sequence acted positively (Ikeda *et al*, 1994) Kawakami *et al.* (1994) also examined the β2 gene and found a *cis* acting element The sequence of -87 to -79 was identified as a positive regulatory element and named the adhesion molecule on glia regulatory element (AMRE) The other isoforms are currently being investigated for similar elements and factors

Phosphorylation of the enzyme by protein kinase A (PKA) or protein kinase C (PKC) can regulate the enzyme's activity by modulating external cation binding (Vasilets and Schwarz, 1994) A highly conserved seryl residue, Ser [943], on the α1 -subunit is the phosphorylation site for PKA while Thr[15] and Ser[16] are the sites for PKC (Beguin *et al*, 1994, Fisone *et al*, 1994) Phosphorylation of the enzyme *in vitro* and in intact cells with the addition of forskolin, cAMP or phorbol 12-myristate 13-acetate (PMA) to transfected COS cells inhibits enzyme activity (Berterello *et al*, 1991, Beguin *et al*, 1994, Fisone *et al*, 1994) Therefore the sodium pump can be regulated in part by signal transduction pathways that utilize PKA or PKC dependent phosphorylation of the α-subunit Manejwala *et al* (1989) suggested that PKA-mediated phosphorylation could play an intrinsic role in mouse blastocoel expansion Whether the phosphorylation regulates the sodium pump or apical sodium channels or both remains to be determined

Differential regulation of α-isoforms by ionic stimuli has been demonstrated (McDonough *et al*, 1992) In rat hypokalemic skeletal muscle, heart and brain, there was a decrease, in response to low K concentrations, in α2 mRNA and a larger decrease in α2 protein levels The decrease in mRNA levels was inadequate to account for the large decrease in protein, implicating increased degradation of α2-type pumps (McDonough *et al*, 1992) Endogenous levels of α1 and β1 subunits in muscle and heart remained unchanged In a hypokalemic outer medullary collecting duct, the opposite effect was observed, α1 subunits increased 3 2 fold and β1 increased 7 5 fold Rats fed a high salt diet, exhibited a 23% decrease in β1 subunit protein and no significant decrease in α1 protein (McDonough *et al*, 1992) It is thought that a high salt diet increases dopamine production which can act as a paracrine substance to inhibit sodium pump activity It is possible that changes in ion concentrations in the oviductal or uterine environment may

also influence sodium pump activity and differential isoform expression during preimplantation mouse development (see chapter 3)

Although there appear to be many methods of regulating Na,K-ATPase gene expression and activity, most of the work described above has utilized cell lines *in vitro* A paucity of information exists on the regulation of the sodium pump during early embryonic development, particularly in the mouse  Recent work by Herrera *et al* (1994) has shown that in the postimplantation mouse embryo (embryonic day 8 5 to 16 5) there is differential spatial and temporal expression of the various sodium pump isoforms  The roles of the various isoforms in different tissues and at different times of development is currently being investigated  One of the goals of my research was to explore the possibility that several different isoforms of the sodium pump might be differentially expressed in the preimplantation embryo

## 1.5 PREIMPLANTATION DEVELOPMENT IN THE MOUSE

Preimplantation development prepares the embryo for eventual implantation (for review see McLaren, 1982, Watson, 1992)  After fertilization, the zygote cleaves several times, partitioning the ooplasm into blastomeres  At the two cell stage of development oogenetic mRNA content reaches a low point and activation of transcription of the embryonic genome occurs (reviewed by Kidder, 1993b)  After the transition, mouse conceptuses will not complete more than one additional cleavage division when RNA or protein synthesis is inhibited, therefore, the gene expression underlying cleavage must be renewed in each cell cycle (Kidder and McLachlin, 1985, Levy *et al* , 1986)

After the third cleavage division in the mouse, the first morphogenetic event compaction occurs  This event is marked by increased contact between blastomeres  The necessary transcriptional events have been sufficiently completed by the mid 4-cell stage before signs of cell flattening have begun to appear (Kidder and McLachlin, 1985, McLachlin and Kidder, 1986)  The embryo becomes very smooth in appearance to the point of being a uniform cellular mass or morula  During the process of compaction the embryo also undergoes cellular polarization (Ziomek, 1987, Wiley *et al* , 1990)  New gene products facilitate establishment of cell polarity in outer blastomeres

At the 16 cell stage, the embryo has two cell layers an outer polar layer surrounding an apolar inner layer of blastomeres (Hogan *et al*, 1986) Free and apposed plasma membranes of blastomeres become distinct Several gene products are involved in compaction and polarization There is a localization of tight junction (Fleming *et al*, 1989, Fleming and Hay, 1991), E-Cadherin (Watson *et al*, 1990b), gap junction proteins (reviewed by Kidder, 1993b, De Sousa *et al*, 1993) between blastomeres, and components of membrane cortical cytoskeleton (Fleming and Johnson, 1988)

After compaction, the second morphogenetic event to occur is cavitation A critical period of transcription, that is necessary for cavitation, takes place just before the process at about 87 to 91 hours post-human chorionic gonadotrophin (post-hCG) (Khidir *et al*, 1995) After cavitation has begun, the process is less tightly coupled to transcription (McLachlin and Kidder, 1986)

Cavitation (blastocyst formation) is mediated by fluid transfer across the outer blastomeres, eventually forming a fluid-filled cavity that varies markedly in size between mammalian species (Watson, 1992, Kidder, 1993b) Cavitation accompanies the formation of the first two embryonic cell lines including the outer differentiated, epithelial trophectoderm and the undifferentiated inner cell mass (Watson, 1992) The blastocyst stage must be attained for the eventual implantation of the embryo into the uterine wall (McLaren, 1982)

Electron probe microanalysis of blastocoelic fluid by Borland *et al*. (1977) showed that there was an increase in concentrations of Na, Cl, K, Ca$^2$, and Mg$^2$ against that of culture medium Active transporters would have to transport these ions against their increasing concentration gradients Manejwala *et al*. (1989) showed that vectorial transport of Na and Cl, but not K, from the medium into the blastocoelic cavity was required for cavitation Sodium ions were presumably entering the apical membrane of trophectoderm cells through Na channels, Na /H exchangers (Manejwala *et al*., 1989) and/or Na /glucose cotransporters (Wiley *et al*, 1990)

A trans-trophectodermal Na gradient is completed by the active transport of Na out of the cell and into the blastocoel via basolaterally localized Na,K-ATPase (Watson and Kidder, 1988) The accumulation of ions in the blastocoel would result in the flow of

water, osmotically, into the cavity (Wiley, 1988) When mouse and cow blastocysts are collapsed in cytochalasin D and allowed to reexpand in culture media containing 1 X 10⁻⁵ M ouabain, the embryos fail to reexpand (DiZio and Tasca, 1977, Betts *et al*, 1996, in press), providing evidence for a role of the sodium pump in cavitation

## 1.6 Na,K-ATPASE AND THE PREIMPLANTATION MOUSE EMBRYO

Northern blot hybridization experiments have detected transcripts encoding the α 1-subunit of the Na,K-ATPase in all stages of mouse preimplantation development (Watson *et al*, 1990a, Gardiner *et al*, 1990b) The α1-subunit mRNA accumulates steadily under the control of the embryonic genome from the late 2-cell stage onward (Watson *et al*, 1990a, Gardiner *et al*, 1990b) and is detected in polysome fractions beyond the 2-cell stage inclusive (MacPhee *et al*, 1994) β1-subunit transcripts were not detected by Northern blotting in 2-, 4-, or 8-cell embryos but were detected in morulae, at the time that the sodium pump was becoming detectable immunocytochemically (Watson and Kidder, 1988), increasing in abundance by the blastocyst stage (Watson *et al* 1990a)

More recently, β1-transcripts have been detected in early cleavage stages of the mouse using the more sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) technique (Kidder, 1993b) Northern blot analysis failed to detect other isoform mRNAs ( α2, α3, β2) in preimplantation mouse embryos (Kidder, 1993b, Watson *et al* 1990a) but authors have postulated their existence based on studies of enzyme activity with the inhibitor ouabain (Van Winkle and Campione, 1991)

Some evidence suggests that regulation of α subunit synthesis during preimplantation development may be imposed downstream of transcription (MacPhee *et al*, 1994) According to immunofluorescence analysis on sectioned embryos the α subunit becomes detectable during the late morula stage when it appears in discrete intracellular foci, later it becomes concentrated in the basolateral plasma membranes of mural trophectoderm, including its extensions covering the inner cell mass (ICM) (Watson and Kidder, 1988, Kidder and Watson, 1990, Watson *et al*, 1990b) Failure to detect the subunit at earlier stages is in contrast to the detection of the α1 mRNA in the 2-cell stage

in fact, the $\alpha 1$ mRNA reaches 60-70% of its blastocyst level by the completion of compaction (Gardiner *et al.*, 1990b, Watson *et al.*, 1990b) The initial immunofluorescence experiments may not have been sensitive enough to detect the $\alpha 1$ subunit in earlier stages It is also possible that fixation and embedding procedures may have limited exposure of the epitope to the antiserum Future work is required to elucidate the conflicting results of mRNA levels and protein detection (see Chapter 3)

## 1.7 OBJECTIVES OF STUDY

This thesis examined expression of Na.K-ATPase genes during mouse preimplantation development It is hypothesized that the enzyme may be a mediator of fluid accumulation during mouse cavitation and disturbance of the sodium pump may effect cavitation during preimplantation development Expression of sodium pump genes was also examined in another mammalian embryo, the horse conceptus, to try and provide evidence of a role for the sodium pump in mediating fluid accumulation during mammalian development In summary, the following questions were examined

1 Where is $\alpha 1$ mRNA located during preimplantation development and is the $\alpha 1$ subunit present in the inner cell mass and polar trophectoderm of blastocyst stage mouse embryos?

2 Where and when are $\alpha 2$, $\alpha 3$, and $\beta 2$ subunits expressed in early cleavage stages of mouse development?

3 Are $\alpha 1$ and $\beta 1$ subunits expressed in horse conceptuses during fluid accumulation and blastocoel expansion?

4 Is the $\beta 1$ subunit important in the functional expression of the Na.K-ATPase during preimplantation development?

# CHAPTER 2

## SPATIAL LOCALIZATION OF α1 TRANSCRIPTS IN MOUSE PREIMPLANTATION EMBRYOS

### 2.1 INTRODUCTION

Na,K-ATPase, the plasma membrane sodium pump, has long been assumed to be a principal mediator of fluid transport during blastocoel formation (cavitation) in eutherians (reviewed by Benos and Balaban, 1990) In blastocysts of several species, fluid transport has been shown to depend on external sodium and to be sensitive to ouabain, a specific inhibitor of Na,K-ATPase (Benos and Biggers, 1981, Overstrom, 1987, Manejwala *et al.*, 1989) One current hypothesis is that an internally directed sodium flux, driven by Na,K-ATPase, causes a corresponding flow of water across the trophectodermal layer  Results showing that Na,K-ATPase activity is localized in membranes lining the blastocoel cavity (i e in the basolateral plasma membranes of trophectoderm cells) are consistent with this hypothesis (Benos and Balaban, 1990)  Given the importance of this enzyme for blastocyst development (and hence for implantation and the establishment of pregnancy), attention has been focused recently on the timing and regulation of expression of the genes encoding its subunits

In the experiments reported in this chapter, regulation of the expression of the Na,K-ATPase α1 subunit in early mouse development has been explored  A wholemount *in situ* hybridization technique has been developed to take advantage of the optical sectioning capability of the laser scanning confocal microscope  This technique has allowed examination of the cellular distribution of α1 mRNA in morulae and blastocysts to assess whether spatially restricted transcript accumulation correlates with the apparent concentration of the α1 subunit in the mural trophectoderm

### 2.2 MATERIAL AND METHODS

*Embryo Collection*

Random-bred CF-1 female mice (Charles River Canada Ltd , St Constant, Quebec) were superovulated with pregnant mare's serum gonadotropin and human

chorionic gonadotropin (both from Sigma Chemical Co , St Louis, MO: 5 i u each, separated by 46-48 hr) and mated with CB6F$_1$/J males (The Jackson Laboratory, Bar Harbor, ME) Cleavage stage embryos on days 1 to 3 were flushed from the reproductive tract using flushing medium 1, whereas, day 4 embryos (morulae and blastocysts) were flushed with flushing medium 2 (Spindle, 1980) Embryos were washed through five drops of Ca$^{2+}$- and Mg$^{2+}$-free phosphate-buffered saline (PBS) containing 3 mg/ml polyvinyl pyrrolidone (PBS-PVP) before fixation for *in situ* hybridization The timing of embryo collection was as follows late morula, 80-84 hr; early blastocyst, 86-101 hr post-human chorionic gonadotropin injection (post-hCG)

*Subcloning and Riboprobe Synthesis*

A 3 2 kb cDNA obtained from Dr Robert Levenson (Yale University, New Haven, Connecticut, USA), encoding the α1 subunit of rat Na,K-ATPase (Herrera *et al.*, 1987), was subcloned into the *Hind* III site of pGEM4 (Promega Corp , Madison, WI) in sense and antisense orientations Procedures for subcloning were essentially those described in Sambrook *et al* (1987) The α1 cDNA was excised from 5 µg of pGEM4 with 15 Units of *Hind* III (Life Technologies, Burlington, Ontario, Canada) in 100 µL of 50 mM Tris-HCl , 10 mM MgCl$_2$, 50 mM NaCl, pH 8 0 and 10 µg of bovine serum albumin (BSA) at 37°C The incubation was terminated by heating at 75°C for 10 minutes Twenty microlitres of 6X loading dye (1 mM EDTA, pH 8 0, 0 25% bromophenol blue, 0 25% xylene cyanol. 50% glycerol ) was added to the sample and the DNA electrophoresed on a 1% agarose gel at 80V

The 3 2 kb cDNA was excised from the gel and the DNA collected using Z-Spin columns (Gelman Science Inc , Montreal, Quebec, Canada) The gel slice was placed in the column, chilled at -20°C for 15-20 minutes, then centrifuged at 13,000 rpm for 20 minutes in a Sorvall MT-2 microcentrifuge The eluent was collected and 1/10 volume of 3 M sodium acetate was added Two volumes of 100% ethanol was added to the solution and the DNA precipitated overnight at -20°C A DNA pellet was collected by centrifugation at 13,000 rpm, air dried and resuspended in 10-20 µL of double-distilled water (ddH$_2$O)

pGEM4 DNA was linearized, for subsequent dephosphorylation, with *Hind* III exactly like above, except that linear plasmid was extracted with two volumes of phenol chloroform then precipitated with two volumes of 100% ethanol. To verify linearity, a portion of the sample was electrophoresed in 1% agarose alongside a 1 kb DNA ladder molecular weight marker (Life Technologies)

Five micrograms of plasmid DNA were dephosphorylated with 30 Units of calf intestinal alkaline phosphatase (CIAP) in 400 $\mu$L of 50 mM Tris-HCl, 0.1 M EDTA, pH 8.5 and 1 $\mu$g/$\mu$L BSA at 37°C for 30 minutes, then at 50°C for an additional 30 minutes. Thirty additional units of CIAP were added to the sample and the incubations repeated. The reaction was terminated by incubation at 75°C for 15 minutes. One tenth volume of 3M sodium acetate was added to the sample, then it was extracted with two volumes of phenol chloroform. DNA was precipitated with two volumes of 100% ethanol, centrifuged at 13,000 rpm for 20 minutes, air dried, and resuspended in ddH2O

One microgram of $\alpha$1 cDNA was ligated to 1 $\mu$g of dephosphorylated pGEM4 with 10 Units of T4 DNA ligase (Life Technologies) in 20 $\mu$L of 50 mM Tris-HCl (pH 7.6), 10 mM MgCl$_2$, 1 mM adenosine triphosphate (ATP), 1 mM dithiothreitol (DTT), 5% w/v polyethylene glycol-8000. The reaction was incubated at 25°C for 24 hours. A control reaction to test the dephosphorylated state of pGEM4, was also utilized exactly like above, with the exception that the cDNA was omitted from the reaction

The ligation reaction was terminated by heating at 65°C for 5 minutes. Forty microlitres of warm TE (10 mM Tris-HCl, 1mM EDTA, pH 8.0) and 1 $\mu$L of 0.5 M EDTA was added to each sample. To twenty microlitres of the mixture was added 100 $\mu$L of HB101 *E. coli* competent cells (Life Technologies). Cells were placed on ice for 45 minutes, heated at 40°C for 45 seconds, chilled 2 minutes on ice and added to 900 $\mu$L of LB media. Cultures were then incubated at 37°C for 1.5 hours on a horizontal shaker. Two hundred microlitres of each sample were plated on to LB-agar plates containing 100 $\mu$g/mL ampicillin (amp). The remaining volume of sample was gently centrifuged and the resulting pellet resuspended in 100 $\mu$L of the supernatant, followed by plating on LB-agar-amp plates. Plates were incubated at 37°C overnight. Bacterial colonies were randomly picked and each one was used to inoculate 4 mLs LB-100 $\mu$g/mL ampicillin. Cultures

were grown overnight at 37°C  Plasmid DNA was isolated using small-scale preparation of DNA procedures (mini-prep) (Sambrook *et al.*, 1987)

To verify orientation of the $\alpha 1$-subunit-containing pGEM4 (pGEM4$\alpha$1), 1 $\mu$g of each plasmid DNA clone was incubated with the restriction endonuclease *Kpn* I in 30 $\mu$L of 20 mM Tris-HCl, pH 7 4, 5 mM $MgCl_2$, 50 mM KCl and 1 Unit RNase A  Samples were incubated overnight at 37°C, the reactions terminated at 65°C, then electrophoresed on a 1% agarose gel

One millilitre of each remaining culture. from the mini-prep procedure, was used to inoculate 250 mL of LB-100 $\mu$g/mL ampicillin  Cultures were grown overnight at 37°C and plasmid DNA was isolated with large scale DNA preparation procedures (maxi-prep) (Sambrook *et al*, 1987)  DNA was further purified with CsCl gradients  DNA pellets were resuspended in 8 0 mL TE buffer, 0 8 mL of 10 mg/mL ethidium bromide, and 8 8 grams of cesium chloride  Mixtures were centrifuged at 8,000 rpm for 30 minutes then transferred to 15 mL Beckman Quickseal ultracentrifuge tubes  Tubes were topped up with light parafin oil  Samples were ultracentrifuged at 56,000 rpm in a Beckman Ti70 1 titanium rotor for 21 hours. DNA bands were removed from the centrifuge tubes with a syringe and DNA extracted 5-8 times with equal volumes of n-butanol  DNA was precipitated at 4°C for two hours with two volumes of 95% ethanol  Following centrifugation at 7,000 rpm, DNA pellets were resuspended in 400 $\mu$L of 3 M sodium acetate. transferred to 1 5 mL microcentrifuge tubes and DNA precipitated with 1 mL 100% ethanol  Following a wash with 70% ethanol. DNA was pelleted. dried and redissolved in TE buffer  Clones were again verified for orientation, within their respective plasmids, exactly like above

The plasmids pSP64-U1b and pSP65-U1b, containing a 0 4 kb cDNA encoding a mouse U1b small nuclear RNA in sense and antisense orientations, respectively, were obtained from Dr  Gilbert A  Schultz (Department of Medical Biochemistry, University of Calgary)

Reagents and the procedure for synthesis of riboprobes containing digoxigenin-11-UTP (DIG-UTP) were contained in the DIG RNA Labelling Kit obtained from

**Table 2.1**

Plasmids Used for Riboprobe Synthesis

| Plasmid | cDNA Length | Linearize with | RNA Polymerase for Transcription | cDNA Orientation |
|---------|-------------|----------------|----------------------------------|------------------|
| pGEM4α1a | 3 2 Kb | *Sma* I | T7 | Sense |
| pGEM4α1b | 3 2 Kb | *Sma* I | T7 | Antisense |
| pSP64U1b | 0 4 Kb | *Eco* RI | SP6 | Sense |
| pSP65U1b | 0 4 Kb | *Hin*d III | SP6 | Antisense |

Boehringer-Mannheim Canada (Laval, Québec) One microlitre of template plasmid DNA, linearized with the appropriate restriction endonuclease (Table 2 1), was transcribed at $37^{\circ}C$ for 2 hours with the appropriate RNA polymerase in a solution consisting of 1 mM each of ATP, CTP, GTP, 0 65 mM UTP, 0 35 mM DIG-UTP, 40 mM Tris-HCl, pH 8 0, 6 mM $MgCl_2$, 10 mM dithioerythritol, 2 mM spermidine, 10 mM NaCl, 1 Unit of RNase inhibitor and containing 10 μCi of 10 5 nmol/mL [$^{35}$S]-CTP to monitor nucleotide incorporation and to calculate riboprobe yield Following the 2 hour incubation, 10 Units of DNase I were added and the solution incubated an additional 15 minutes

*Northern Blotting*

The specificity of the antisense riboprobes was confirmed by probing northern blots of total RNA from adult organs (Fig 2 1) Total RNA samples were obtained from Daguang Zhu 5-10 μL, representing 10 μg each of rat brain, heart, kidney, liver, C6 glioma cells and Cx-43 transfected C6 glioma cell total RNA were added to a premix of 4 μL of 10X MOPS buffer (4 2% (w/v) 3-(N-morpholino) propanesulfonic acid, 0 05 M sodium acetate, 0 01 M EDTA, pH=8 0), 7 μL of 37% formaldehyde, and 20 μL deionized formamide 8 μL of loading dye was added to each following heating at $55^{\circ}C$ for 15 minutes Samples were electrophoresed on 1 2% agarose-1% formaldehyde gels at 100V for 3 hrs in 1X MOPS buffer

Gels were washed three times with $ddH_2O$, then washed in 1 5 M NaCl, 0 15 M tri-sodium citrate, pH 7 0 (10X SSC) for 45 minutes Nylon membranes (Hybond-N, 0 45 μm, Amersham Life Sciences, Mississauga, Ontario) of 11 x 14 cm were soaked in diethylpyrocarbonate-treated (DEPC) $ddH_2O$, then soaked in 3 0 M NaCl, 0 3 M tri-sodium citrate (20X SSC) for 10 minutes RNA was transferred to nylon membranes by capillary elution for 20 hours utilizing Whatman 3MM paper (VWR Canlab) Blots were exposed to ultraviolet light for 5 minutes

Blots were soaked in 0 75 M NaCl, 50 mM $NaH_2PO_4$, 5 mM EDTA, pH 7 4 (5X SSPE) then prehybridized for 4 hours at $42^{\circ}C$ in hybridization solution consisting of 5 mLs of 5X SSPE, 50% deionized formamide, 5X Denhardt's, 10% dextran sulphate, 100 μg/mL denatured salmon sperm DNA and 1% sodium dodecyl sulphate (SDS)

[32]P-labelled riboprobes were synthesized in a reaction containing 40 mM Tris-HCl pH 7 5, 6 mM MgCl$_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 20 units RNase inhibitor, 0 125 mM each of ATP, GTP and UTP, 10 µM CTP, 1 µg template cDNA, 0 165 µM ^{32}P-α-CTP, and 20 units of appropriate RNA polymerase The reaction was incubated at 37°C for 2 hours, then 5 units of RQ1 DNase was added and the reaction incubated for an additional 15-30 minutes One microlitre of each transcription reaction was diluted to 500 µL with ddH2O and 5 µL spotted on to GF/F filters (Whatman, VWR Canlab) for determination of total and trichloroacetic acid (TCA) precipitatable counts per minute (cpm)

The labelled probe was added to 15 mls of hybridization solution and mixed thoroughly Blots were hybridized in a Hybrid-Ease chamber at 42°C for 40-48 hours Blot were then rinsed briefly, but vigorously, with prewarmed (42°C) 2X SSC, 0 5% SDS to remove most of the unhybridized probe The rinse was repeated and blots incubated for 30 minutes at 42°C A third wash was then performed at 65°C followed by a wash for 30 minutes in 0 5X SSC, 0 5% SDS at 65°C The latter wash was repeated twice more for 1 hour each and then blots were wrapped in cellophane and exposed to X-ray film

*Dot Blotting*

As explained in the Results section. it is important to monitor the digoxigenin content of the sense and antisense riboprobes This monitoring was accomplished using the Genius[TM] RNA Dot Blotting System from Boehringer Mannheim (Laval, Quebec) After dotting serial dilutions (1 ng, 100 pg, 10 pg, 1 pg) of each riboprobe preparation onto a nylon membrane, the probes were crosslinked to the membrane by irradiation for 5 minutes on a UV transilluminator The membranes were rinsed briefly in Genius Buffer 1 0 (100 mM Tris-HCl pH 7 5, 150 mM NaCl), then incubated in Buffer 1 0 containing 1% milk powder (Genius Buffer 2 0) for 30 minutes The membranes were then incubated in a 1 5000 dilution of alkaline phosphatase-conjugated anti-digoxigenin for 30 minutes at room temperature, followed by two 15 minute washes in 30 ml of Buffer 1 0 The membranes were equilibrated briefly in 30 ml of Genius Buffer 3 0 (100 mM Tris-HCl pH 9 5. 100 mM NaCl, 50 mM MgCl$_2$) then incubated in 10 ml of colour substrate solution

(45 µl NBT, 35 µl X-phosphate in Buffer 3 0) until the color reaction had occurred
Color reactions were stopped with a 5 minute soak in 10 mM Tris-HCl, 1 mM EDTA
(Fig 2 2)

*Wholemount, fluorescent in situ hybridization (FISH)*

The application of this technique was based on the method of Conlon and Rossant
(1992) for postimplantation embryos, the most important modification being that the
digoxigenin-labeled riboprobes were detected by means of an anti-digoxigenin-fluorescein
antibody (Boehringer-Mannheim) Unless otherwise stated, all steps of the procedure
were performed at room temperature

### Fixation and pre-hybridization treatments

The embryos were fixed for 3 hr in 3% paraformaldehyde, 0 5% glutaraldehyde in
phosphate buffered saline (PBS), then rinsed through PBS containing 0 1% Triton X-100
(PBT) followed by extraction with the same solution for 20 min at 4"C They were then
treated with 10 µg/ml proteinase K in PBT for 15 minutes with agitation, washed twice in
fresh 2 mg/ml glycine in PBT for 5 minutes, and then refixed with 4% paraformaldehyde,
0 2% glutaraldehyde in PBS for 30 minutes with constant agitation Following a rinse in
100 mM triethanolamine buffer (pH 8 0), the embryos were treated three times for 5
minutes each with 0 25% acetic anhydride in the same buffer, then once for 20 minutes
with 0 1% sodium borohydride in PBT Following these treatments, embryos for
ribonuclease controls were rinsed with RNase buffer (500 mM NaCl 10 mM PIPES pH
7 2, 0 1% Triton X-100) and incubated twice for 30 minutes each time at 37"C in the same
buffer containing 20 µg/ml RNase A and 100 U/ml RNase T₁ All embryo groups were
then prehybridized for 1 hr at 50"C in hybridization buffer (50% formamide, 0 75 M NaCl,
10 mM PIPES pH 6 8, 1mM EDTA, 100 µg/ml yeast tRNA, 0 1% bovine serum albumin,
and 1% sodium dodecyl sulphate)

## Hybridization.

Hybridization was carried out at 50°C in hybridization buffer containing the appropriate full length riboprobe at a concentration of 1 μg/ml Hybridization was for 34 hr with constant agitation

## Post-hybridization wash and RNase treatment

All posthybridization treatments were done with constant agitation Embryos were rinsed briefly through wash 1 [300 mM NaCl, 1% SDS in PE buffer (10 mM PIPES pH 6 8, 1 mM EDTA)] at 50°C, then washed in the same solution for up to 20 hr at 60°C They were rinsed briefly at 50°C with wash 1 5 (50 mM NaCl, 0 1% SDS in PE buffer), then washed two more times each for 30 minutes After two brief rinses in RNase buffer the embryos were incubated twice for 30 minutes at 37°C in the same buffer containing 100 μg/ml RNase A and 100 U/ml RNase $T_1$ (both enzymes from Boehringer-Mannheim)

## Post-RNase washes and anti-DIG treatment

After a final rinse to remove the RNases, the embryos were rinsed briefly in wash 2 (50% formamide, 300 mM NaCl, 1% SDS in PE buffer) then washed in this buffer for 45 minutes at 50°C Following another wash with wash 3 (50% formamide, 150 mM NaCl, 0 1% Triton X-100 in PE buffer) for 30 minutes at 50°C, they were washed twice in wash 4 (500 mM NaCl, 0 1% Triton X-100 in PE buffer), the second time for 20 minutes at 70°C, then cooled on ice The embryos were washed briefly in PBT then blocked for 3 hr at 4°C with 2% fetal calf serum (FCS) in PBT A 1 50 dilution of anti-digoxigenin-fluorescein in blocking solution was added to the embryos and incubation was carried out overnight at 4°C Following a 1 5 hr wash in PBT, the embryos were rinsed 5 times successively through PBT, and then held in this solution for 5 hr The embryos were mounted in 18 μl of FITC-Guard mounting medium (Testog, Chicago, IL) and observed with a Bio-Rad MRC 600 confocal laser scanning microscope

## 2.3 RESULTS

*Is the apparent concentration of the α subunit in mural trophectoderm determined by restricted mRNA abundance?*

According to immunofluorescence evidence, the catalytic subunit of Na/K-ATPase is concentrated in the mural trophectoderm of blastocysts If this is truly its distribution, then this gene can be regarded as a marker of the trophectoderm differentiation pathway (Watson and Kidder, 1988; Watson *et al.*, 1990b, Kidder and Watson, 1990) In order to determine if this situation . associated with spatially restricted α1 mRNA accumulation, a wholemount FISH technique was applied to morulae and blastocysts Antisense riboprobe specificity to target mRNA was confirmed by probing northern blots of total RNA from adult tissues and cell lines (Fig. 2 1)

As a test of the specificity of the *in situ* hybridization, the distribution of U1b small nuclear transcripts, which are restricted to interphase nuclei in preimplantation embryos (Lobo *et al*, 1988), was examined Subsequently, in each experiment with α1 riboprobes, some of the embryos were used for hybridization with U1b riboprobes For both the U1b and α1 hybridizations, a sense riboprobe transcribed from the same cDNA provided a further control for nonspecific probe binding For this control to be valid, the DIG cont. of sense and antisense riboprobes had to be comparable. DIG content was assayed by dot-blotting This procedure proved to be essential It was found for example, that SP6 transcripts had considerably less DIG-UTP than T7 transcripts Therefore, sense and antisense transcripts had to be synthesized using the same polymerase As shown in Fig 2 2, it was possible to obtain sense and antisense riboprobes with similar DIG contents

U1b class small nuclear RNA is an abundant nuclear transcript involved in 5' mRNA splicing (Maniatis and Reed, 1987) Through the course of 10 experiments in which late morulae and blastocysts were examined, embryos hybridized with the antisense U1b riboprobe (n=135) exhibited a strong and consistent nuclear signal (Fig 2 3A-C) This hybridization signal was greatly reduced by treatment of the embryos with ribonuclease (n=97, Fig 2 3D-F) A weak cytoplasmic hybridization signal was obtained with the sense riboprobe (n=119, Fig 2 3G-I) These results confirmed the findings of Lobo *et al.* (1988) using radioactive probes on embryo sections, and demonstrated that

**FIGURE 2.1.** Testing specificity of antisense riboprobes for target mRNAs on northern blots of total RNA from rat brain, heart, kidney, liver, C6 glioma cells and connexin 43 transfected C6 glioma cells (C6-13) (lanes 1-6 respectively) **A.** Sodium pump $\alpha 1$ riboprobes hybridized to the appropriate ~4 kb mRNA species in tissues examined **B.** U1b snRNA antisense riboprobes hybridized to the appropriate 0 2 kb mRNA species although they hybridized to a lesser degree to heart, C6 and C6-13 cell line total RNA A longer exposure detected the appropriate mRNA species in all samples (data not shown) Sodium pump $\alpha 1$ and U1b sense riboprobes did not hybridize to target mRNAs (data not shown)

A

1 2 3 4 5 6

9.49 =
7.46 =
4.40 —
2.37 —
1.35 —

0.24 —

B

1 2 3 4 5 6

9.49 =
7.46 =
4.40 —
2.37 —
1.35 —

0.24 —

**FIGURE 2.2** Determination of the DIG content of riboprobes by means of dot-blotting. Serial dilutions of the antisense and sense riboprobes and two control RNAs (one DIG-labeled the other not, supplied by Boehringer Mannheim) were reacted with the anti-DIG antibody. A,B- sense and antisense U1b riboprobes, respectively. C,D- sense and antisense $\alpha 1$ riboprobes, respectively. E,F- labeled and unlabeled control RNAs, respectively

|        | 1 ng | 100 pg | 10 pg | 1 pg |
|--------|------|--------|-------|------|
| A      | ●    |        |       |      |
| B      | ●    |        |       |      |
| C      | ●    | ●      | ●     |      |
| D      | ●    | ●      | ●     |      |
| E      | ●    | ●      |       |      |
| F      |      |        |       |      |

**FIGURE 2.3** Distribution of J1b RNA in the morula and early blastocyst stages as revealed by wholemount FISH  A,B,C- embryos hybridized with the antisense riboprobe, **D,E,F**- embryos treated with RNase before hybridization with the antisense riboprobe, **G,H,I**- embryos hybridized with the sense riboprobe  The left column (**A,D,G**) shows morulae, the center and right columns show blastocysts  The scale bar indicates 50 μm
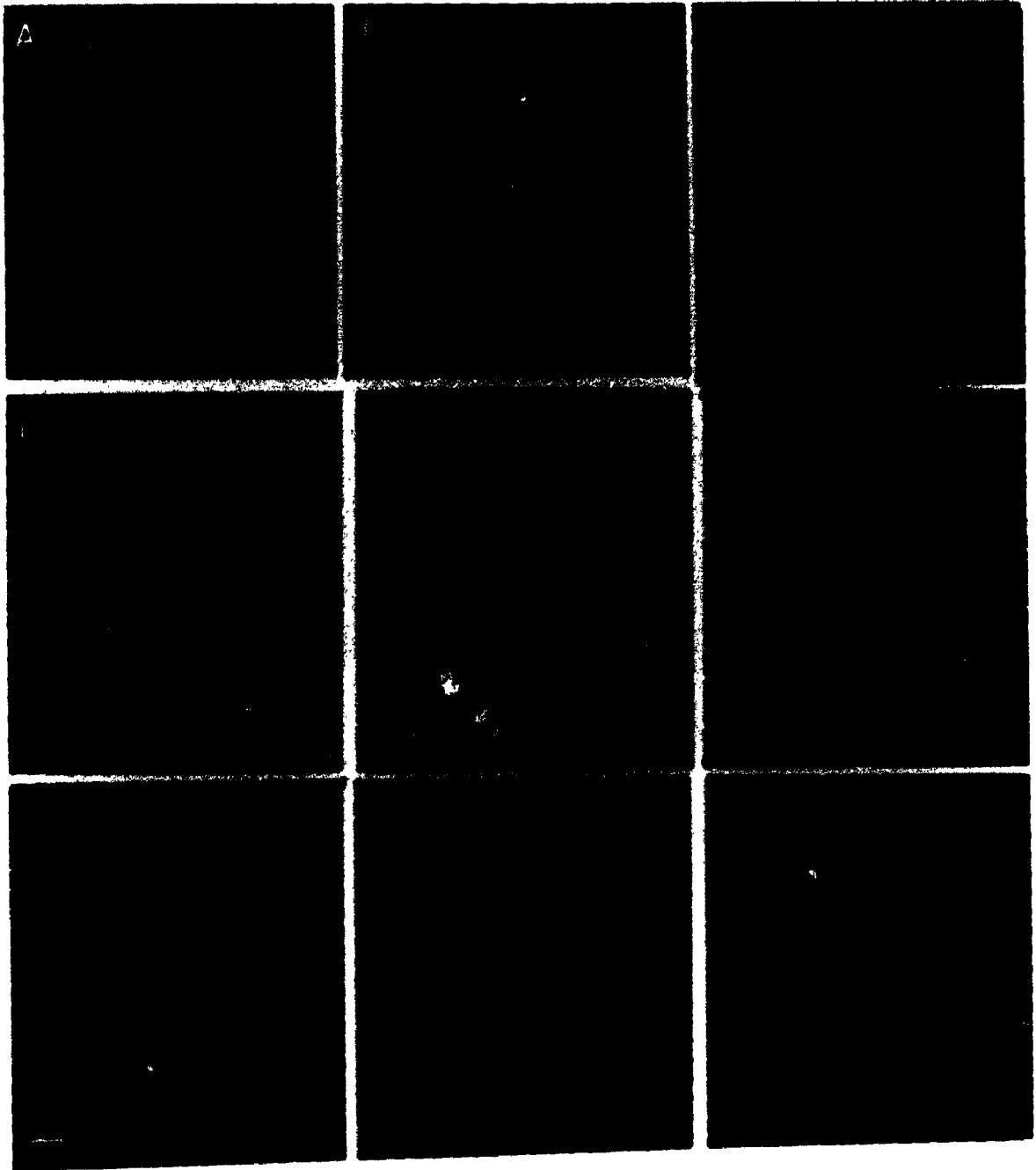
the wholemount FISH technique can provide accurate information on the spatial distribution of transcripts, with good signal/noise ratios and excellent subcellular resolution The only consistent sign of nonspecific background was the nucleolar staining that could be seen in RNase control embryos and those hybridized with the sense probe (Figs 2 3,2 4) This nucleolar staining appears to be a property of the anti-DIG antibody, since it was present even when riboprobes were omitted during the hybridization step

Analysis of the distribution of Na,K-ATPase α1 transcripts was performed on three separate batches of embryos, using two different riboprobe preparations Care was taken to ensure the same amount of compression under the coverslip for all embryos observed, which was accomplished using the specimen thickness data computed by the microscope software Care was necessary because coverslip compression was found to be a variable that affected apparent signal intensity Late morulae and blastocysts (n 74) hybridized with the antisense α1 riboprobe showed a consistent cytoplasmic signal in all blastomeres (parts A-C of Fig 2 4) As with the U1b riboprobes, RNase pretreatment reduced the hybridization significantly (n=28, Fig 2 4 D-F), and there was little hybridization with the sense riboprobe (n=54, Fig 2 4 G-I) The absence of an enrichment of α1 transcripts in mural trophectoderm demonstrates that transcription of the α1 gene is not limited to the mural trophectoderm

## 2.4 DISCUSSION

Previous studies of the timing and distribution of Na,K-ATPase expression during mouse preimplantation development have indicated that synthesis of this enzyme is regulated both temporally and spatially (Watson and Kidder, 1988, Watson et al. 1990a Gardiner et al., 1990b) It is clear that this regulation is not imposed at the level of mRNA accumulation α1 transcripts accumulate well in advance of the time of first appearance of α subunit immunoreactivity and, as shown here, are not concentrated in the mural trophectoderm, as the α subunit itself appears to be based on immunofluorescence experiments(Watson and Kidder, 1988) Data also make it clear that mRNA recruitment for translation does not play a significant role in the timing of α subunit appearance (MacPhee et al, 1994) A potential conclusion is that the appearance of
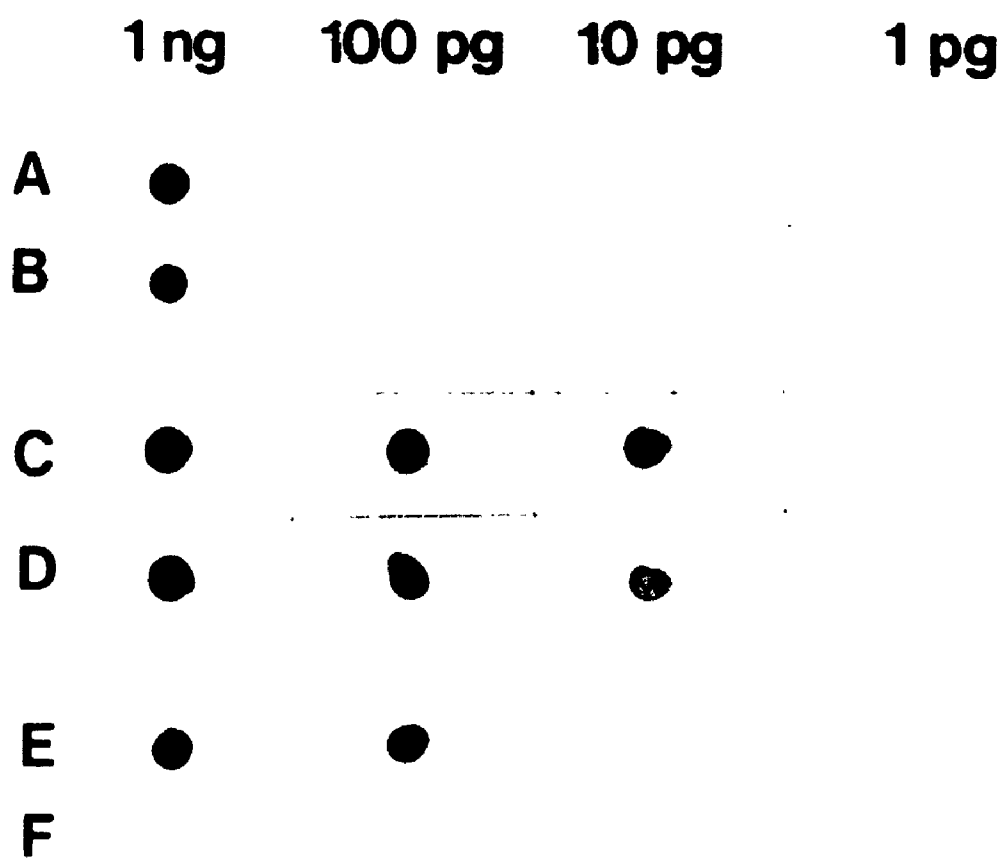
**FIGURE 2.4** Distribution of Na,K-ATPase $\alpha$1 mRNA in the morula and early blastocyst stages as revealed by wholemount FISH  A,B,C- embryos hybridized with the antisense riboprobe, D,E,F- embryos treated with RNase before hybridization with the antisense riboprobe, G,H,I- embryos hybridized with the sense riboprobe  The left column (A,D,G) shows morulae, the center and right columns show blastocysts  The scale bar indicates 50 μm
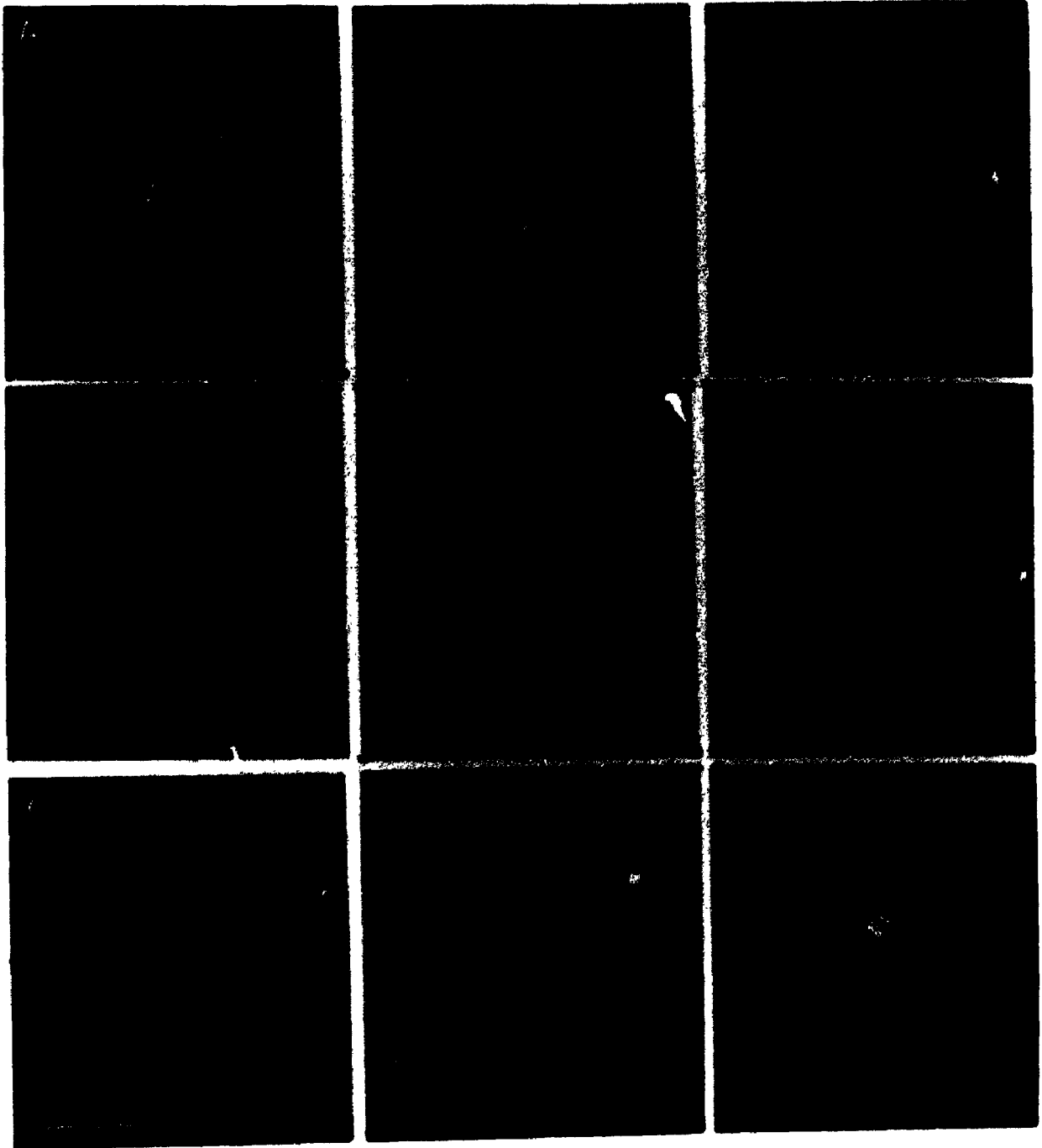
immunocytochemically detectable α subunits is delayed until the late morula stage and immunoreactivity restricted to mural trophectoderm not because of a lack of α1 mRNA or limits on its translation, but because the nascent α subunits are either unstable or incompletely processed so as to be unrecognizable by some antibodies. It is also possible that the previous work showing the concentration of the α-subunit in the mural trophectoderm (Watson and Kidder, 1988), which utilized embryo embedment and sectioning, may not have given an accurate description of the α1 expression pattern. This could be due to differential exposure of epitopes in the section (See chapter 3).

It is possible that an insufficient supply of nascent ß subunits may limit the accumulation of mature α subunits prior to the late morula stage. This hypothesis is based on the known requirement for nascent ß subunits in functional expression of α subunits (reviewed by Geering, 1990b, 1991b) and the fact that, although ß1 transcripts are present throughout preimplantation development, their abundance is at least an order of magnitude lower than that of α1 transcripts prior to the late morula stage (Watson *et al.*, 1990a, Kidder, 1993a,b).

Findings must be considered in the context of the total α and ß subunit content and Na,K-ATPase activity of preimplantation mouse embryos. In contrast to the results of immunofluorescence experiments, western blot analysis has revealed that the total α subunit content does not change appreciably (on a per embryo basis) throughout preimplantation development (Gardiner *et al.*, 1990b). Furthermore. the total Na,K-ATPase activity increases only about two-fold. although most of this increase occurs after the morula stage and represents enzyme in membranes exposed to the blastocoel (Van Winkle and Campione, 1991). The most likely explanation for this is that much of the α subunit content (and enzymatic activity) of the embryo prior to cavitation is accounted for by stable, oogenetic α/ß heterodimers that went undetected in earlier immunofluorescence experiments (Watson and Kidder, 1988, see Chapter 3). Indeed, results consistent with the presence of Na,K-ATPase isozymes in addition to α1ß1 were reported by both Gardiner *et al.* (1990) and Van Winkle and Campione (1991). whereas neither Gardiner *et al.* (1990b) nor Watson *et al.* (1990a) detected mRNAs for any other α or ß subunit isozymes. If much of the oogenetic enzyme were to be degraded to make way for new,

embryonically encoded and basolaterally targeted enzyme at the onset of cavitation, then the total Na,K-ATPase activity of the embryo might not change appreciably According to the above hypothesis, this influx of new Na,K-ATPase would be triggered by up-regulation of transcription of the ß subunit gene

The expression of Na,K-ATPase $\alpha$ and ß subunit genes has been examined in later developmental stages and adult organs as well, and the results have often been similar to the ones just described  For example, $\alpha 1$ mRNAs are much more abundant than ß1 mRNAs during early stages of fetal lung development, with the abundance of ß1 mRNAs increasing to approach that of $\alpha 1$ mRNAs just before birth (O'Brodovich *et al*, 1993) In general, $\alpha$ subunit mRNA abundance exceeds that of ß subunit mRNA in developing and adult organs such that ß subunit mRNA content appears to determine the total Na,K-ATPase activity (Gick *et al*, 1993) Interestingly, post-transcriptional regulation of $\alpha$ subunit expression has been demonstrated during brain development (Corthesy-Theulaz *et al*, 1991)

# CHAPTER 3

# EXPRESSION OF Na,K-ATPASE α AND ß SUBUNIT ISOFORM GENES

## 3.1 INTRODUCTION

The majority of studies investigating the role of Na,K-ATPase in cavitation have been applied to the preimplantation mouse or rabbit embryo (Watson and Kidder, 1988, Gardiner *et al.*, 1990a,b, Watson *et al.*, 1990a,b, MacPhee *et al.*, 1994) It is essential to investigate the onset of these events in additional mammalian species, such as the horse, to provide insight into the mechanisms that govern mammalian preimplantation development in general

In the mouse and rabbit, blastocyst formation involves the establishment of ion concentration gradients, specifically of sodium and chloride ions (Borland *et al* 1976), across the epithelium, thereby facilitating the osmotic accumulation of water The Na,K-ATPase, integrated in the basolateral membrane surface of trophectoderm cells is believed to play a major role in this event (Watson and Kidder, 1988, Chapter 1). Epithelial junctional complexes regulate leakage of blastocyst fluid and maintain apical and basolateral plasma membrane domains (Fleming *et al*, 1989) Fluid transported by way of water-to-solute coupling is isotonic or slightly hypertonic in comparison to serum (Diamond and Bossert, 1967), Na⁺ and Cl⁻ concentrations in rabbit (days 6-10, Lewis and Lutwak-Mann, 1954, days 5-7, Borland *et al*, 1976) and mouse blastocyst fluid (Borland *et al*, 1977) are similar to the serum concentrations of these ions

Unlike the mouse, the horse has a maximally expanding blastocyst between day 7 and day 16 when its diameter increases from approximately 200 μm to 25 mm (Biggers, 1972) This, together with a considerable turgidity (Ginther, 1986), indicates that the horse conceptus is highly efficient in accumulating and retaining water Interestingly, however, the blastocyst fluid, from day 10 to at least day 25, is markedly hypotonic compared to serum (Waelchli *et al*, 1996) This is reflected by buoyancy of the blastocyst in phosphate-buffered saline While in the rabbit and mouse, implantation coincides with the formation of the parietal endoderm, in the horse, the formation of the endoderm on day 7 and 8 (Enders *et al*, 1993) precedes the first signs of trophoblastic cell invasion (at

day 34) of the endometrium by almost four weeks  Thus, the horse blastocyst is bilaminar during the major part of its expansion stage and the acquired fluid has to cross two epithelial layers

Sodium pump α1 and β1 subunit isoform genes are known to be expressed in preimplantation mouse development (Watson *et al*, 1990a)  With the advent of techniques like reverse transcriptase polymerase chain reaction (RT-PCR), that are more sensitive than *in situ* hybridization and northern blotting, many tissues and organs that were once thought to express single subunit isoforms of the sodium pump are now known to express a multitude of isoforms (Ahn *et al*, 1993, Herrera *et al*, 1994, Clapp *et al*, 1994)  Watson *et al* (1990a) and Kidder (1993b) were not able to detect α2, α3, or β2 isoform mRNAs by northern blotting during preimplantation stages of mouse development  A fourth alpha subunit, α4, has been discovered in mouse testis (Shamraj and Lingrel, 1994)  Subsequently, these results dictate that a more thorough and sensitive investigation of the expression patterns of the various isoform mRNAs be conducted

In this chapter, an earlier analysis of Na,K-ATPase isoform gene expression in the mouse preimplantation embryo (Watson and Kidder, 1988, Watson *et al*., 1990a,b) has been extended to include all of the presently characterized Na,K-ATPase subunit gene transcripts and proteins  This chapter also attempts to describe the role of the Na,K-ATPase in the accumulation of low osmolality blastocoel fluid in the maximally expanding horse embryo  This component of the thesis is the result of a collaboration with Dr Keith Betteridge and Dr Ruedi Waelchli at the University of Guelph  RT-PCR and immunocytochemistry were utilized in horse embryos to detect sodium pump α and β transcripts and subunit proteins, respectively

## 3.2 MATERIALS AND METHODS

*Mouse Embryo Collection*

Random bred CF1 female mice (Charles River Canada Ltd , St Constant, Quebec) were superovulated and mated as previously described (see Chapter 2) Day 2-3 (4-cell. 8-cell. compacting 8-cell stages) embryos were removed from oviducts with flushing medium 1, while day 4 (morulae and blastocysts) embryos were removed from uteri with flushing medium 2 (Spindle, 1980) The embryonic stages and times post-hCG used in experiments were as follows oocytes, 18 hr, 2-cell, 48 hr, 4-cell, 60 hr, 8-cell, 65 hr, 8-cell compacting, 72 hr, morula, 84 hr; blastocysts, 90-92 hr Embryos used for immunocytochemistry were pooled and washed five times in calcium, magnesium free PBS containing 0 3 mg/mL polyvinylpyrrolidone (PBS-PVP)

*Mares and Collection of Conceptuses*

Seven mares from the Equine Research Centre at the University of Guelph were utilized for experiments Animals were housed and used in accordance with the regulations of the University of Guelph's Animal Care Committee

Follicular growth and uterine characteristics, diagnosis of ovulation, and artificial insemination were monitored and/or performed by Dr R Waelchli (University of Guelph) Since the ovulation could have occurred anytime during a 48-hour interval between 2 examinations, the day when it was detected was designated Day 0 +/- 1 Conceptus tissues were also collected by Dr R Waelchli via transcervical flushing of intact (Days 8-16) or collapsed embryos (Days 16-31) Transcervical recovery was performed by uterine lavage with Dulbecco's phosphate-buffered saline solution (PBS), pH 7 4 (GIBCO-BRL)

The capsule was removed from intact conceptuses and the tissue was washed several times with PBS Under a dissecting microscope, pieces measuring at least 5 mm by 5 mm from bilaminar yolk sac (Days 8-31) and vascularized trilaminar yolk sac (Days 16-25) were separated with a scalpel blade Day 8 and Day 9 conceptuses (bilaminar yolk sac) were left intact For fixation, the tissues were placed in phosphate buffered 4% formaldehyde for 6 to 24 hours The fixed tissues were dehydrated with a series of 15 minute washes in 70%, 95%, 100% isopropanol, washed 2 X 15 minutes in xylene and

embedded in paraffin (University of Guelph Histology Labs, Guelph, Ontario) For RT-PCR, embryos were fixed in 70% ethanol in PBS then transported to the lab and stored at -20°C

*Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)*

### RNA Isolation

Total RNA was extracted according to Rappolee *et al* (1989) and Valdimarsson *et al.* (1993) Pools of 100 murine embryos or 20 mg of horse conceptus, mouse kidney or testes were lysed at room temperature in 100 µL of solution D (Chomczynski and Sacchi, 1987) in the presence of 20 µg of *E. coli* rRNA Embryo lysates were layered on to 100 µL of 5 7 M CsCl in 0 1 M EDTA, pH 7 5 and ultracentrifuged at 80,000 rpm for 4 hours at 20°C utilizing a TLA-100 titanium ultracentrifuge rotor and a Beckman TL-100 ultracentrifuge RNA pellets were dissolved in 2 5 M ammonium acetate, precipitated in absolute ethanol, centrifuged, dried, and re-suspended in sterile MilliQ water

### Reverse Transcription

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 of Dr Patricia Watson (Lawson Research Institute, University of Western Ontario) RNA pellets were re-suspended in 11 µL of sterile MilliQ water and incubated with 1 µg of oligo (dT)$_{12-18}$ for 10 minutes 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 hr Reactions were terminated by heating at 94°C for 4 minutes and flash cooling on ice Following the RT reactions, mouse embryo cDNA samples were at a final concentration of 5 embryo equivalents/µl

## Amplification of α- and ß-subunit isoform cDNAs

PCR was carried out in a total volume of 25 microlitres All assays were repeated at least twice on embryos from two different embryo collections One microlitre of RT preparation, representing 5 embryo equivalents in the mouse embryo preparations, 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 of dNTPs, 1 mM MgCl$_2$, and 0 4 µM of sequence-specific primers (4 µM for ß$_2$ primers) Reactions were overlaid with 25 microlitres of paraffin oil and amplified with a Perkin Elmer-Cetus DNA Thermal Cycler

Each cycle of PCR consisted of 1 minute denaturation at 94"C, 30 seconds annealing at 54-60"C for mouse isoforms or 58"C for horse isoforms, and 1 minute primer extension at 72"C Mouse α2, α3, and α4 isoforms were amplified for 40, 34, and 40 cycles respectively while ß2 was amplified for 38 cycles Horse α1 and ß1 isoforms were amplified for 40 cycles 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

Primer pairs 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, mouse and horse cDNA sequences (Shull *et al.*, 1986, Young *et al.*, 87, Martin-Vasallo *et al.*, 1989, Kano *et al.*, 1989, Gloor *et al.*, 1990) and the sizes of the expected PCR products are shown in Table 3 1 The primer pairs were designed to produce isoform-specific RT-PCR products and to bracket an intron(s) for discrimination of products from genomic DNA and cDNA (see Table 3 1) To confirm identity, each DNA product was subjected to cleavage with an appropriate diagnostic restriction enzyme (Table 3 1) and/or each product was cleaned with a QIAquick Spin PCR purification kit (Qiagen, Chatsworth, CA, USA) and directly sequenced by the dye termination method with an ABI Prism 377 DNA Sequencer (Genalytic, Department of Zoology, University of Guelph, Guelph, Ontario)

Direct sequencing produced sequences that encompassed the vast majority of the PCR amplicons, but not the entire amplicon Sequencing in this manner, utilizing the PCR

**Table 3.1**

Na.K-ATPase subunit isoform PCR primer sequences

| Subunit isoform | Primer sequences | Amplicon position and size (bp) | Genomic DNA size (bp) | Diagnostic enzyme | Fragment Sizes (bp) |
|---|---|---|---|---|---|
| α1 | 5' primer= ACCTGCTGGGACTCCGCGTG 3' primer= AGGGGAAGGCACAGAACCACCA | Exons 18-22 or horse cDNA = 336[1] | 4246 | Ssp I | 220 116 |
| α2 | 5' primer= GGCTGCTTGGGATCCGCCTTGA 3' primer= AGGGGAAGGCACAGAACCACCA | 2731-3066 of rat cDNA = 335[1] | 2946 | Iva I | 267 68 |
| α3 | 5' primer= ACCTGGTGGGCATCCGGCTCAA 3' primer= AGGGGAAGGCACAGAACCACCA | 2755-3091 of rat cDNA = 336[1] | 2946 | Bcl I | 179 157 |
| α4 | 5' primer= GGAGTTGAAGAAGGAAGTGG 3' primer= AGCCAGTGATGATGACCACG | 114-452 of rat cDNA = 338[1] | 3478 | Hae III | 242 96 |
| β1 | 5' primer= TTCAGCCCAGAAGGACGACATG 3' primer= AGGGAAGCCGTAGTATCCGCCCA | 378[1]* | 6346 | Hind III | 266 112 |
| β2 | 5' primer= GCCTTACAACGACTCCATCCA 3' primer= CGGCATTCTACATTCACCTCC | 805-1246 of rat cDNA = 441[1] | 1664 | Hae III | 291 150 |

[1] Shull et al. 1986. Young et al. 1987. Martin-Vasallo et al. 1989. Kano et al 1989 Gloor et al. 1990. Shamraj and Lingrel. 1994

* No published sequence information is available for the horse β1 cDNA sequence Therefore the genomic DNA size listed is from the rat

primers for the sequencing reactions, can only approach 10-50 bases away from the 5' and 3' ends of the amplicons (A Holliss, Department of Zoology, University of Guelph, Guelph, Ontario, Canada, personal communication) dependent on the purity of the templates

*Immunocytochemistry*

### Mouse Embryo

#### *Diethylene Glycol Disterate (DGD) Embedding*

Embryos were fixed in 4% paraformaldehyde-0 2% glutaraldehyde or 4% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 1 mM $MgCl_2$, pH 6 9) Preimplantation stages were processed for DGD embedding according to Valdimarsson and Huebner (1989) Embryos were dehydrated with a graded series of ethanol (70%, 80%, 95%) for 15 minutes in each wash, then twice in 100% ethanol for 30 minutes Samples were placed in BEEM capsules for subsequent addition of reagents and embryos were kept at the bottom of the capsules by centrifugation at 450 X g

Samples were equilibrated in butanol with three, 15 minute washes of ethanol/butanol 2 1, 100% ethanol n-butanol, 1 1, 100% ethanol n-butanol, 1 2, 100% ethanol n-butanol Embryos were then washed in two, 15 minute washes of 100% n-butanol followed by infiltration in butanol/DGD at $60^0$C, 2 1 n-butanol DGD for 1 hour, 1 1 n-butanol DGD for 1 hour, and 1 2 n-butanol DGD for 1 hour Groups of 50-100 mouse embryos were embedded in 100% DGD overnight at $60^0$C Blocks were polymerized at room temperature and 2-5 μm sections cut with a Sorval MT-2 microtome

For immunocytochemistry, DGD sections were dried at $40^0$C on to glass slides coated with poly-L-lysine Slides were soaked in three, 1 hour changes of n-butanol and DGD removed from the tissue sections with 3 changes, 3 minutes each, of transition fluids 3 1, 1 1, 1 3 n-butanol 100% ethanol Embryo sections were rehydrated through 95% o, 70% o, and 50% ethanol by immersion for 3 minutes each During the 70% ethanol soak, a second 70% ethanol soak containing 1 mg/mL $NaBH_4$ was utilized Sections were soaked in PBS for 3 minutes then in PBS containing 1% bovine serum albumin (BSA) for

12 hours at 4"C  Primary and secondary antisera incubations were performed overnight at 4"C, post-antisera washes were in PBS/BSA at room temperature and sections were mounted in FITC-Guard (Testog, Inc , Chicago, Illinois)

### *Wholemount Indirect Immunofluorescence with Paraformaldehyde Fixation*

Embryos were washed once with PHEM buffer then fixed for at least 1 hour to overnight in 1% paraformaldehyde in PHEM buffer according to Valdimarsson *et al* (1991)  Preimplantation stages were washed 4 times in PHEM buffer, permeabilized 20 minutes with PHEM buffer containing 0 1% Triton X-100, washed twice in PBS, permeabilized 20 minutes in PBS containing 0 1% Triton X-100 (PBT) and blocked for at least 1 hour in PBT containing 1% bovine serum albumin (BSA)  Embryos were incubated overnight with primary antisera in blocking buffer then washed 6 times with PBT for 10 minutes each, except the last wash which was for 5 hours  Samples were treated with secondary antisera in blocking buffer for 1 hour followed by 6 washes in PBT as described  Embryos were mounted in 15-18 µl of FITC-Guard

### *Wholemount Indirect Immunofluorescence with Methanol (MeOH) Fixation*

Preimplantation stages were fixed and processed for immunocytochemistry according to Becker and Davies (1994)  Embryo stages were fixed in 2 1 PBS/MeOH, 1 1 PBS/MeOH (50%) for 3 minutes each and then 2 1 PBS/MeOH again for 3 minutes  Embryos were blocked in 0 01% Triton X-100, 1X PHEM, 0 1 M Lysine and 1% goat serum for 45 minutes and then washed in PBS-PVP for 5 minutes  Primary antiserum diluted in PBS, was added to various preimplantation stages and incubated overnight at 4"C  Treatment groups were washed 3 times, for 10 minutes each, in PBS/PVP followed by addition of secondary antiserum diluted in PBS  Embryos were incubated in secondary antiserum for 1 hour at 4"C then washed 3 times, for 5 minutes each, with PBS-PVP  Embryos were mounted on to glass slides in FITC-Guard (Testog, Inc , Chicago Illinois) and viewed with a Bio-Rad MRC-600 laser scanning confocal microscope

### Horse Embryos

Horse embryos were processed and embedded in paraffin by the Histology Laboratory at the University of Guelph (Guelph, Ontario, Canada) Sections of 4 μm were placed on glass slides coated with 2% aminopropyltriethoxy-silane (APTEX, Sigma Chemical Co, St Louis, Missouri) in acetone and dried for 24 hours at 60$^{0}$C The sections were deparaffinized with two washes in SlideBrite™ (S&S Co of Georgia Inc, Albany, GA) for 3 minutes each and then rehydrated with a series of washes, 2 minutes each, in 100%, 95% and 70% isopropanol, followed by soaking in PBS Sections of horse kidney were used as positive controls For negative controls, the primary antiserum was replaced with non-immune goat serum

*Antibodies*

For horse immunocytochemistry and Western blotting, a rabbit polyclonal antiserum (UBIα1) raised against a specific peptide of the rat Na/K-ATPase α1-subunit (Shyjan and Levenson, 1989, Zlokovic *et al.*, 1993, Cameron *et al*, 1994) (Table 3 2) was purchased from Upstate Biotechnology Incorporated (Lake Placid, NY) A second rabbit polyclonal antiserum (NASEα1) raised against a specific peptide of the α1-subunit (Pressley, 1992, Becker and Davies, 1995, Koster *et al*, 1996,1995) was a gift from Dr Tom Pressley (Texas Technical University, Lubbock, TX, USA) A rabbit polyclonal antiserum raised against the dog kidney Na,K-ATPase β1-subunit (Blanco *et al*, 1995a,b, Koster *et al*, 1996) (ASKARI β1) was a gift from Dr Amir Askari (Medical College of Ohio, Toledo, OH, USA)

For mouse immunocytochemistry and Western blotting, NASEα1, ASKARIβ1 and UBIβ2 (Shyjan *et al*, 1990) antisera were utilized Monoclonal antisera raised against the sodium pump α1 subunit (CAPLAN α1), β1 subunit (CAPLAN β1), α2 subunit (McB2) and α3 subunit (RDI α3) were utilized as well as a polyclonal antiserum raised against the α3 subunit (CAPLAN α3) (Table 3 2) These antisera were gifts of Dr Michael Caplan (Yale University, New Haven, Connecticut, USA) and Dr Kathleen Sweadner (Massachussetts General Hospital, Boston, Massachussetts, USA) All primary

**Table 3.2**

A Summary of the Antibodies Used in Immunocytochemistry (IC) and Western Blotting

| Name | Type | Recognition Site | Used for Western blotting | Used for Immunocytochemistry | | Recommended Dilutions for IC |
|---|---|---|---|---|---|---|
| | | | | Sections | Wholemount | |
| UBI α1 | Pc[1] | Rat a a 338-513 | | √(X)[3] | √(X) | 1 200 |
| UBI α2 | Pc | Rat a a 335-519 | | √(X) | | 1 200 |
| UBI α3 | Pc | Rat a a 320-514 | | √(X) | √(X) | 1 200 |
| UBI β1 | Pc | Rat a.a 152-304 | | √(X) | √(X) | 1 200 |
| UBI β2 | Pc | Rat a a 63-285 | √(X) | | √ | 1 200 |
| NASE α1 | Pc | Rat a a 489-499 | √ | √(X) | √(X) | 1 100 |
| HERED α2 | Pc | Rat a a 489-500 | √ | √(X) | √(X) | 1 100 |
| TED α3 | Pc | Rat a a 489-500 | | √(X) | √(X) | 1 100 |
| ASKARI β1 | Pc | Dog whole β1 | √ | √ | √(X) | 1 100 |
| CAPLAN α1 | Mc[2] | Dog a a 3-16 | √ | | √ | 1 100 |
| CAPLAN α3 | Pc | Rat a a 2-14 | √ | | √ | 1 50 |
| CAPLAN β1 | Mc | Dog β1 subunit | | | √ | 1 50 |
| McB2 α2 | Mc | Rat N-terminus | | | √ | 1 4 |
| RDI α3 | Mc | Dog α3 subunit | | | √ | 1 100 |
| McTub | Mc | Sea Urchin α-tubulin | | √ | √ | 1 10 |

[1]Polyclonal Antiserum

[2]Monoclonal Antiserum

[3]Check mark indicates that the antiserum was used for the described experiments If an X is present in brackets this signifies that no fluorescent signal (imunocytochemistry) or detection of protein on Western blots was observed with the antibody

antibodies were used for immunocytochemistry at recommended dilutions (Table 3 2)
These antisera have been extensively used for western blotting and to a more limited
extent for immunocytochemistry (Uruyama *et al*, 1989, Gottardi and Caplan, 1993, Van
Why *et al*, 1994, Pietrini *et al*, 1992, 1994, Munzer *et al*, 1994, Blanco *et al*,
1994,1995a, Koster *et al*, 1995, 1996)

Fluorescein conjugated rabbit antiserum raised against mouse IgG and fluorescein
conjugated goat antiserum raised against rabbit IgG (ICN Immunochemicals, Montreal,
Quebec) were used as secondary antisera at recommended dilutions of 1 50 No
preimmune sera was available for the antisera described above

### *SDS-PAGE and Western Blot Analysis*

A 2 cm horse embryo of 0 150 g, 0 02 g of mouse brain, kidney, liver, or 650
mouse morulae and blastocysts were homogenized in an equal volume of 2X SDS loading
buffer (100 mM Tris-HCl, 200 mM dithiothreitol, 4% SDS, 0 2% bromophenol blue, 20%
glycerol) A last wash of PBS/PVP, from mouse embryo collections, was also utilized as a
negative control Samples were then left at room temperature for 10 minutes, not boiled
(Dr K Sweadner, personal communication), centrifuged 10 minutes at 10,000 rpm at 4°C
and the supernatant recovered 3 0 μg of mouse kidney, 30 0 μg of mouse brain and liver
total protein, total protein from 650 mouse embryos, and 25 uL of horse embryo protein
were loaded on to a 4% polyacrylamide-0 1% SDS stacking gel and electrophoresed in
Tris-glycine electrophoresis buffer (25 mM Tris, 250 mM glycine, pH 8 3, 0 1% SDS) at
50 V until the loading dye front reached the 9 0% polyacrylamide-0 1% SDS resolving gel
(12% polyacrylamide was used for analyzing ß1 subunits in horse samples) The samples
were then electrophoresed at 100V

Gels were processed and blotted according to procedures outlined in the enhanced
chemiluminescence kit from Boehringer-Mannheim (Laval, Quebec, Canada) Gels were
soaked in transfer buffer (39 mM glycine, 48 mM Tris base, 0 037% SDS) for 20 minutes
PVDF membranes were soaked in methanol for 1 minute then deionized water for 3
minutes followed by transfer buffer for 3-5 minutes Proteins were electroblotted to PVDF
for 2 hours at 250-400 mA at 20°C Fresh transfer buffer was added to the blotting tank

after the first hour Membranes were then rinsed in PBS to remove residual acrylamide and air dried Membranes were re-wetted in methanol prior to antisera incubations and development

Polyacrylamide gels were checked for efficiency of transfer by staining in 0 5°o Coomasie Brilliant Blue, 50% methanol, 10% glacial acetic acid for 45 minutes, then destaining in 10% glacial acetic acid, 10% isopropanol Gels were fixed in 40°o methanol, 20% glacial acetic acid overnight then vacuum dried to Whatman 3MM filter paper

Blots were washed for 3 minutes in Tris buffered saline (TBS) followed by blocking in TBS containing 0 1% Tween-20 (TBST) and 1% Boerhinger Mannheim blocking solution for 1 hour Incubations were performed with gentle agitations The membranes were then incubated in primary antibodies (at recommended dilutions), diluted 1 100 (NASEα1, HEREDα2) or 1 500 (CAPLAN α3, β1, UBIβ2 ) in 0 5°o blocking solution, for 1 hour The blots were then washed twice for 10 minutes each in TBST at room temperature and then washed twice with 0 5% blocking solution for 10 minutes each time Blots were incubated for 30 minutes with peroxidase-labelled (POD) secondary goat or sheep antisera raised against rabbit or mouse IgG respectively Blots were washed four times, 15 minutes each time, in large volumes of TBST then placed in seal-a-meal™ bags with premixed and prewarmed (25°C) detection reagent consisting of substrate solution A and starting solution B (Boehringer-Mannheim Biochemicals) in a ratio of 100 1 Blots were incubated in the premixed reagent for 60 seconds at room temperature, excess reagent drained, and the membranes exposed to Kodak XAR x-ray film

## 3.3 RESULTS

*Detection of Na,K-ATPase α- and β-subunit isoform transcripts during murine preimplantation development.*

Transcripts of the α2-isoform gene could only be detected in mouse oocytes (Fig 3 1), although the transcript abundance is probably very low as this result was inconsistent both within and between cDNA preparations Transcripts encoding the α3-subunit were detected throughout murine preimplantation development (Fig 3 1) There was an apparent increase in abundance of this mRNA from the 4-cell stage onwards, suggesting

**FIGURE 3.1:** Detection of α- and ß-subunit isoform transcripts in murine preimplantation embryos  Each lane represents the α- and ß-subunit RT-PCR products derived from total RNA from the equivalent of 5 embryos  Transcripts encoding Na/K-ATPase α2, α3, α4, and ß2-subunits were investigated with expected amplicon sizes of 335, 336, 338, and 441 bp, respectively  Lanes are L= DNA ladder, - = negative control (no cDNA), + = positive control (tissue cDNA), O  ovulated oocytes, 2= 2-cell embryos, 4= 4-cell embryos, 8= 8-cell embryos, 8C  8-cell compacting embryos, M= morulae, BL= blastocysts  Positive tissue controls were rat midbrain cDNA for α2, α3, and ß2-subunits and mouse testis cDNA for the α4-subunit  Transcripts encoding Na/K-ATPase α3 and ß2-subunit isoforms are present throughout preimplantation development, while transcripts for the α2-subunit were only found in oocytes  Transcripts for the α4-subunit were not detected in any murine embryonic stage
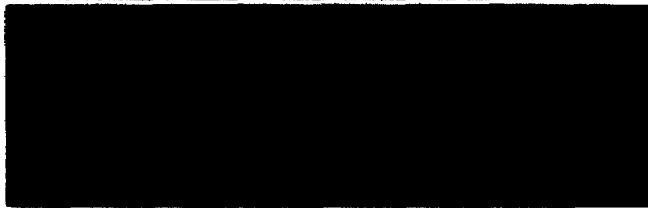
its derivation from both maternal and embryonic gene transcription  The identity of the α3 amplicon was confirmed with restriction endonuclease digestion analysis  For example, the restriction enzyme *Bcl* I cut the amplicon into the predicted 178 and 158 bp fragments (Table 3 1, Fig 3 2)  Transcripts of the α4-isoform gene were not detected in any of the preimplantation stages examined (Fig 3 1)  However, an α4-amplicon of predicted size was obtained from a control mouse testis RT preparation  The identity of the amplicon was confirmed by conducting the appropriate diagnostic restriction enzyme digestion (Fig 3 2)  The 441 bp β2 amplicon was also detected in RNA samples throughout murine preimplantation development (Fig 3 1)  Again, the apparent increase in β2 mRNA after the 4-cell stage indicated that the gene may be embryonically transcribed  Amplicon identity was confirmed with the restriction enzyme *Hae* III (Table 3 1, Fig 3 2)  α3 and β2 amplicon identities were also confirmed by direct sequencing and compared to the appropriate rat and mouse sequences from Genbank, utilizing the Genetics Computer Group (GCG) computer program GAP (Figs 3 3, 3 4)

*Detection of Na,K-ATPase α1 and β1-subunit mRNAs during horse development*

Both α1 and β1 mRNAs were detected throughout horse conceptus stages of day 12-28 (Fig 3 5)  The analyses were conducted on two different batches of embryos  Restriction enzyme analysis indicated the amplicons were the appropriate DNA sequences  *Ssp* I cut the α1 amplicon into the predicted 220, 116 bp pieces, while H*ind* III cut the β1 sequence into the predicted 246, 112 bp fragments (Fig 3 5)  Sequencing further verified the identity of the amplicons and the sequences were compared to the appropriate horse or rat sequences from Genbank (Figs 3 6, 3 7)  The horse α1 amplicon was found to be 97 6% identical with the corresponding region, exons 19-22, of the published horse α1 sequence (Kano *et al*, 1989) and the β1 amplicon was found to be 83 5% identical with the corresponding region of the rat β1 sequence (Mercer *et al*, 1986, Martin-Vasallo *et al*, 1989)

**FIGURE 3.2** Restriction endonuclease analysis of RT-PCR amplicons Mouse sodium pump isoform amplicons were digested with appropriate restriction endonucleases (see Table 3 2) to verify their identity  A  Mouse testes α4 amplicon digested with *Hae* III produced two bands of 242 and 96 base pairs (bps)  Only the 242 bp piece of DNA is visible on the gel  **B**  Mouse blastocyst α3 amplicon digested with *Bcl* I produced 179 and 157 bp fragments  **C**  Mouse oocyte α2 amplicon digested with *Ava* I produced 267 and 68 bp bands  **D**  Mouse blastocyst β2 amplicon digested with *Hae* III produced two bands of 291 and 150 bps  Bands are shown with arrows  Left lane of each gel represents the 1 kb DNA ladder while the right lane of every gel represents the digested PCR amplicons
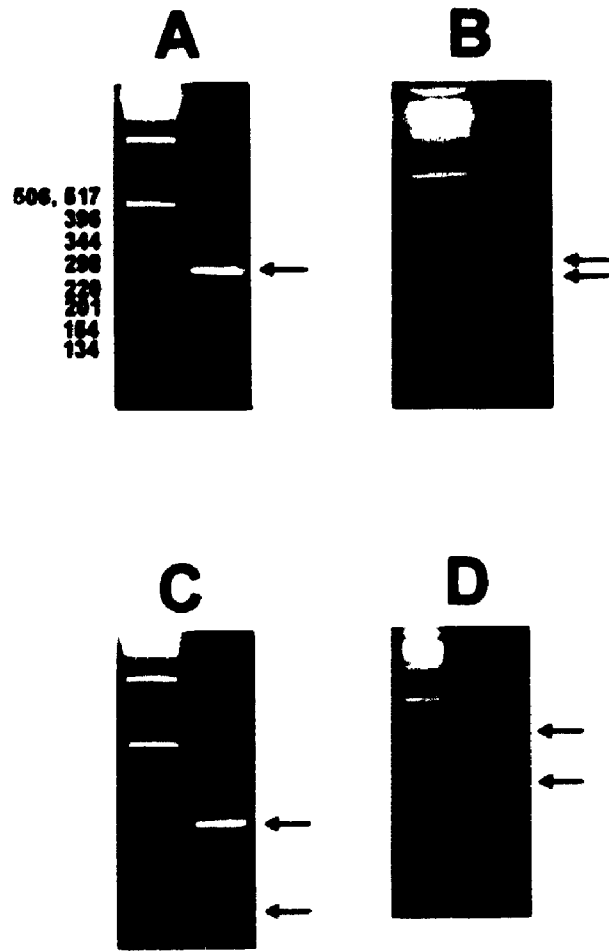
**FIGURE 3.3** Verification of the identity of mouse sodium pump α3 isoform PCR amplicon by direct sequencing The amplicon sequence (1-302 bases) was compared to the rat α3 sequence (2751-3100 bases, Genbank Accession # M14513) with the program GAP The amplicon had 90 1% sequence identity with the rat α3 sequence

```
                                             .
  .  ............................TGGGAGACGCATGTCAA 17
                                          ||      |||'|.
...  CC'GGTAACCTGGTGGGCATCCGGCTCAACTGGGATGACCGCACTGTCAA 2800

   .  TGACTAG..AAACAGTTATGGGCAGCAGTGGACTTATGAGCAGAGGAA.G 64
       .           |||  ||  ||||||||'||||||||||||||||||||.| |
...  'GACCTGGAAGACAGCTACGGGCAGCAGTGGACTTATGAGCAGAGGAAGG 2850

  ..  T'GTAGAGTTCACATGCCACACAGCCTTCTTTGTGAGTATCGTGGTGGTC 114
          |||||.|||| | |||||||||||||.|| ||||| ||| |||||.|||
295.  TGGTTGAGTTCACGTTC.CACACAGCCTTCTTCGTGAGCATAGTGGTGGTC 2900

  i1'  CAGTGGGCTGACCTGATCATCTGCAAGACCAGGAGGAACTCCGTCTTCCA 164
      '.||||| '|.  |||||||||  |||||||'||||||| |||||||||||
...  'AGTGGGCTGACCTGATCATTTGCAAGACCAGGAGGAATTCCGTCTTCCA 2950

  .'5  GCAGGGCATGAAGAATAAGATCTTGATCTTCGGCTTGTTTGAGGAGACGG 214
      |||||||'|'||||||||||||| .|||||||||| ||||||||||| |||'
2'6'I  GCAGGGCATGAAGAATAAGATCTTGATCTTCGGACTGTTTGAGGAAACGG 3000

  i1'  CCCTCGCTGCCTTCCTGTCCTACTGCCCAGGCATGGATGTGGCCCTTCGC 264
      ||||||||||||'|||||||||||||||||||||||||||||||||||||
3.Di  CCCTCGCTGCCTTCCTGTCCTACTGCCCAGGCATGGATGTGGCCC'TCGC 3050

  .'5  ATGTACCCTCTCAAGCCCAGCTGGTGGTTCTGTTCCCC............ 302
      |||.'||'' |||||||||||||||||||||||||||||| ||
4'51  ATGTACCCGCTCAAGCCCAGCTGGTGGTTCTGTGCCTTCCCCTACAGTTT 3100
```

**FIGURE 3.4** Verification of the identity of the mouse sodium pump β2 isoform PCR amplicon The amplicon sequence (1-340 bases) was compared to the mouse β2 sequence (951-1288 bases, Genbank Accession #X56007) with GAP The amplicon had 99 4% sequence identity with the mouse β2 isoform

```
951  CAGAAGAATGATGTCTGCCGTCCAGGGCGATATTATGAGCAACCTGATAA 1000
     ||||:|||||:|||||||||||||||||||||||:|||:||||||||||| :||
  1  .AGAAGAATGATGTCTGCCGTCCAGGGCGATATTATGAGCAACCTGATAA 49

1001 TGGGGTTCTGAACTACCCAAAACGTGCCTGCCAGTTCAACCGGACCCAAC 1050
     ||:::||||:||||||||||||||||||||||||:||||||||||||||||
  50 TGGGGTTCTGAACTACCCAAAACGTGCCTGCCAGTTCAACCGGACCCAAC 99

1051 TGGGCGATTGCTCTGGCATTGGGGACCCTACCCACTATGGTTACAGCACC 1100
     ||||:|||||||||||||||||||||||||||||||||||||:::||||||
 100 TGGGCGATTGCTCTGGCATTGGGGACCCTACCCACTATGGTTACAGCACC 149

1101 GGGCAGCCCTGTGTCTTCATCAAAATGAATCGGGTCATCAACTTCTATGC 1150
     ||:   |||||||||||||||||||.||||||||||||||||||||::   :||
 150 GGGCAGCCCTGTGTCTTCATCAAAATGAATCGGGTCATCAACTTCCTTGC 199

1151 AGGGGCAAACCAGAGCATGAATGTCACTTGTGTTGGCAAG.GAGATGAAG 1199
     ::::|||||::|||||||||||||||||||||||:|||:|||||| ||||:||||
 200 AGGGGCAAACCAGAGCATGAATGTCACTTGTGTTGGCAAGAGAGATGAAG 249

1200 ATGCTGAGAACCTTGGCCACTTTGTCATGTTCCCTGCTAATGGCAGCATT 1249
     ||||||||||:||||||||||||||||||||||||||||||||||||||||
 250 ATGCTGAGAACCTTGGCCACTTTGTCATGTTCCCTGCTAATGGCAGCATT 299

1250 GATCTGATGTACTTTCCCTACTATGGCAAAAAGTTCCAT.......... 1288
     |||||||||:|||||||||||||||||||||||||||||||
 300 GATCTGATGTACTTTCCCTACTATGGCAAAAAGTTCCAT          340
```

**FIGURE 3.5. A** Detection of α1 (top panel) and β1 (lower panel) isoform amplicons by RT-PCR in 12, 13, 14, 15, and 28 day (lanes 1-5 respectively) horse conceptuses W= water blank, K= horse kidney cDNA, G= horse kidney genomic DNA, L=1 kb DNA ladder, from top to bottom of ladder 517, 506 (one band), 396, 344, 298, 220, 201 base pairs (bp) The bottom panel has two additional ladder bands of 154 and 134 bp **B** Diagnostic restriction endonuclease analysis of RT-PCR amplicons Left panel α1 isoform UC= uncut cDNA amplicon, C= α1 amplicon digested with S*yp* I producing two bands of 237, 99 bp Right panel β1 isoform UC= uncut amplicon, C= β1 amplicon digested with H*ind* III producing two bands of 266, 112 bp L= 1 kb Ladder
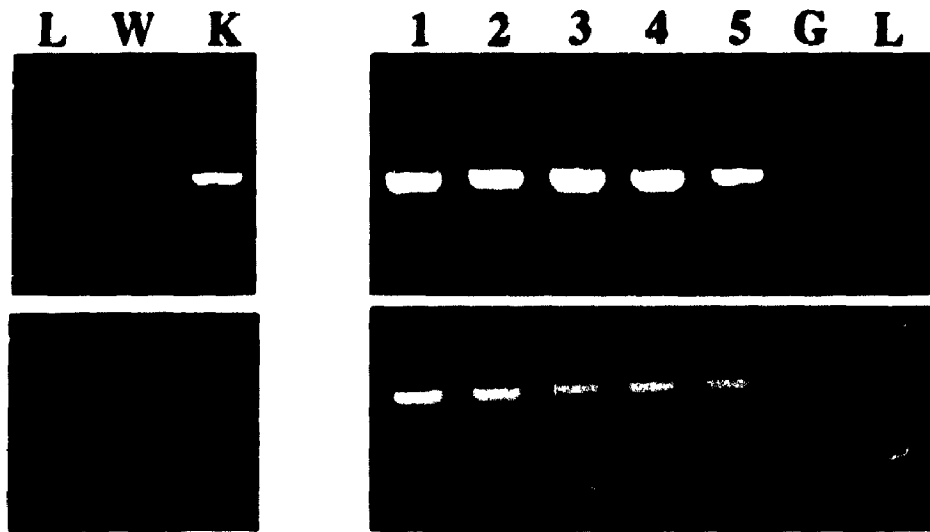
A

L W K    1 2 3 4 5 G L

B

UC C L    L UC C
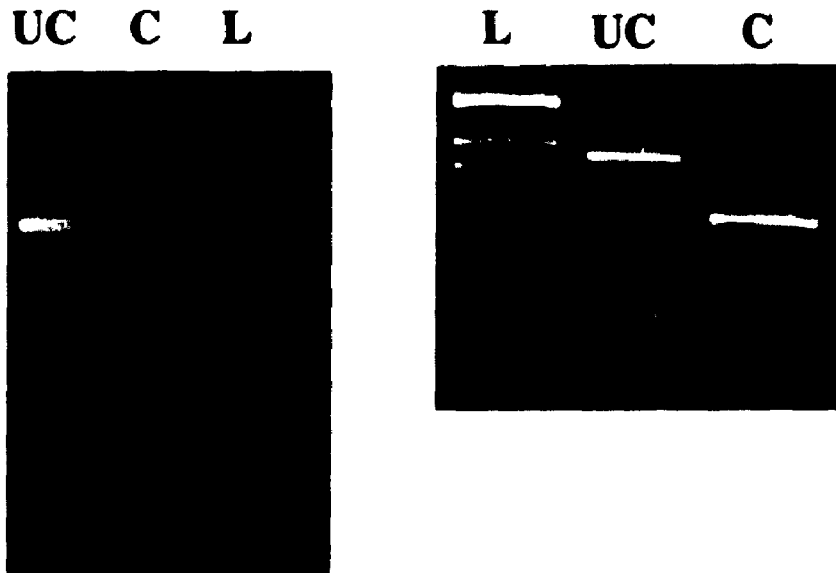
**FIGURE 3.6:** Verification of horse sodium pump α1 isoform amplicon identity The amplicon (1-288 bases) was compared to the published horse α1 sequence from Genbank (651-1000 bases, Accession # X16790-X16794) using the GCG program GAP The amplicon had 97 6% sequence identity with the Genbank sequence

```
651 GAGAATGGCTTCCTCCCAATTCACCTGCTGGGACTCCGCGTGGACTGGGA 700

  1 ..................................................G 1

701 TGACCGCTGGGTCAACGACGTGGAGGACAGCTACGGGCAGCAGTGGACTT 750
    ||||||||||||||||||||||| |||||||||||||||||||||||||||
  2 ATGACGCTGGGTCAACGACGTGGAAGACAGCTACGGGCAGCAGTGGACTT 51

751 ACGAACAGAGGAAAATCGTGGAGTTCACCTGCCACACAGCATTCTTCGTC 800
    | |||||||||||||||||||||||||||||||||||||||||||||||||
 52 ACGAACAGAGGAAAATCGTGGAGTTCACCTGCCACACAGCATTCTTCGTC 101

801 AGTATCGTGGTGGTACAGTGGGCCGACTTGGTCATCTGCAAGACCAGGAG 850
    ||||||||||||||||||||||||||||||||||||||||||||||||||
102 AGTATCGTGGTGGTACAGTGGGCCGACTTGGTCATCTGCAAGACCAGGAG 151

851 GAACTCAGTCTTCCAGCAGGGGATGAAGAACAAGATCCTAATATTTGGCC 900
    |||||||||||||||||||||||||||||||||||||||||||||  ||||
152 GAACTCAGTCTTCCAGCAGGGGATGAAGAACAAGATCCTAATATTTGGCC 201

901 TCTTCGAAGAGACGGCCCTTGCTGCCTTCCTTTCCTACTGCCCTGGAATG 950
    ||||||||||| ||||||||||||||||||||||||||||||||||||||
202 TCTTCGAAGAAACGGCCCTTGCTGCCTTCCTTTCCTACTGCCCTGGAATG 251

951 GGTGTGGCCCTGAGGATGTATCCCCTCAAGCCTACCTGGTGGTTCTGTGC 1000
    ||||||||||||||||||||||||||||||||||||||
252 GGTGTGGCCCTGAGGATGTATCCCCTCAAGCCTACCT.............. 288
```

**FIGURE 3.7** Verification of horse sodium pump ß1 isoform identity by direct sequencing The amplicon (1-324 bases) was compared to the published rat ß1 sequence (801-1197 bases; Genbank Accession # J02701) with the computer program GAP The amplicon had 83 % sequence identity with the rat sequence

```
   1 ..................................GATGTGGCAGTGTGCCCA  18
                                       |||||||| ||||||
 801 TTCGGCCCAGAAGGACGACATGATTTTCGAGGATTGTGGCAGTATGCCCA 850

  19 GCGAACTCAAAGAAACGAGGAGAATTTAACAACGAACGAAGGAGAGCGAA  68
     | |||| ' ||| | || |||||' || ||  ' ||||| |||.||||| |
 851 GTGAAC.CCAAGGAGCGGGGAGAGTTCAATCATGAACG.AGGAGAGCGCA 898

  69 AAGTCTGCAGGTTCAAGCTTGAGTGGTTGGGAAATTGCTCTGGAATAAAT 118
     | || ||:|||||||||||||||| ||| |||| || ||||||||  | |||
 899 AGGTGTGCAGGTTCAAGCTTGACTGGCTGGGGAACTGCTCTGGTCTCAAT 948

 119 GATGAAACTTACGGCTACAAAGGAGGGCAAACCATGTGTCATTATAAAGC 168
     ||||||  ||||||||||||| ||||| || || ||| |||||||| ||||
 949 GATGAATCCTACGGCTACAAA.GAGGGGAAGCCCTGTATCATTATCAAGC 997

 169 TCAACCGAGTTCTGGGCTTCAAACCTAAGCCTCCTAAGAATGAGTCCTTG 218
     |||||||| | ||||||||||||||||||||||||| |||||||| ||||||
 998 TCAACCGAATGCTGGGCTTCAAACCTAAGCCTCCCAAGAATGAATCCTTG 1047

 219 GAGACTTACCCAGT...GATGAAGTACAGCCCGTACGTCCTGCCTGTTCA 265
     |||||||||||| |   ||||||||| | || ||||||| |||'|| ||
1048 GAGACTTACCCTCTGACGATGAAGTATAATCCAAACGTCCTACCTGTCCA 1097

 266 GTGCACTGGCAAGCGTGATGAAGATAAGGAAAAGATTGGAAACGTGGAGT 315
     :||||||||||||||| ||||| ||||||||| || |||||||| | ||||
1098 GTGCACTGGCAAGCGCGATGAGGATAAGGATAAAGTTGGAAACATAGAGT 1147

 316 ATTTTGGAC.................................  324
     | ||'|||
1148 ACTTTGGGATGGGCGGATTCTATGGCTTTCCTCTGCAGTACTATCCCTAC 1197
```

*Immunocytochemistry with Mouse Embryos*

### DGD Sections

A number of antisera were tested for use with DGD sections of mouse embryos Surprisingly, only a few antisera produced a detectable amount of signal in the tissue sections UBIα1 isoform specific polyclonal antiserum (Table 3 2) did not produce any detectable signal above background in two different experiments utilizing two DGD blocks, each containing 50-100 embryos NASEα1 also did not produce detectable signal in three different experiments even though the same antiserum detected α1 subunits in basolateral domains of mouse kidney tubules (Fig 3 14) TEDα3 and HEREDα2 did not detect any of the corresponding subunits in mouse embryo sections

Askari β1 detected β1 subunits in basolateral domains of mouse mural trophectoderm and a very low level in the cytoplasm of inner mass cells and blastomeres of morulae (Fig 3 9) That antibody also detected β1 subunits in basolateral domains of mouse kidney tubules McTub localized α-tubulin in mouse kidney tubules (Fig 3 8)

UBI α1 and β1 antisera were also tested on cryosections of mouse kidney, with and without 0 1% Triton X-100 as a permeabilizing detergent Both antisera did not detect the corresponding subunits in mouse kidney tubules

Mouse blastocysts, fixed exactly as described for DGD embedding, were also embedded in polyethylene glycol (PEG), according to Watson and Kidder (1988), to determine whether the composition of the embedding medium could effect localization of sodium pump α1 subunits by NASE In four experiments, no detectable levels of α1 subunits were detected in PEG sections of blastocysts No detectable levels of β1 subunits were found with the Askari antiserum on PEG embedded tissue even though this antiserum recognized β1 subunits in DGD sections of blastocysts

**FIGURE 3.8.** Detection of sodium pump β1 subunits in mouse kidney DGD sections **A** kidney incubated with Askari β1. **B** secondary control goat antiserum raised against rabbit IgG **C** phase contrast image of kidney section **D** kidney incubated with McTub to detect α-tubulin **E** secondary control secondary rabbit antiserum raised against mouse IgG Scale Bar= 25 μm
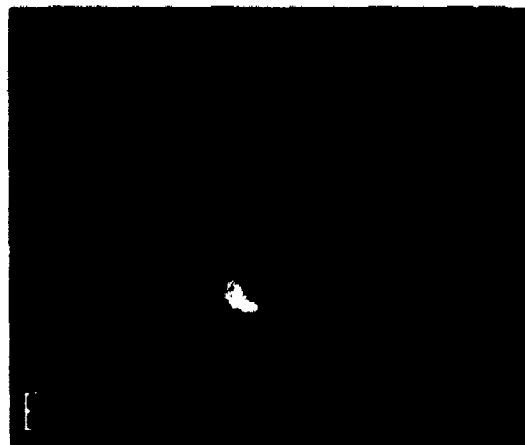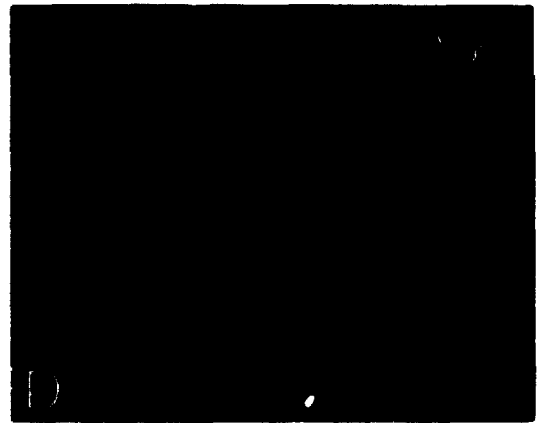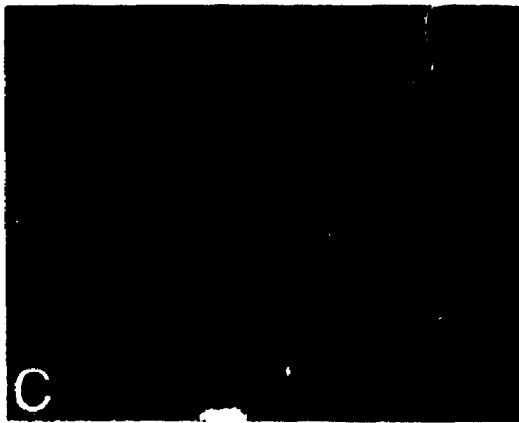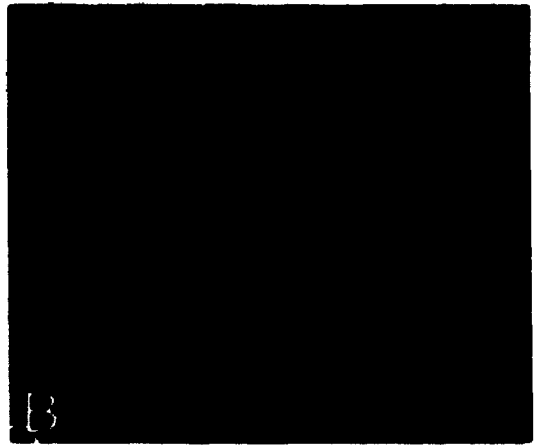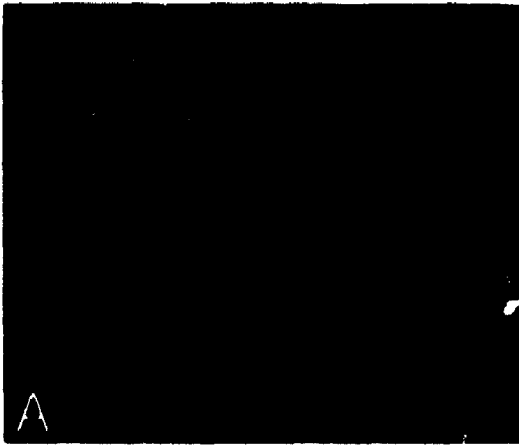
**FIGURE 3.9.** Detection of sodium pump ß1 subunits in DGD sections of mouse blastocysts with Askari ß1  **A** blastocyst  **B** morulae  **C** blastocyst incubated with secondary goat antiserum raised against rabbit IgG  Scale Bar= 25 μm

## Wholemount Indirect Immunofluorescence with Paraformaldehyde Fixation

The purpose of utilizing this procedure was to try to utilize the laser scanning confocal microscope's higher resolution imaging capabilities UBI$\alpha$1 and B1 were tested to detect the corresponding isoforms in mouse blastocysts and late morulae No membrane staining was observed in any of the embryos with this procedure $\alpha$ tubulin was detected in all embryos examined

To test whether increased fixation, over that of 1% paraformaldehyde, could increase detection of subunits in membranes of mouse preimplantation stages. blastocysts and morulae were fixed with 4% or 2 5% paraformaldehyde in PHEM buffer Increased fixation did not produce any detectable membrane staining

### Methanol Fixation

No sodium pump $\alpha$ isoforms or B1 subunits were detected in methanol fixed blastocysts or morulae with NASE$\alpha$1, HERED$\alpha$2, TED$\alpha$3 or Askari$\beta$1 antisera In case the Triton X-100 concentration was too high. over extracting blastomere membranes. another experiment tested 0 01% Triton X-100 extraction with the antisera above and McTub antiserum Sodium pump subunits were not detected, although $\alpha$-tubulin was detectable in microtubules of mouse embryos examined The lack of detectable $\alpha$1 subunits was surprising because Becker and Davies (1994), using the identical immunocytochemical procedure and NASE$\alpha$1, observed general cytoplasmic localization but no membrane staining of $\alpha$1 subunits throughout mouse blastocysts Higher concentrations of NASE and Askari antisera were tested (1 20) but no $\alpha$1 or B1 subunits were detected in blastocysts

With the localization of $\alpha$-tubulin throughout the mouse embryo after extraction with 0 01% Triton X-100. future experiments utilized the same detergent concentrations Utilizing CAPLAN $\alpha$1. $\alpha$1 subunits were detected from 4-cell stage embryos to blastocysts (Fig 3 10) In 4-cell embryos (n=61) fluorescence was localized to blastomere

**FIGURE 3.10.** Detection of α1 subunits of the sodium pump in membranes of mouse preimplantation stages **A** 4-cell **B** 8-cell **C** compacting 8-cell embryo **D** morula **E** blastocyst An embryo incubated in rabbit antiserum raised against mouse IgG served as a secondary control and can be seen in **Fig. 3.12. panel G.** Note that there is little or no staining of α1 subunits in the apical membranes of the mural and polar trophectoderm of blastocysts (arrows) Scale Bar 25 µm
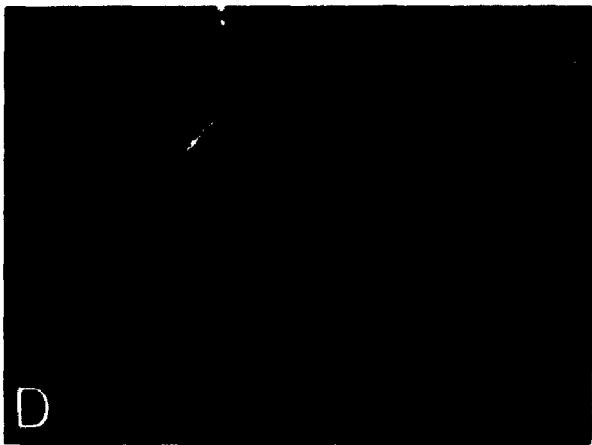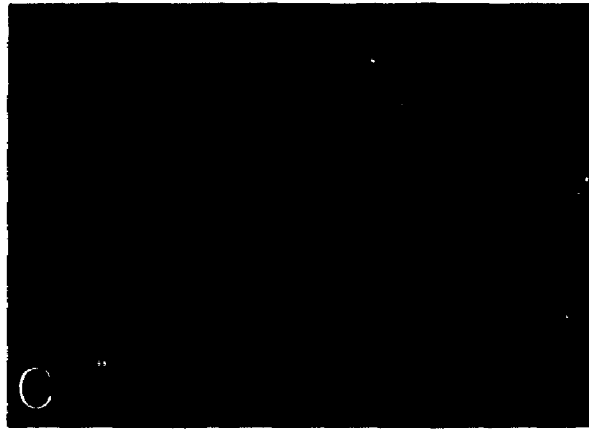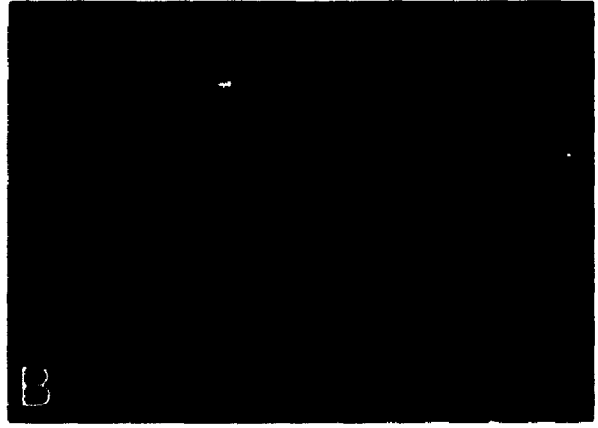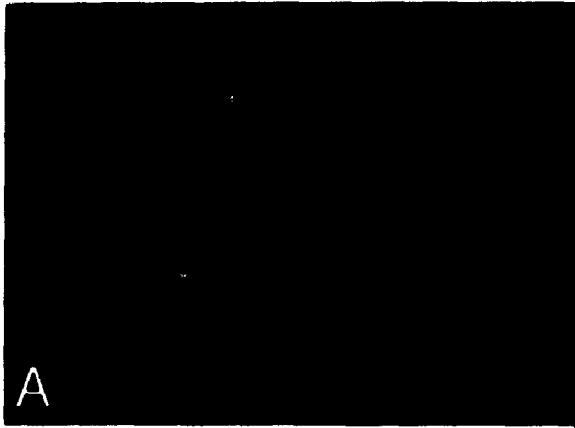
membranes that were in contact or close apposition to one another In 8-cell embryos (n=31), α1 subunits were detected throughout blastomere membranes and as embryos compacted (n=10) and became morulae (n=25), fluorescence became more intense but still localized throughout blastomere membranes In blastocysts (n=43), fluorescence became localized to basolateral domains of mural trophectoderm, surrounding the blastocoel, and to membranes of inner cell mass Little or no fluorescence was detected in apical membranes of mural or polar trophectoderm

The experiments with 4-cell to compacting 8-cell embryos, described above, utilized 50% methanol as a fixative One experiment with 4-cell stage embryos utilized 100% methanol as fixative The embryos still exhibited the same localization pattern for α1 subunits, but the embryos appeared shrunken and in a false state of compaction Therefore, all subsequent immunocytochemical experiments with methanol utilized 50% methanol to minimize shrinkage

α2 subunits were not detected in 4-cell, 8-cell, morulae or blastocysts (n 22, 25, 25, 18 respectively) with McB2 monoclonal antisera Utilizing RDIα3, α3 subunits were not detected in membranes of any preimplantation stages (4-cell, n= 18, 8-cell, n 22, morulae, n=31, blastocysts, n=41), however, faint cytoplasmic staining was observed in all stages (Fig 3 11) A few nuclei stained brightly in each embryo and may represent non specific primary antibody binding since nuclear staining was absent in secondary control embryos

Utilizing CAPLAN β1, β1 subunits were not detected in 4-cell, 8-cell, or 8-cell compacting mouse embryos (n= 25, 17, 17, respectively) but became detectable in apposing membranes and in more limited extent cytoplasmically in late morulae (n 35) (Fig 3 12) The isoform later became localized around the blastocoel and in apposing membranes of the inner cell mass of blastocysts (n= 26) No detectable fluorescence was observed in secondary controls of the above experiments (4-cell n 42, 8-cell n 35 8-cell

**FIGURE 3.11.** Detection of α3 subunits in the cytoplasm of preimplantation stages **A** 4-cell **B** 8-cell **C** morula **D** blastocyst Note the non-specific nuclear staining in some regions of the embryos (arrows) An embryo incubated in rabbit antiserum raised against mouse IgG served as a secondary control and can be seen in **Fig. 3.12, panel G.** Scale Bar = 25 μm
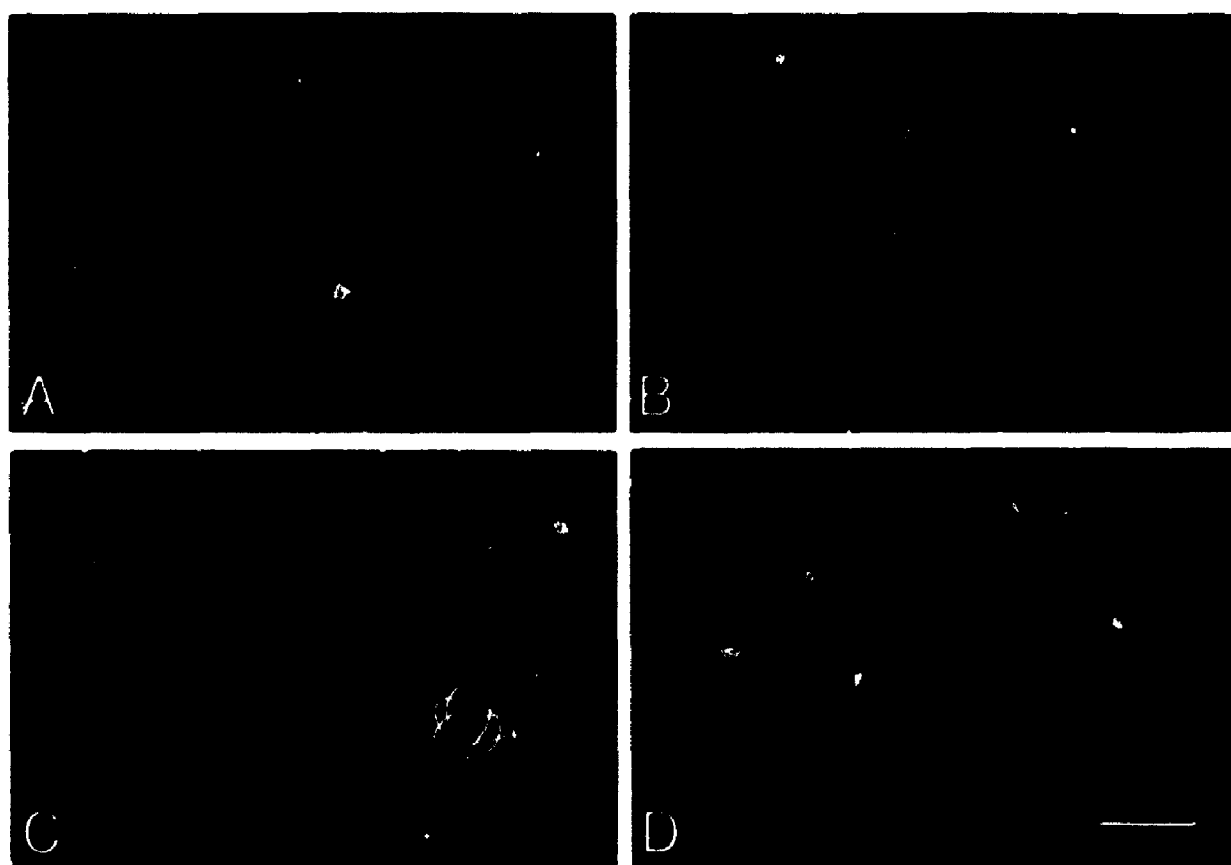
**FIGURE 3.12.** Detection of β1 subunits in preimplantation stages of mouse embryos **A** 4-cell **B** 8-cell **C** compacting 8-cell **D** morula **E-F** blastocysts **G** secondary control  rabbit antiserum raised against mouse IgG  Note that β1 subunits become more discernible in basolateral membranes of the older blastocyst (**F**)  Subunits are also detected in membranes of morulae and inner cell mass of blastocysts (arrows)  Scale bar = 25 μm
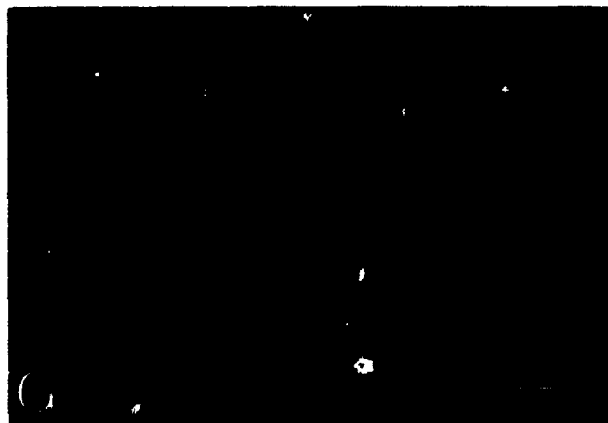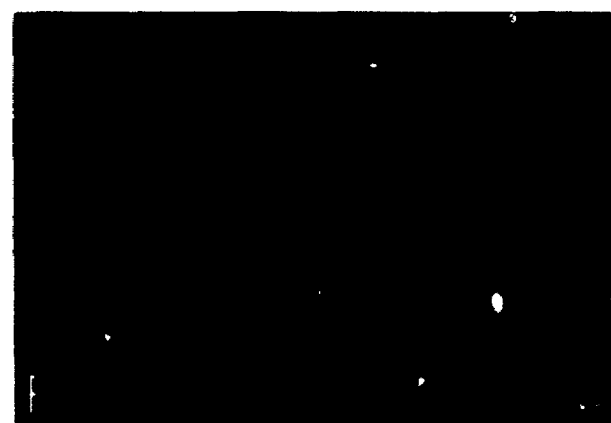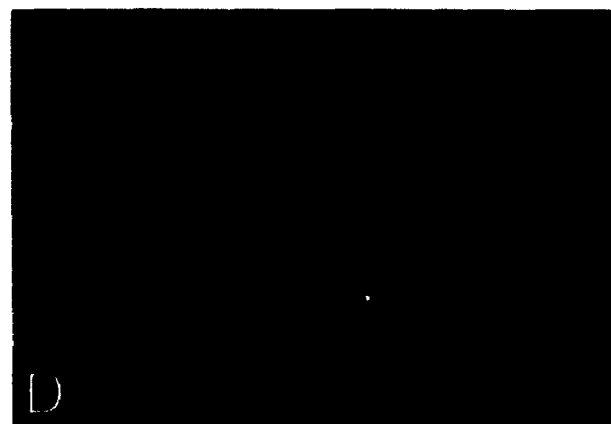
compacting n=24, morulae n=31, blastocysts n=40) incubated only with rabbit antisera raised against mouse IgG

β2 subunits were not detected in membranes of any preimplantation stages (4-cell, n 17, 8-cell, n= 19, morulae, n= 16, blastocysts, n= 18) compared to secondary controls of goat antisera raised against rabbit IgG (n= 11, 17, 26, 21 respectively), although some faint cytoplasmic fluorescence and some nuclear staining was detectable in preimplantation stages (Fig 3 13) The nuclear staining may again represent non specific primary antibody binding This same antiserum localized β2 subunits in DGD sections of mouse brain (D Betts, personal communication)

*Immunocytochemistry with Horse Embryos*

Utilizing NASE, α1 subunits were detected in 10 μm paraffin sections of two 16 day old horse conceptuses as a ring of fluorescence surrounding the expanding blastocoel (Fig 3 14) Upon closer examination, the fluorescent signal was detected in the basal regions of the trophoblast cell layer and developing endodermal cell layer

Utilizing Askari β1, β1 subunits were also detected in the same region of the horse conceptus sections examined (Fig 3 14) A more detailed analysis utilizing the same primary antiserum, an additional α1 antiserum (UBIα1) and a Histostain-SP[TM] streptavidin-peroxidase immunohistochemical kit (Zymed Laboratories) has indicated that both α1 and β1 subunits are found throughout the trophoblast and endoderm layers (Dr R Waelchli, personal communication)

*Western Blot Analysis*

### Mouse Embryos

α3 subunits (approximately 100-115 kDa) were detected in mouse brain and faintly detected in blastocysts and morulae (Fig 3 15) with CAPLANα3 The antiserum

**FIGURE 3.13.** Detection of ß2 subunits in mouse preimplantation stages  A  4-cell  **B**  8-cell  **C**  morula  **D**  blastocyst  **E**  secondary control  goat antiserum raised against rabbit IgG  Faint cytoplasmic staining was observed in embryo stages and some nuclear staining (arrows)  Less fluorescent signal was observed in the mural trophectoderm  Scale Bar = 25 µm
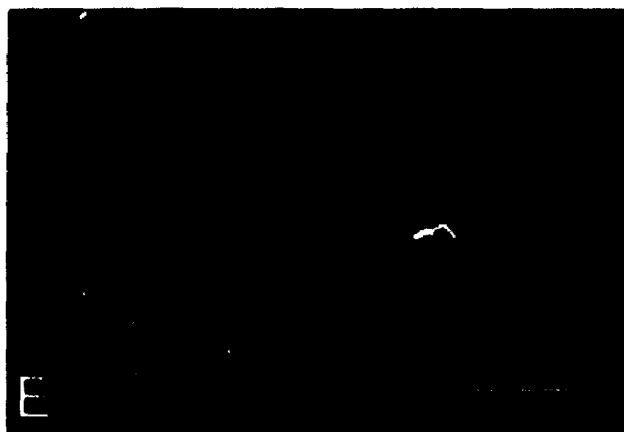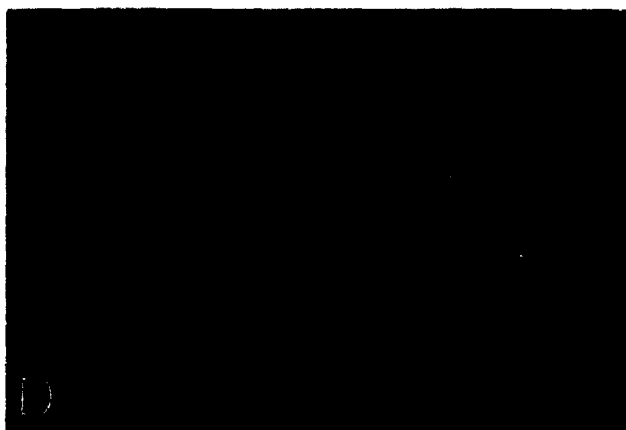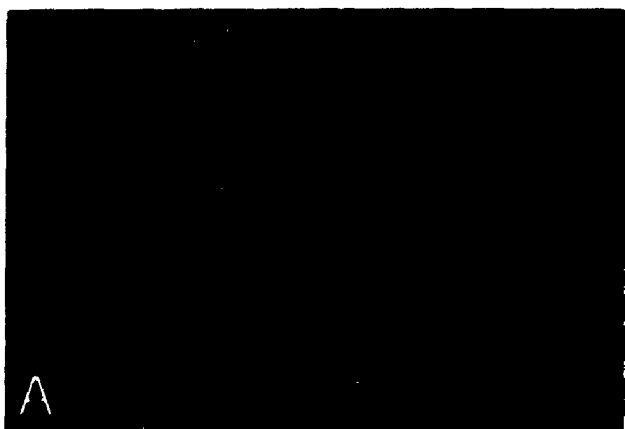
**FIGURE 3.14.** Localization of sodium pump α1 and β1 subunits in horse conceptuses  **A**  mouse kidney tubules incubated with NASE α1  **B**  phase contrast of mouse kidney tubules  **C**  day 16 horse conceptus incubated with NASE α1  **D. F**  secondary controls  goat antiserum raised against rabbit IgG  **E**  day 16 horse conceptus incubated with Askari β1  **G**  phase contrast of **E**  Note the presence of α1 and β1 subunits in the basal regions of trophoblast overlying the endoderm (arrows)  Scale Bar = 25 μm for **A, B, E, F, G**  Scale Bar = 25 μm for **C, D**
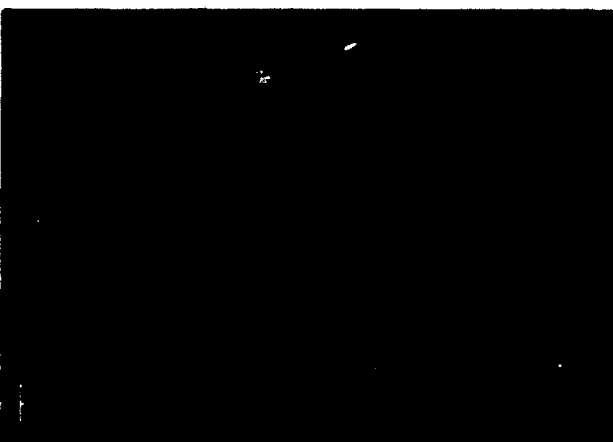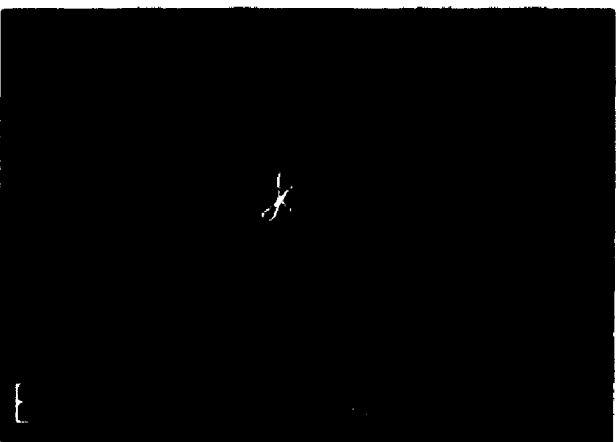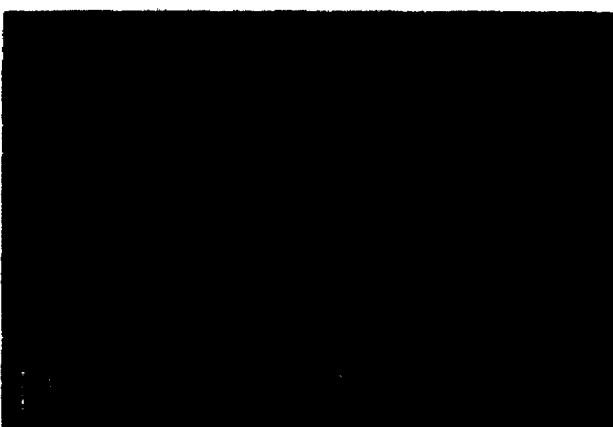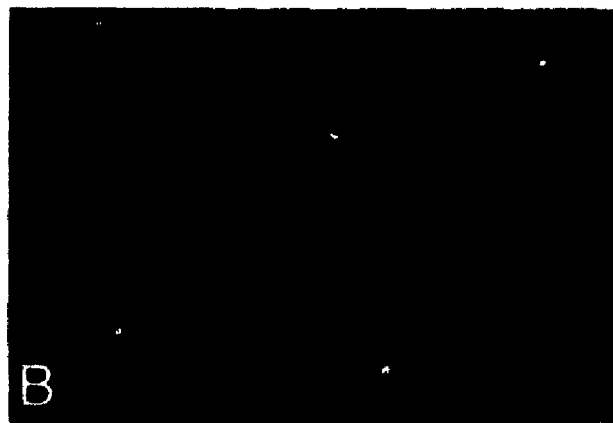
**FIGURE 3.15.** **A** Western blots of total protein from mouse kidney, brain, liver, embryo washes, and 650 blastocysts/morulae (lanes 1-5 respectively) Blots were incubated with CAPLAN α3 and HERED α2 antiserum Antisera detected proteins at approximately 115 kDa **B** Western blots of total protein from mouse kidney, brain, liver or day 16 horse conceptus (lanes 1-4 respectively) Blots were incubated with NASE α1 or Askari β1 Antisera detected glycosylated β1 subunits at approximately 45-65 kDa and partially or non-glycosylated subunits at approximately 30 kDa

**A**

1 2 3 4 5

200.0 —
97.4 —
68.0 —

43.0 —

α3

200.0 —
97.4 —
68.0 —

43.0 —

α2

**B**

1 2 3 4

200.0 —
97.4 —
68.0 —

43.0 —

α1

200.0 —
97.4 —
68.0 —
43.0 —

29.0 —

18.4 —

β1
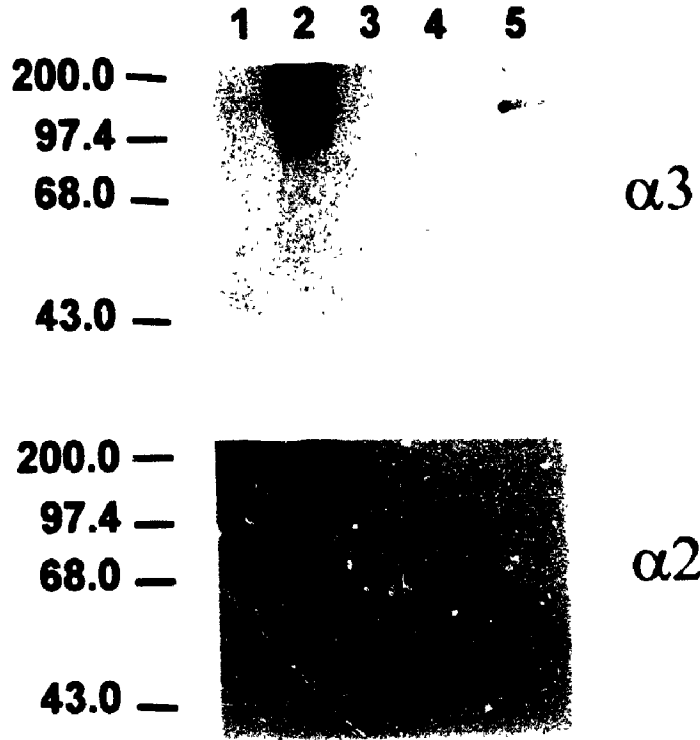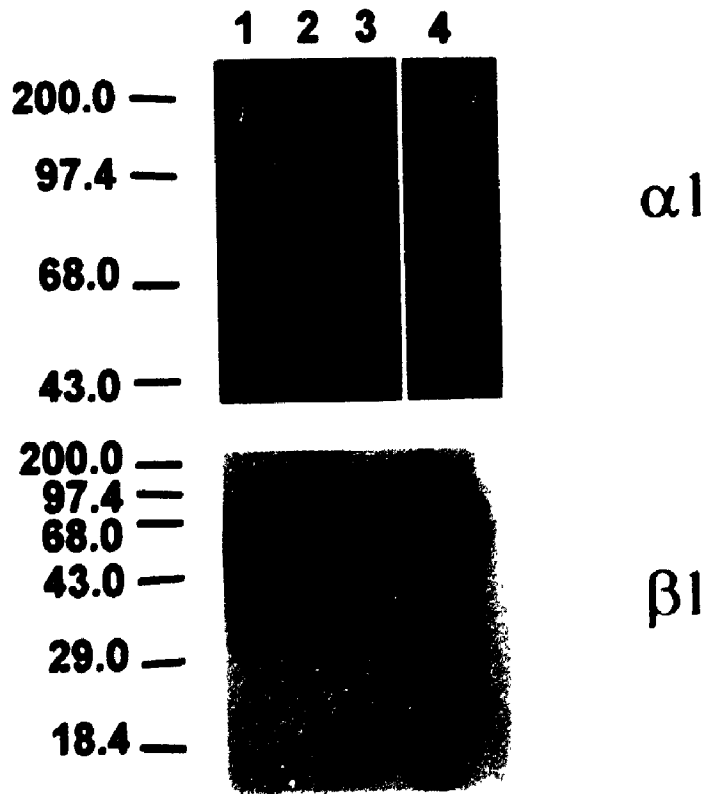
did not detect the α3 isoform in kidney. liver or the last wash control α2 subunits

(approximately 100-115 kDa) were detected in mouse brain and in no other tissue or

embryo sample (Fig 3 15) The presence of ß2 subunits is unknown UBIß2 antiserum

failed to detect any appropriate protein after prolonged exposure (1 hour) to x-ray film. in

mouse kidney, brain. liver, and mouse blastocyst/morulae Background chemiluminescence

was high with this antiserum

### Horse Embryos

α1 subunits were detected in mouse kidney controls and in a horse embryo total

protein sample (Fig 3 15) α1 subunits were not detected in mouse brain or liver ß1

subunits of approximately 50-65 kDa, were detected in mouse kidney and brain controls

as well as horse embryo total protein (Fig 3 15) In the kidney, brain, and liver controls,

non- or partially glycosylated ß1 subunits were also detected around 32-40 kDa

## 3.4 DISCUSSION

*Expression of Multiple Sodium Pump Isoform mRNAs and proteins in the Mouse*

In this study, an earlier analysis of Na.K-ATPase isoform expression in murine

preimplantation embryos has been updated to include all sodium pump isoforms The

oligodeoxynucleotide primers were designed through use of mouse and rat cDNA

sequences (Gloor *et al.*, 1990, Martin-Vasallo *et al*, 1989. Young *et al.*, 1987, Shull *et

al.*, 1986) Their ability to amplify specific murine embryo cDNAs was verified in all cases

by observing the expected size of the amplified DNA fragment in two replicates of murine

early developmental embryo stages, cleaving each amplicon with a diagnostic restriction

enzyme to produce DNA fragments of expected size, and direct sequencing

Na.K-ATPase is present in most (if not all) mammalian cell membranes. where it

functions to regulate cell volume and ion concentration Na.K-ATPase activity has been

measured in mouse ova (Powers and Tupper, 1975) and two cell embryos (Powers and

Tupper, 1977). but the enzyme is not detectable by immunofluorescence until the late

morula stage using an antiserum raised predominantly against the α1 isozyme of rat kidney

(Watson and Kidder, 1988) With the onset of cavitation, the Na,K-ATPase

immunofluorescence pattern undergoes a dramatic change appearing as a fluorescent ring encircling the blastocyst cavity, restricted to the basolateral plasma membrane domains of the mural trophectoderm (Watson and Kidder, 1988) In contrast Gardiner *et al* (1990b) reported the presence of Na,K-ATPase α- and ß-subunit polypeptides throughout early murine development They detected three α-subunit protein bands, on western blots, that remained at constant levels during the preimplantation period The authors utilized a rabbit anti-guinea pig Na,K-ATPase antiserum that cross reacted with α1, α2 and ß subunits They could not discriminate whether the three bands observed represented α subunit isoforms

Van Winkle and Campione (1991) were unable to detect more than a 2-fold increase in Na,K-ATPase activity from early cleavage stage embryos to the blastocyst stage Since the various Na,K-ATPase isozymes display different inhibitory sensitivities to ouabain (specific inhibitor of Na⁺-pump activity), the general conclusion from these published data is that additional isozymes of the Na,K-ATPase may be expressed during early mammalian development For these reasons it was essential to characterize the expression of all of the presently reported isoforms of the Na,K-ATPase subunits during murine preimplantation development

Northern blot analyses employing cDNAs encoding three α-subunit isoforms (α1, α2, α3) of the Na,K-ATPase suggested that only the α1- (ouabain "insensitive") isoform is expressed during murine preimplantation development (Watson *et al.*, 1990a,b) However, I have determined that multiple α-isoforms of the Na,K-ATPase are expressed throughout early murine development by applying the more sensitive technique of RT-PCR α3 mRNAs were detected throughout the first week of murine development, although failure to detect them by northern blotting indicates their very low abundance α3 subunits in morulae/blastocyst total protein were faintly detected with a much longer exposure to x-ray film than for detection of the subunit in mouse brain total protein The relative insensitivity of mouse cavitation to ouabain implies that the highly ouabain sensitive α3-isoform is not a dominant factor during mouse cavitation

Transcripts encoding the α4-isoform were not detected in murine early embryos This result is not completely unexpected, since the expression of this recently

characterized Na -pump isoform is predominantly confined to the testes (Shamraj and Lingrel. 1994) Due to the absence of an antibody against the $\alpha4$ subunit. it was not possible to determine the presence or absence of the protein in preimplantation embryos $\alpha2$ mRNA was not detected in any preimplantation stages except for oocytes and this detection varied among three different oocyte preparations The isoform was also not detected in mouse morulae/blastocysts on western blots indicating no role for this isoform during mouse cavitation

Following preimplantation development. Herrera *et al* (1994) detected generalized co-expression of $\alpha1$ and $\alpha2$ isoform transcripts in the 9 5 - 10 5d post-coital mouse embryo By 15 5 - 16 5 days post-coitus, differential expression of $\alpha1$. $\alpha2$, and $\alpha3$-transcripts becomes evident in developing organs The results in the preimplantation embryo raise the possibility that two Na,K-ATPase $\alpha$ subunit isoforms ($\alpha3$, $\alpha1$) are co-expressed in the early mammalian embryo prior to tissue specific differentiation

Northern blot analysis has revealed that transcripts encoding the mouse $\beta1$-isoform are first expressed in the late morula with the level increasing by the blastocyst stage (Watson *et al*. 1990a) The $\beta1$-subunit mRNA has. however, been detected in early cleavage stages by RT-PCR (Kidder. 1993a) These results suggest that the timing of expression of the $\beta1$-subunit is closely coordinated with the onset of cavitation and may be important in regulating Na,K-ATPase activity and thus blastocyst formation Although the $\beta2$ isoform mRNA was detected throughout preimplantation development (Fig 3 1). the mRNAs encoding the $\beta2$-isoform do not likely accumulate to an appreciable level in the mouse as they were not detected by northern blot analysis (Kidder, 1993b) Determination of the presence or absence of the subunit in morulae/blastocyst total protein was inconclusive Two different blots were utilized to try to detect the protein In both cases. after prolonged exposure. no $\beta2$ protein was detected and high background was observed with this antiserum This analysis, therefore. requires a new antiserum raised against the $\beta2$ subunit I have been unable to obtain aliquots of a second polyclonal antiserum raised against this subunit. also known as AMOG (see Chapter 1)

A $\beta2$ null mutant mouse appears to suffer no ill effects during preimplantation development (Magyar *et al*.. 1994) Therefore. the $\beta1$-isoform is still the most reasonable

candidate for regulating the expression of functional enzyme units in the trophectoderm coordinate with the onset of cavitation Gardiner *et al* (1990b) detected a 2 fold increase in ß1 subunit protein levels between the 2-cell stage and embryos beginning a morula to blastocyst transition, followed by a 9-fold increase in fully formed blastocysts The initial increase during the 2-cell stage may not be detectable by immunofluorescence compared to the blastocyst stage My immunofluorescence results suggest this is true since I detected ß1 subunits initially in morulae then a stronger fluorescent signal in blastocysts

The expression pattern of sodium pump subunit isoforms has recently been examined in the bovine preattachment embryo (Betts *et al* , 1996, in press) $\alpha1, \alpha2, \alpha3$, and ß2 transcripts were detected from the 1-cell zygote to blastocyst stages However, ß1 transcripts were only detectable from the morula stage onward This expression pattern is similar to that in the mouse, where ß1 transcripts were not detectable on northern blots prior to the morula stage, but accumulated thereafter at the same time that the enzyme became detectable by immunocytochemistry (Watson *et al* , 1990a,b) Thus, in both species. expression of the ß1 subunit is closely coordinated with the onset of cavitation The two species are different, however, because unlike the mouse, $\alpha2$ transcripts were detected throughout bovine preattachment stages and the $\alpha3$ subunit was detected in apical membrane domains of the trophectoderm (Betts *et al* , 1996, in press)

The expression of Na/K-ATPase genes has also been examined in later developmental stages of the mouse and adult organs Usually the abundance of $\alpha$-subunit mRNA exceeds that of ß-subunit mRNA suggesting that ß-subunit mRNA content may determine the total Na/K-ATPase activity (McDonough *et al.*, 1992, Gick *et al.*, 1993) During fetal lung development, for example, $\alpha1$ subunit mRNA is more abundantly expressed than ß1 mRNA (O'Brodovich *et al.*, 1993) The presence of multiple Na-pump isoforms in adult rodent and human tissues is well documented $\alpha1, \alpha2$, and $\alpha3$ isoforms are co-expressed in organs such as brain, eye, heart, and skeletal muscle (Rose and Valdes, 1994) while ß1 and ß2 isoforms are co-expressed in brain, heart, spleen and lung (Schmalzing and Gloor, 1994) Ahn *et al* (1993) and Clapp *et al* (1994) detected co-expression of $\alpha$ and ß-isoform mRNAs throughout the kidney by *in situ* hybridization and

RT-PCR Thus. with the use of sensitive mRNA phenotyping by RT-PCR, it is very likely that additional tissues will be found to express multiple isoforms of sodium pump subunits

## Multiple Isoforms and Preimplantation Development

With the detection of multiple subunit isoforms, what roles do the various possible isozymes of the sodium pump have during preimplantation development? Different isozymes do display distinct ion affinities and Na and K concentrations can influence isoform expression Jewell and Lingrel (1991) found that rat $\alpha 1$- and $\alpha 2$-containing sodium pump molecules, expressed in HeI a cells, have similar apparent affinities for Na , but that $\alpha 3$-containing enzyme has a 2-3 fold lower affinity for this ion McDonough *et al* (1992) found that low K concentrations decrease $\alpha 2$ mRNA levels and protein levels by $38\%$ and $82\%$, respectively, in hypokalemic skeletal muscle When K concentrations were restored, the mRNA and proteins also rose to pre-hypokalemic levels

If the ionic environment surrounding the mouse preimplantation embryo (i e oviduct or uterus) were to change suddenly or for a prolonged period of time during development, the potential use of different sodium pump isozymes might be advantageous Borland *et al* (1977) measured the concentrations of a variety of ions in the oviduct during the early period of embryonic development (2-cell stage) For example, Na and K concentrations ranged from 139-154 mM and from 23 4-25 5 mM, respectively, in the oviduct 11-36 hours post coitus, but uterine fluid 1-4 days post coitus contained 114 mM Na and 31 6 mM K Although uterine fluid ionic concentrations have not been determined during blastocyst development, mostly due to very low levels of fluid just prior to implantation (Borland *et al*, 1977), significant changes in ionic composition might effect sodium pump isoform expression

The ß-subunit may be involved in stabilizing the correct transmembrane folding of the $\alpha$-subunit (Geering, 1991, McDonough *et al.*, 1993) rather than being involved directly in the ion transport process However. different ß-isoforms (ß1 or ß2) combined with various $\alpha$-isoforms may produce modified membrane conformations and therefore influence enzyme kinetic properties For example, when the mouse ß2 isoform was expressed with $\alpha 1$ subunits in *Xenopus* oocytes, lower apparent K affinity was observed

than with the β1 isoform (Schmalzing *et al.*, 1992) The β subunit can also influence the extracellular K activation of the enzyme (Jaisser *et al*. 1992) and can be actively involved in the formation or stabilization of the K occluding complex of the enzyme (Lutsenko and Kaplan, 1992)

Sodium pump isoform expression can also be differentially regulated by hormones (Horowitz *et al.*, 1990, Orlowski and Lingrel, 1990, see Chapter 1) and α-isoform genes contain 5' specific sequences that are potential *trans* acting factor and hormone binding sites (Kawakami *et al.*, 1994, Rose and Valdes, 1994) Whether any of these sequences could operate during preimplantation development to generate the mRNA phenotypes discussed above, remains to be determined

In summary, expression of mRNAs encoding the α2 (in oocytes), α3, and β2-subunit isoforms of the Na,K-ATPase during preimplantation murine development has been demonstrated, but α2 subunits were not detected in morulae and blastocysts and detection of β2 subunits is uncertain With the detection of α3 subunits in the same embryo stages, the findings suggest the possibility of up to two different α/β isoform pairs, and therefore, potentially two distinct Na,K-ATPase isozymes in early mammalian embryos Further research should be directed at determining the functional role(s), if any, the second isozyme (α3β1) could play during early mammalian development, specifically in mediating morphogenetic events such as blastocyst formation

### Expression of α1 and β1 isoforms in horse conceptuses

The horse has a maximally expanding blastocyst (Biggers, 1972) with the diameter of the embryo increasing from ~200 μm at day 7 of development to 25 mm at day 16 There is also substantial turgidity of the expanding blastocyst (Ginther, 1986, see Introduction) However, the blastocyst fluid after day 10 to at least day 25 is markedly hypotonic compared to serum (Waelchli *et al*, 1996), unlike mouse blastocoel fluid (Borland *et al*, 1977) With the considerable expansion of the horse blastocoel, it was important to determine whether the sodium pump might have a role in horse embryo blastocoel formation and expansion

The detection of α1 and β1 isoform mRNAs in 12-28 day old horse conceptuses parallels data in the mouse (Watson et al., 1990a,b, Gardiner et al., 1990b), rabbit (Gardiner et al., 1990a) and cow (Betts et al., 1996, in press) for the presence of sodium pump transcripts during blastocyst development and expansion in the mouse Sequencing confirmed the identity of the horse α1 sequence and provided new sequence information on the horse β1 transcript Immunocytochemistry indicates that the two subunits are localized somewhat basally in trophoblast cells, between trophoblast cells and underlying endoderm cells This pattern is consistent with a possible duty to pump sodium ions into the growing blastocoel

With the detection of the α1 and β1 subunits in day 16 horse conceptuses on western blots, it is possible that the sodium pump could be acting as a mediator of fluid accumulation during this stage of development However, this enzyme is found in membranes of almost all animal cells and the fact that the blastocoel fluid is hypotonic relative to serum may argue against a significant role for this enzyme during horse blastocyst expansion It is also possible that ions accumulating in the expanding blastocoel may be quickly recycled back into the trophoblast cells (Dr S Caveney, personal communication) resulting in a blastocoel fluid that is hypotonic Water channels, known as aquaporins, may also be present in trophoblast cells to move water into the blastocoel Nevertheless, an important point must be made The concentrations of ions in the uterine fluid during this period of expansion is unknown The uterus is almost dry in nature during this time of blastocoel expansion (Dr R Waelchli, personal communication) so collection of uterine fluid is very difficult The uterine fluid ionic composition in the mouse has not been determined for a similar reason (Borland et al., 1977) If the uterine fluid were much more hypotonic compared to serum (i e isotonic or hypertonic compared to blastocoel fluid), it may be possible for expansion to occur utilizing a model of active ion transport into the blastocoel to create an osmotic gradient to drive water into the expanding blastocyst

With the tremendous expansion of the blastocyst, it is also puzzling to determine where such a considerable amount of water could originate There are lipid constituents and considerable numbers of mitochondria in the horse endoderm (Enders et al., 1993)

that might account for some water production through ß-oxidation of fatty acids

Numerous vacuoles are present between the endoderm and trophoblast (Enders *et al*,

1993) and it possible these regions are sites for fluid to initially accumulate  Work is

currently being done to further define the sodium pump's role in horse blastocyst

expansion (Dr  R  Waelchli, personal communication)

# CHAPTER 4

# ANTISENSE OLIGODEOXYNUCLEOTIDE TARGETING OF Na,K-ATPASE β1 TRANSCRIPTS

## 4.1 TARGETING THE β1 SUBUNIT OF Na,K-ATPASE

Na,K-ATPase β1 subunit transcripts were not detected by northern blotting in 2-, 4-, or 8-cell mouse preimplantation embryos (unlike the α1-subunit), but were detected in morulae, at the time that the sodium pump was first detected immunocytochemically (Watson and Kidder, 1988), and increased in abundance by the blastocyst stage (Watson *et al*, 1990a) Recently, β1 transcripts have been detected in early cleavage stages of the mouse using the more sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) technique (Kidder, 1993b, Chapter 1)

It is possible that the β1 subunit is the trigger for the functional maturation and transport of the enzyme to the plasma membrane (Watson *et al*, 1990a, Geering *et* al, 1991, Kidder, 1993b) To address the importance of this subunit during mouse preimplantation development, the β1 subunit was targeted with antisense phosphorothioate oligodeoxynucleotides (ODNs) during embryo culture

## 4.2 ANTISENSE TECHNOLOGIES

Antisense techniques have been used to study a variety of problems (for review see Krieg, 1992) The examination of specific events during development, in particular, has been a focus of research (Erickson *et al*, 1993)

Three different classes of antisense reagents exist  ODNs, RNA and ribozymes
Antisense ODNs are short (usually less than 30 bases) nucleotide sequences that are
complementary to specific intracellular target RNAs  The ODNs can be introduced into
cells by microinjection (Bevilacqua *et al*, 1988) or applied externally with or without the
use of a carrier such as a cationic liposome (Bennett *et* al, 1992, Ao *et al*, 1991)  Effects
of these reagents are usually transient (for review see Colman, 1990)  Antisense RNAs can
be synthesized *in vitro*  These can be injected into cells and embryos (Bevilacqua *et al*,
1989), but usually they are produced through the introduction of the encoding, antisense
DNA gene into cultured cells or germ lines  Antisense RNA production can be transient or
constitutive depending on the promoter driving the synthesis  Ribozymes are antisense
RNAs with enzyme activity that can cut RNA at preselected sites

The experiments described in this chapter utilized ODNs  ODNs can be unmodified
(i.e phosphodiester backbone) or modified (i e phosphorothioates)  The mode of action
of ODNs is described below  The very first successful demonstration of antisense targeting
*in vivo* occurred in 1978 (Zamecnik and Stephenson, 1978)  Synthetic unmodified ODNs,
added to culture media, inhibited Rous Sarcoma Virus proliferation in cell culture

Regardless of which antisense reagent is used, the specificity and efficacy of the
reagent depends on several factors (Colman, 1990)  intracellular access to target RNA,
secondary structures of reagent and target, uniqueness of the target sequence, strength of
complementary base pairing, mode of inhibition, resistance to endogenous nucleases and
the ability of the reagents to enter a cell(s)  Another factor that appears to be important is
the length of ODN used  Shorter ODNs can be delivered more efficiently into cells than
longer ones (Lappalainen *et al*, 1994) and they can also be more specific in targetting
complementary sequences  The longer the ODN the greater the chance of non-specific
interaction with non-target sequences (Woolf *et al*, 1992)  Several investigators have now
recommended that ODNs of 15 bases in length are ideal for experiments (reviewed by
Slavkin, 1995)

A problem arose early in the development of antisense ODN technology
Unmodified ODNs were often but not always (Holt *et al*, 1988) degraded by nucleases.
present in culture media or in the cytoplasm, that could degrade phosphodiester linkages
(Erickson, 1993) Concentrations of ODNs had to be increased to compensate for this
continual loss  One of the modifications or derivatives that was designed involved ODNs
with phosphorothioate (P-S) backbones  These P-S oligos were effective at lower
concentrations and much more resistant to nucleases (Lappalainen *et al*, 1994)  However,
P-S oligos appear to have increased toxicity, some of which may be due to their ability to
bind proteins, they have lower cellular uptake and a lower melting temperature (Ghosh
and Cohen, 1992, Lappalainen *et al*, 1994)  Toxicity seems to occur particularly when
four consecutive G nucleotides occur in the ODN (Yaswen *et al*, 1992)

### Antisense Reagents and Development

Early antisense experiments in developmental biology focused on *Drosophila* using
*in vitro* synthesized antisense RNA to study the *Kruppel* gene (Rosenberg *et al*, 1985,
Cohen *et al*, 1988)  Cabrera *et al.* (1987) injected antisense RNA complementary to a
portion of *wgless* mRNA  These experiments utilized *Drosophila* embryos at the
syncytial stage when antisense RNA could diffuse throughout the embryo

Early stages of mouse development have also been used for antisense experiments
Antisense ODNs (amidate modified) to epidermal growth factor receptors (EGF-R) have
been shown to decrease the onset of cavitation in preimplantation mouse embryos (Brice
*et al*, 1993)  Rappolee *et al.* (1992) found that unmodified 30 µM antisense ODNs
targeting the initiation codon of insulin-like growth factor-II (IGF-II) specifically
decreased immunodetectable IG II antigen when added to embryos at the 2-cell stage
and cultured to the blastocyst stage  The amount of IGF-II mRNA was also reduced by
the ODN compared to controls and the overall result in the embryo was a decrease in the
onset of blastocyst formation  Antisense RNA to cadherins (Ao and Erickson, 1992) and
to connexins (Bevilacqua *et al*, 1989) have been microinjected into mouse
preimplantation stages and the results have indicated roles for these molecules in mouse
embryo compaction

An advantage of utilizing preimplantation stage embryos for antisense experiments is that they can be cultured in the presence of ODNs This approach can work because cultured cells have frequently shown cellular uptake of ODNs (Erickson *et al*, 1993) Successes in inhibiting gene expression have been reported in preimplantation embryos cultured with antisense ODNs (Paria *et al*, 1992, Brice *et al*, 1993) Unfortunately there have been documented examples where antisense technology is not the most practical (Ao *et al*, 1991, Lappalainen *et* al, 1994)

## 4.3 MATERIALS AND METHODS

*Embryo Collection*

Random-bred CF-1 female mice (Charles River Canada Ltd, St Constant, Québec) were superovulated and mated as previously described (see Chapter 2) Four cell stage embryos of 60 hrs post hCG injection (day 2) were flushed from the reproductive tract using flushing medium 1 (Spindle, 1980) Embryos were cleaned and ready for aliquoting at 64-66 hours post hCG

*Antisense Oligodeoxynucleotides (ODNs)*

Phosphorothioate 15-mer ODNs were synthesized by the Core Molecular Biology Facility, Department of Biochemistry, University of Western Ontario ODNs were derived from published rat cDNA sequences (Shull *et al.*, 1986, Young *et al.*, 1987) and were designed to target the translational start site (AUG) of the sodium pump β1 subunit mRNA Two sets of ODNs were designed Set #1 and set #2 were used for culture experiments without Lipofectin® and with Lipofectin®, respectively The first set flanked the AUG codon while the second set targeted the β1 sequence immediately 5' to it and including the AUG

Set #1 Sense 5'-GCCACCATGGCCCGC-3'

Antisense 5'-GCGGGCCATGGTGGC-3'

Random 5'-GGGGGCGAGTGCCCT-3'

Set #2 Sense 5'-GGAGCAGCCACCATG-3'

Antisense 5'-CATGGTGGCTGCTCC-3'

Random 5'-CACTTGTCGGCGGCT-3'

ODNs were precipitated with absolute ethanol, dried, resuspended in ddH₂O, quantified by spectrophotometry, then aliquoted into 1 nmole quantities Set #1 ODNs were used for experiments without a cationic liposome as a carrier ODNs were added directly to culture media (see below) ODNs were checked for significant hairpin secondary structures, self complementarity and significant differences in annealling temperatures with the aid of Oligo® Primer Analysis Software (National Biosciences. Inc . Plymouth. MN, USA)

*Antisense Experiments without Carrier*

### Determination of Culture Conditions for Antisense Experiments

Four well known mouse embryo culture media (Table 4 1). CZB (Chatot *et al*, 1989). KSOM (Biggers *et al*, 1993). MTF (Gardner and Sakkas, 1993). and SECM (Spindle, 1980), were tested for their ability to support development of cavitating embryos and blastocysts (Fig 4 1) 4-cell embryos were washed in fresh flushing medium and then pooled in 0 8 mls of fresh CZB, KSOM. MTF or SECM culture medium in organ culture media dishes prior to random aliquoting of 25 embryos into each of the various culture groups Experiments were done blindly and in triplicate Embryos were cultured in 25 μL drops of culture medium in 35 mm Petri dishes, overlayed with Mineral oil (Sigma Chemical Co . St Louis. MI). in an atmosphere of 5% CO₂ in air Embryos were scored, by observation with a dissecting microscope, for developmental stage every three to six hours (Fig 4 1) Embryos were scored as cavitating when small vacuoles became present within the embryo, early blastocysts when the blastocoel was less than 50% of the embryo, and late blastocysts when the blastocoel was greater than 50% of the embryo

### Titration of ODN Concentrations

4-cell mouse embryos were flushed and washed as above. Embryos were pooled in 0.8 mL of CZB culture medium then randomly aliquotted into culture drops containing ODNs (Set #1 only) or without ODNs (Control). 1 nmole quantities of lyophilized ODNs were reconstituted in 10 μL of ddH$_2$O to give 100 μM stock solutions. For one culture drop of CZB/ODNs, 10 μL of 2X CZB was added to appropriate volumes of ODNs (e.g. 0.5 μL for 2 μM) and the remaining volume made up with ddH$_2$O to a final volume of 20 μL. 20-25 4-cell embryos were added to each treatment culture drop in 5 μL of CZB medium. Embryo culture drops were placed in 35 mm Petri dishes and cultured under mineral oil as previously described. Experiments were done blindly and embryos were scored for developmental stage and signs of cavitation every 3-6 hours.

*Antisense Experiments with Lipofectin®*

Two micrograms (2 μL) of Lipofectin® reagent (Life Technologies, Burlingto Ontario, Canada) were added to 2 μL of ddH$_2$O and 4 μL of 2X CZB culture medium to give a final Lipofectin® concentration of 0.25 μg per μL of CZB Lipofectin® solution (solution A).

**Table 4.1**

The components of culture media tested for their ability to support blastocyst development Concentrations are expressed in mM unless otherwise stated

|  | SECM | CZB | MTF | KSOM |
|---|---|---|---|---|
| NaCl | 84 10 | 81 62 | 114 19 | 95 00 |
| KCL | 4 78 | 4 83 | 4 78 | 2 50 |
| KH₂PO₄ | 1 19 | 1 18 | 1 19 | 0 35 |
| CaCl₂·2H₂O | 0 00 | 1 70 | 1 71 | 1 71 |
| MgSO₄·7H₂O | 1 19 | 1 18 | 1 19 | 0 20 |
| NaHCO₃ | 22 50 | 25 12 | 25 00 | 25 00 |
| Na-lactate | 19 71 | 31 30 | 4 79 | 10 00 |
| Na-pyruvate | 0 25 | 0 27 | 0 37 | 0 20 |
| ⁻a-lactate | 1 71 | 0 00 | 0 00 | 0 00 |
| glucose | 0 00 | 5 55 | 3 40 | 0 20 |
| Na₂-EDTA | 0 00 | 0 11 | 0 00 | 0 01 |
| glutamine | 0 00 | 1 00 | 0 00 | 1 00 |
| BSA (mg/mL) | 3 00 | 5 00 | 4 00 | 1 00 |
| phenol red (µg/mL) | 10 00 | 10 00 | 10 00 | 10 00 |
| pennicillin (units/mL) | 100 00 | 100 00 | 100 00 | 100 00 |
| streptomycin (µg/mL) | 50 00 | 50 00 | 50 00 | 50 00 |

Culture experiments with Set #2 ODNs or Lipofectin® alone, testing 0 25 µM, 0 5 µM and 1 0 µM, were done blindly in duplicate or triplicate determinations All embryos were cultured at 37°C in a 5% $CO_2$ atmosphere Lyophilized ODNs were reconstituted in 100 µL of ddH₂O to give a stock concentration of 10 µM For one culture drop of CZB Lipofectin® ODNs solution (solution B) the following volumes were prepared in polystyrene test tubes 10 µL of 2X CZB, 1µL of appropriate ODNs (0 5 µM) or ddH₂O for controls, and 6 5 µL of ddH₂O 0 5 µL of solution A was added to the 17 5 µL of solution B (to produce solution C) and 20-25 4-cell mouse embryos added to the culture drop in 2 µL of culture medium Solution C was left to incubate at 37°C in 5% $CO_2$ for approximately an hour before addition of embryos The final Lipofectin® concentration of 6 25 µg/ml/drop, used in these culture experiments, was previously determined by Dr Daguang Zhu and Dr Gerald M Kidder Embryos were cultured in solution C for 5 hours in a humidified chamber (125 mm Petri dish and Whatman filter paper moistened with PBS) without oil overlay, then they were washed in fresh CZB medium and cultured for the remainder of experiments in fresh CZB medium, containing no ODNs or Lipofectin®, but overlaid with mineral oil. Embryos were periodically scored for developmental stage, particularly for signs of cavitation A cavitating embryo was scored as an embryo containing fluid filled vacuoles or a blastocoel

*Statistical Analysis*

Statistical analyses were performed with GraphPad Prism™ Software (GraphPad Software Inc , 1994) and Statgraphics Statistical software Statistical tests used to determine significance were analysis of variance (ANOVA) and Tukey multiple range tests Levels of significance used for testing were $p < 0 10$ and $p = 0 05$ The use of both significance levels was necessitated by the inherent variabilty of age among mouse embryos of the same morphological stage (see below for more details)

## 4.3 RESULTS

*Optimization of Culture Conditions for Antisense Experiments*

Embryos were scored for incidence of cavitation between the culture times of 90
and 111 hours post-hCG (Fig 4 1) Significant differences in the number of cavitating
embryos in different culture media were obtained between CZB and KSOM media at 99
hrs post-hCG (p · 0 10) No other significant differences were observed between CZB
and any other medium at remaining culture times All culture media produced greater than
90% blastocysts by the end of the culture period Due to CZB consistently producing
more cavitating embryos, though not necessarily statistically more, it was chosen for use in
subsequent antisense experiments

*Antisense Experiments without Carrier*

In culture experiments with triplicate determinations, no statistically significant
differences in the number of cavitating embryos were observed between antisense and
sense, random or control cultured embryos (see Fig 4.2 for example) Concentrations
between 2 and 40 μM were tested and the diameters of blastocoels at 111-116 hrs post
hCG were measured with an ocular micrometer and an Olympus dissecting microscope
No significant differences in diameter were observed between treatment groups (Table
4 2) except in two experiments testing 20 μM and 40 μM ODNs, respectively Random 20
μM ODN-incubated embryos had significantly larger diameters than the remaining
treatment and control groups (p < 0 05) Control embryos (no ODNs) in a 40 μM titration
experiment had significantly greater diameters than ODN treated embryos.

**FIGURE 4.1.** Determination of culture conditions for antisense experiments Mouse preimplantation embryos were cultured in four different culture media CZB, KSOM, MTF, and SECM (n=3 for each medium tested) Significantly more cavitating embryos (p<0 10) were observed in CZB medium than in KSOM medium at 99 hrs post-hCG (*) Error bars represent standard error of the mean (SEM)
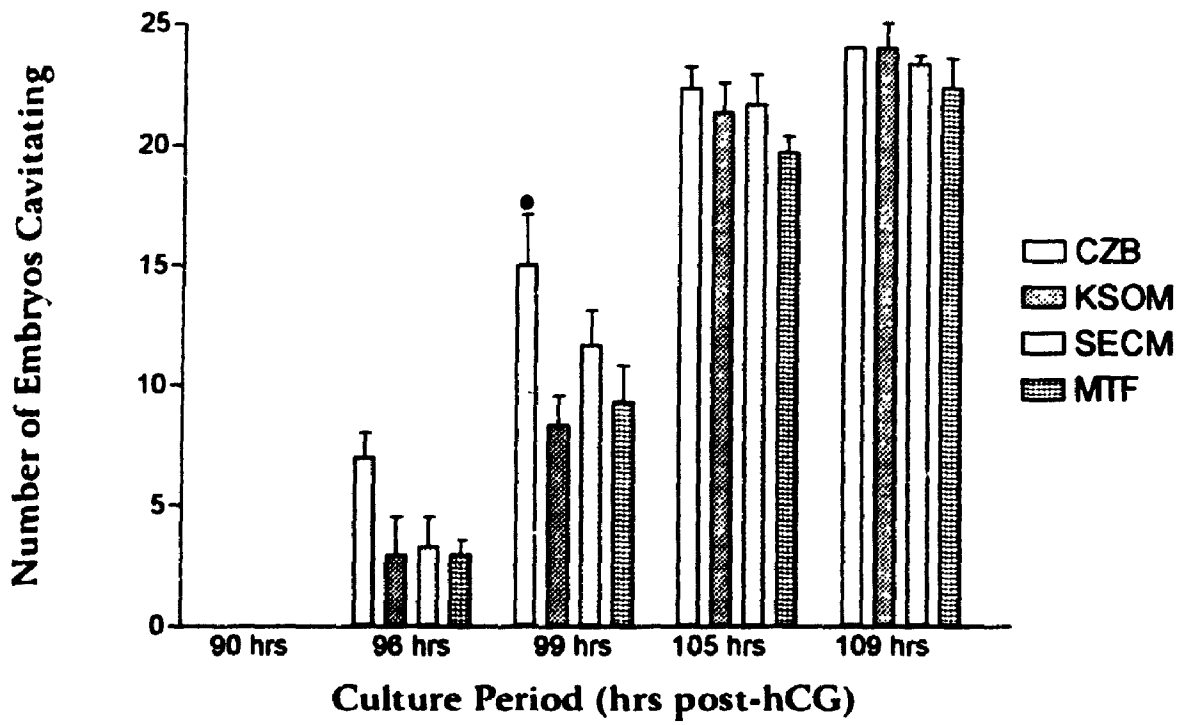
**FIGURE 4.2.** An example of an antisense culture experiment, without Lipofectin®, demonstrating no significant differences (p >0 10) in the number of cavitating embryos cultured in 20 μM ODNs (n=3 for each treatment group) Error bars represent standard error of the mean (SEM)
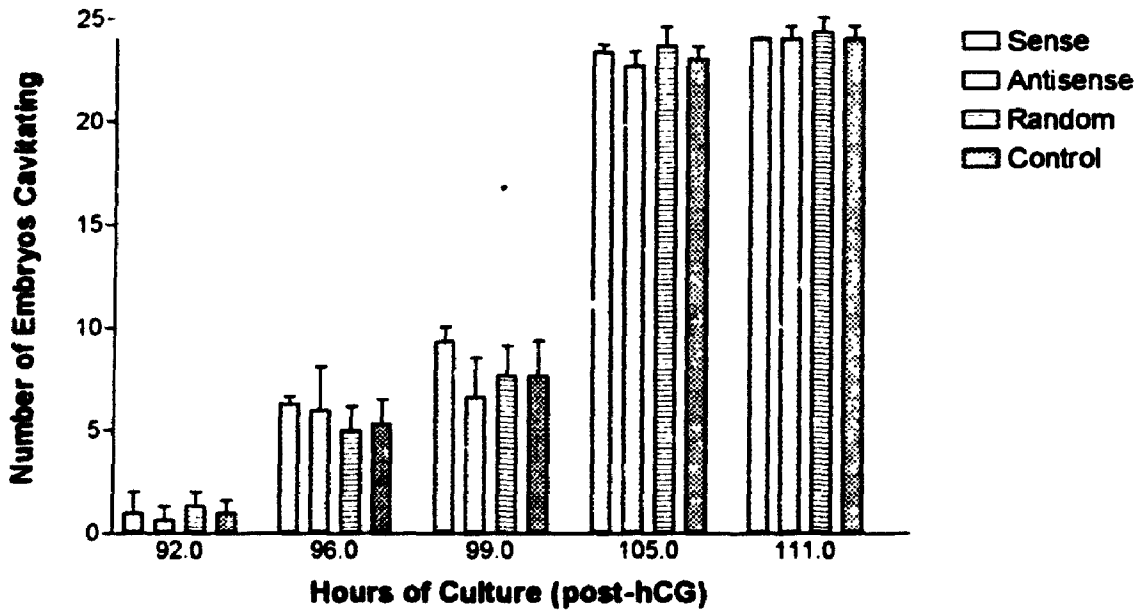
**Table 4.2**

Average blastocoel diameters in mouse blastocysts exposed to various oligodeoxynucleotides Diameters are in μm +/- standard deviation in parentheses Each row in the table represents a separate experiment with noted exceptions

| Treatment Concentration | Sense | Antisense | Random | Control (no oligo) |
|---|---|---|---|---|
| 2 μM | 82 (22) | 85 (21) | 79 (23) | 81 (24) |
| 3 μM | 98 (20) | 102 (23) | 100 (24) | 98 (27) |
| 4 μM | 111 (18) | 112 (18) | 108 (18) | 112 (22) |
| 5 μM[1] | 91 (22) | 95 (22) | n/d | 94 (19) |
| 10 μM[1] | 92 (22) | 92 (19) | n/d | 94 (19) |
| 15 μM[1] | 93 (25) | 91 (19) | n/d | 94 (19) |
| 20 μM | 89 (17) | 88 (24) | n/d | 98 (15) |
| | 98 (19) | 94 (23) | 84 (24)[2] | 96 (20) |
| | n/d | 86 (17) | 87 (18) | 81 (23)[3] |
| 40 μM | 79 (24) | 72 (18) | n/d | 98 (15)[2] |
| | n/d | 88 (17) | 87 (18) | 81 (23)[3] |

[1]The three concentrations were tested within the same experiment with the same control group (no oligo)

[2]Significantly different from other treatment groups at the same concentration of ODNs ($p < 0.05$)

[3]An experiment tested 20 and 40 μM concentrations with the same control (no oligo) treatment group

The time of culture when half of the embryos had cavitated (t 1/2) was also determined, and in experiments with triplicate determinations no statistically significant differences were observed between treatment groups at any titrated concentrations (Table 4 3)

*Antisense Experiments with Lipofectin®*

In two experiments. 0 25 μM and 1 0 μM ODN concentrations were titrated for effects on cavitation No large differences in the number of cavitating embryos, between treatment groups, were apparent in the 0 25 μM titration experiment In an experiment titrating 1 0 μM concentrations, antisense treatment produced significantly more cavitating embryos (p · 0 10) than control embryos at 102 hrs post hCG of culture (Fig 4 3) Lipofectin controls produced significantly fewer (p< 0.10) cavitating embryos than ODN treatments at 100 hrs post-hCG of culture No other significant differences were observed at other time points

In experiments testing 0 5 μM, antisense treatment produced significantly fewer cavitating embryos (p · 0 10) compared to random ODN or lipofectin control embryos (Fig 4 4) These results were observed in two different experiments utilizing either 4-cell or 8-cell embryos In both experiments, significant differences were observed within the same window of time at 99 to 102 hrs post-hCG

## Table 4.3

A summary of time points when half the embryos were cavitating (t1/2 in hours) during embryo culture in oligodeoxynucleotide (ODN) treatment groups No significant differences were observed in t1/2 times within experiments with triplicate determinations Numbers in parentheses are standard deviations

| ODN Concentration Tested | Sense | Antisense | Random | Control (no ODN) |
| --- | --- | --- | --- | --- |
| 2 µM | 102 7 (1.5) | 101 3 (1 5) | 103 0 (1 2) | 103 0 (2 9) |
| 3 µM | 100 7 (0 6) | 100 7 (1 5) | 100 0 (0) | 102 0 (2 5) |
| 4 µM | 100 3 (2 3) | 100 3 (1 2) | 101 0 (0 6) | 99 0 (1 5) |
| 5 µM[1] | 100 0, 101 0 | 98 0, 100 0 | n/d | 102 0, 99 0 |
| 10 µM[1] | 100 0, 99 0 | 101 0, 99 0 | n/d | 102 0, 99 0 |
| 15 µM[1] | 100 0, 101 0 | 102 0, 100 0 | n/d | 102 0, 99 0 |
| 20 µM[2] | 100 0, 101 0 | 100 0, 98 0 | n/d | 98 0, 101 0 |
|  | 100 0 (0 5) | 100 6 (0 6) | 101 0 (1 0) | 100 5 (0 9) |
|  | n/d | 96 0, 96 0 | 97 0, 96 0 | 96 0, 97 5 |
| 40 µM[2] | 99 0, 103.0 | 102 0, 102 0 | n/d | 98 0, 101 0 |
|  | n/d | 96 0, 96 0 | 94 0, 95 0 | 96 0, 97 5 |

[1]The three concentrations were tested within the same experiment with the same control group (no oligo)

[2]An experiment tested 20 and 40 µM concentrations with the same control (no oligo) treatment group

**FIGURE 4.3.** An antisense culture experiment. utilizing Lipofectin® as a carrier. testing 1 0 µM ODNs Antisense treatment produced significantly more cavitating embryos (p ˂0 10) than control embryos (*) at 102 hours post-hCG of culture while Lipofectin® controls produced signifcantly fewer (p ˂0 10) cavitating embryos than ODN treatments (#) at 100 hours post-hCG (n=3 for each treatment group) Error bars represent standard error of the mean (SEM)
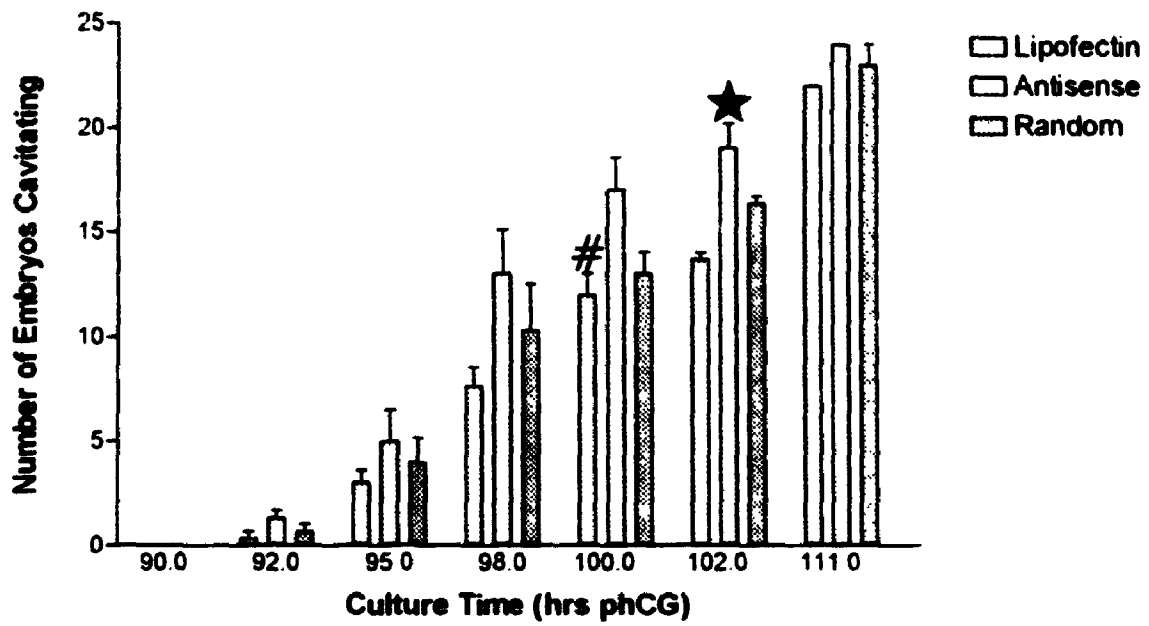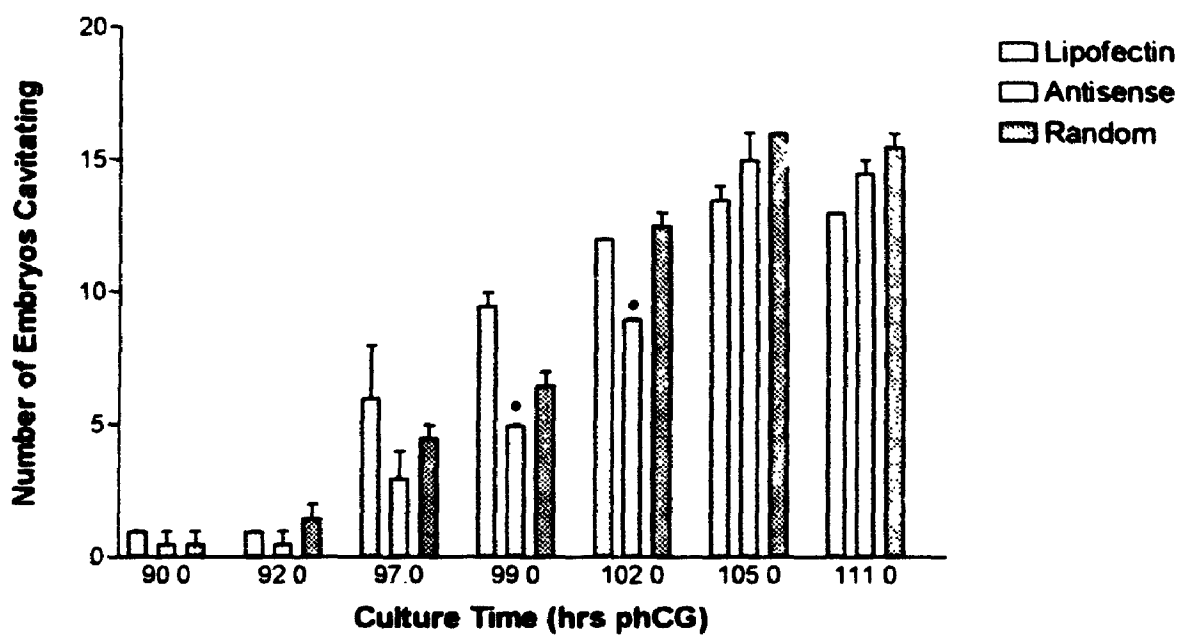
**FIGURE 4.4.** Antisense culture experiment, with Lipofectin®, testing 0 5 μM ODN effects on 4-cell mouse preimplantation embryos  Antisense treatment produced significantly fewer (●) cavitating embryos (p ·· 0 10) compared to random or Lipofectin® treated embryos (n=3 for each treatment group)  Error bars represent standard error of the mean (SEM)

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## 4.4 DISCUSSION

Two theories exist as to how antisense ODNs act on target mRNAs (Krieg, 1992). The binding of the ODN to the target mRNA produces a duplex that may activate endogenous RNAse H activity, degrading the mRNA in the hybrid DNA RNA complex Binding of an ODN to its complementary target may also interfere sterically with ribosome assembly, decreasing the amount of target mRNA that is translated The site of action chosen for most antisense experiments is the site of, or flanking, the translational start codon AUG Targeting to this region appears to give the best results (Krieg, 1992, Colman, 1990) although reports by Slavkin (1995) and Munroe (1988) suggest that mRNA splice sites are good targets for antisense nucleic acids In the experiments described above, the ß1 mRNA of the sodium pump was targeted with phosphorothioate ODNs to try to disturb cavitation in the mouse preimplantation embryo

*Antisense Experiments Without Carrier*

In experiments testing 2-40 µM concentrations of ODNs, no significant differences between treatment groups were observed in the number of cavitating embryos A question that arises is could significant amounts of ODNs be degraded by nucleases during the ODN treatment period, thereby preventing significant targeting of ß1 mRNA? It is possible that ß1 transcripts could reaccumulate after ODN degradation Several lines of evidence refute the possibility that ODNs could be completely degraded before they could affect cavitation Campbell *et al* (1990) demonstrated that phosphorothioate ODNs can be relatively stable in a variety of media and extracts at 37 °C Half lives of 7 ± 1h, 14 ± 2h, and 19 ± 7h have been reported for antisense ODNs in HeLa cell postmitochondrial extract, RPMI 1640 medium with 10% fetal bovine serum and rat cerebrospinal fluid, respectively

Previous work by Kidder and McLachlin (1985), using α-amanitin and cycloheximide (inhibitors of transcription and translation, respectively) in embryo culture medium, has also shown that the initial phase of fluid accumulation is tightly coupled to transcription and translation. mRNAs reach sufficient concentrations 5-7 hr prior to the event of fluid accumulation at 93-96 hr post-hCG. Translation products, sufficient to support the event, are available just preceding the first signs of cavitation

Recently, Khidir *et al* (1995) utilized lysolecithin to permeabilize mouse embryos and α-amanitin to reexamine the period of transcription that is critical for cavitation. They found that when transcription was inhibited between 87 and 91 hr post-hCG, there was a significant delay in the onset of cavitation. The delay was greater after 2 hrs of α-amanitin treatment compared to 1 hr treatment. Within the window of time, addition of α-amanitin at 89 hr post hCG maximally inhibited cavitation. Since it appears unlikely that phosphorothioate ODNs could be degraded completely in embryo culture med um, by 89 hr post hCG, antisense ODNs should be present in some concentration at times of culture that are critical for cavitation to proceed

The fact that ODNs did not inhibit cavitation may also suggest that a small but critical amount of β1 subunit (oogenetic and/or zygotic) could complex with α1 subunit and this amount could be sufficient for cavitation to occur. A small amou f α1/β1 heterodimer may be sufficient to initiate cavitation and blastocyst development and only when the vast majority of β1 subunit mRNA is destroyed or unavailable for translation, will cavitation be significantly effected

The result of 20 μM random ODN-incubated embryos having significantly larger diameters than any other treatment group or controls in an experiment (Table 4 2) is puzzling. There is inherent variability present in culture experiments due to the fact that flushed 4-cell embryos are not all exactly the same age post-fertilization. Therefore it is possible that a group of 4-cell embryos can be chosen randomly and the majority of the embryos could be older than the remainder of the embryo pool. This could account for the greater blastocoel diameters in one particular treatment group. To circumvent such a problem in the future, experiments should be expanded to increase the number of embryos in each drop, thus guarding against outlying values

The greater diameters of control embryos in an experiment titrating 40 μM concentrations of ODNs indicate that the concentrations are beginning to approach toxic levels ODNs are known to have non-sequence specific effects that cause toxicity (Erickson, 1993) Brice *et al* (1993) found that amidate modified ODNs were toxic to mouse preimplantation at 30 μM Pampfer *et al* (1995) concluded that addition of phosphorothioate ODNs at concentrations greater than 10 μM was detrimental to mouse blastocysts *in vitro*. The authors scored cell numbers in the inner cell mass and trophectoderm and found significant decreases in cell numbers at 13-14 μM concentrations Quattrone *et al* (1994) found that 10 μM concentrations of unmodified ODNs were toxic to CCRF-CEM/VLB cells Therefore, significantly greater blastocoel diameters in control preimplantation embryos over ODN treatment groups is consistent with potential ODN toxicity

With experiments utilizing no carriers, lack of effects on cavitation indicate that targeted disruption of β1 mRNA might be difficult, particularly if effects are dependent on phosphorothioate CDN uptake Lack of observable effects with antisense ODNs is not uncommon Ao *et al* (1991) reported that injection of unmodified 20-mer antisense ODNs complementary to the initiation codon of β-glucuronidase gene failed to inhibit gene expression in preimplantation mouse embryos. When experiments were done with radiolabelled ODNs added to culture medium, 0 0058% of the total ODN in the culture droplet (22 μM) was taken up by the embryos Colige *et al*. (1993) found that antisense ODNs directed against a portion of the gene for proα1 (I) chains of type I procollagen (COL1A1) were ineffective in inhibiting expression in mouse 3T3 cells with concentrations of ODNs up to 25 μM. Only when 10 μg/mL Lipofectin® was utilized to increase cellular uptake was the antisense ODN effective

*Antisense Experiments with Lipofectin®*

The mechanism of ODN entry into cells mediated by liposomes is not well understood  One hypothesis is the membrane fusion theory where the cationic liposomes fuse with the plasma membrane and release ODNs into the cytoplasm  There is also some evidence that ODN-receptors exist on the cell surface (Yakubov *et al*, 1989) and uptake of ODNs is dependent on time and concentration (Lappalainen *et al*, 1994, Yakubov *et al*, 1989)

Cationic liposomes can deliver ODNs efficiently into cells because they complex the anionic ODNs on their surface with electrostatic interactions  Lappalainen *et al* (1994) found that liposomes increase delivery of 23-mer ODNs into the cytoplasm and nucleus of CaSki cells  Intracellular endonucleases and exonucleases can degrade ODNs in cells  It is thought that liposomes can help increase ODN stability in cells by improving their delivery  Increasing the amount of intact ODNs in cells may increase the half-life of ODNs because nucleases could become saturated demonstrating classical Michaelis-Menton kinetics

In an experiment testing $1.0\ \mu M$ ODN concentrations, lipofectin control embryos at 100 hr post-hCG were not cavitating to the same extent as other treatment groups ($p < 0.10$)  A greater number of cavitating embryos was observed at 102 hr post-hCG ($p < 0.10$) in antisense treatment than in remaining treatment groups  Reasons for these two results are unclear  At both times post-hCG, $1.0\ \mu M$ ODNs may be saturating the liposome  Less inhibition has been observed with Lipofectin® and high concentrations of ODN due to the saturation preventing fusion with cell membranes (Colige *et al*, 1993, Chiang *et al*, 1991)  Alternatively, these two measurements are outlying values and increasing embryo numbers per culture droplet may alleviate this problem in the future

In experiments testing 0 5 µM concentrations of phosphorothioate ODNs, significantly fewer cavitating embryos (p< 0 10) were seen at 99 and 102 hr of culture post hCG in antisense incubated mouse embryos compared to random or control treatment groups The concentration that produced effects on cavitation is consistent with two recent reports of antisense experiments utilizing cationic liposomes as carriers Lappalainen *et al* (1994) and Colige *et al* (1993) utilized phosphorothioate ODNs in the 0 1 to 0 4 µM range These concentrations also serve as examples that cationic liposomes may increase the potency of ODNs Bennett *et al* (1992) found that N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA®) increased ODN potency greater than 1000 fold compared to experiments without carrier

No significant differences were observed, at other time points, in the number of cavitating embryos among various treatment groups Diameters were not measured in experiments utilizing cationic liposomes The two significant time points in the above experiments were only significant at the p<0 10 level The antisense effect on cavitation was subtle and by 105 hr post hCG no significant differences were observed Therefore it was unlikely that blastocoel diameters would have been significantly different between groups following 102 hr post hCG Furthermore, measuring blastocoel diameters at the specific time point of 99 or 102 hr post hCG would not have distinguished the cavitating morulae from the early blastocysts because cavitating morulae are very difficult or almost impossible to accurately measure vacuole diameters

These results suggest that when ß1 mRNA is targeted with antisense phosphorothioate ODNs, a subtle effect can be observed in the number of cavitating embryos This effect is consistent with the hypothesis that synthesis of the ß1 subunit is required for cavitation during mouse preimplantation development (Kidder, 1993b, Watson *et al*, 1990a) However, several changes should be made to improve the targetting of the ß1 mRNA in mouse embryos Uptake studies need to be completed to determine whether uptake is consistent among embryos in a treatment group The number of embryos per treatment group needs to be increased to decrease the potential of outlying values in populations of 4-cell embryos that are not equivalent in age, but equivalent in morphological stage If the effects on cavitation can be increased then RT-PCR of mouse embryos would be practical to specifically show loss of the targeted region of the mRNA Furthermore, immunocytochemistry could be utilized to specifically show a decrease in the amount of ß1 subunit present in antisense treated embryos If future antisense experiments are unable to magnify the effect on cavitation, a second method to target ß1 expression may be utilized The ß2 mRNA coding region could be "knocked-in" to the ß1 mRNA coding region to replace it during mouse preimplantation development This strategy could address two questions The potential role of the ß2 subunit in the preimplantation embryo and the effect of removing ß1 gene expression from mouse preimplantation development

# CHAPTER 5
## SUMMARY AND FUTURE DIRECTIONS

In summary, the results of the present study show

1 Na,K-ATPase $\alpha1$ mRNA is distributed throughout mouse morulae and blastocysts

2 Na,K-ATPase $\alpha1$ subunits are present in membranes of all preimplantation stages of mouse development while $\beta1$ subunits are detectable in membranes beginning at the morulae stage

3 Na,K-ATPase $\alpha3$ and $\beta2$ isoform mRNAs are present in preimplantation stages while $\alpha4$ is not detectable $\alpha2$ isoform mRNA is only detectable in oocytes

4 Na,K-ATPase $\alpha3$, $\alpha2$ and $\beta2$ subunits are not detectable, by immunofluorescence, in membranes of mouse preimplantation embryos

5 The sodium pump $\alpha3$ subunit can be detected in total protein from 650 blastocysts and morulae, unlike the $\alpha2$ subunit Results for $\beta2$ are inconclusive and future research will require a new antiserum directed against this subunit

6 As a result of a collaboration, sodium pump $\alpha1$ and $\beta1$ mRNAs and subunit proteins were found to be detectable in horse conceptuses during blastocoel expansion

7 Initial results from experiments targeting the sodium pump $\beta1$ subunit with antisense ODNs suggest that this subunit could be important for initiation of cavitation in the preimplantation mouse embryo

The detection of $\alpha1$ mRNA throughout mouse morulae and blastocysts correlates with the detection of the $\alpha1$ subunit in membranes of morulae and membranes of mural

and polar trophectoderm and inner cell mass  The ß1 subunit was detected beginning at the morula stage where it was present in membranes  The protein then becomes localized to membranes in blastocysts in a fashion similar to the α1 subunit  The localization of the two subunits in similar regions agrees with published data illustrating that the subunits are dependent on one another for proper intracellular processing, transport to the membrane and functional maturation of the enzyme (Ackermann and Geering, 1990, Geering *et al*, 1989, Geering, 1991)  The lack of detection of the ß1 subunit until the morula stage also correlates with northern blot and RT-PCR data (Watson *et al*, 1990a, Kidder, 1993a), showing very low levels of ß1 mRNA until the morula stage when the transcript level surges  This result strengthens the hypothesis that the sudden increase in the amount of the ß1 subunit at the morula stage may be the trigger for cavitation to proceed as a result of more sodium pump heterodimers becoming localized to membranes of preimplantation embryos

Earlier work by Watson *et al* (1990b) and Watson and Kidder (1988) indicated that the sodium pump α subunit is restricted to the basolateral membranes of the mural trophectoderm lining the blastocoel.  Their experiments utilized 1 μm thick polyethylene glycol sections for immunofluorescence, not whole mounted embryos and a laser scanning microscope described in the present study  Immunofluorescence with sections only allows access to potential epitopes on the surface of the sections, therefore the immunofluorescence described in the present study may be more sensitive  The authors also utilized a different rabbit antiserum directed against the whole α subunit of rat kidney

Detection of sodium pump α2, α3, and ß2 subunit mRNAs and α3 subunits during early mouse development may allow the embryo to adapt in some way to changes in its environment during development (see Chapter 3)  The embryos' environment *in vivo*, particularly during implantation, may be important in regulating sodium pump activity  Regulating activity could affect the ability of the blastocyst to hatch from its zona pellucida, a requirement for implantation to occur  However, it is important to remember that these mRNAs were not detected by northern blotting (Watson *et al*, 1990a) so it is difficult to determine whether detection by RT-PCR is meaningful  Detection of the α3 subunit on a western blot may indicate a more meaningful role for this isoform compared

to the others  The $\alpha$3 isoform has 2-3 fold lower affinity for sodium ions than the other $\alpha$ isoforms (McDonough *et al*, 1992), but whether this will be significant during mouse blastocyst formation is unknown and should be investigated

Targeting the ß1 subunit mRNA with antisense phosphorothioate ODNs produced a subtle effect on cavitation  Whether this effect can be increased is unknown  Future work with this targeting system requires an analysis of ODN uptake characteristics of mouse preimplantation embryos and perhaps the use of another carrier like lysolecithin (Khidir *et al*, 1995)  Studying uptake characteristics appears to be difficult (H  Jones, personal communication)  Another way to target the ß1 subunit may also shed some light on the functional potential of the ß2 subunit during mouse preimplantation development  A "knock-in" of the ß2 subunit coding region into the ß1 coding region may be productive, helping to determine more specifically the role of the ß1 subunit and determining whether another isoform (ß2) can replace the original and still support preimplantation development

In conclusion, the present study has further defined the expression of sodium pump genes during mouse preimplantation development  It has produced greater evidence that the $\alpha$1 and ß1 isoforms are the predominant isoforms during this developmental period and that the ß1 isoform may be the critical subunit for initiation of cavitation  This study has also indicated that the $\alpha$3 isoform may have a role during preimplantation development, although not likely as a mediator of cavitation

# REFERENCES

Ackermann, U., Geering, K  1990  Mutual dependence of Na,K-ATPase α- and ß-subunits for correct posttranslational processing and intracellular transport  FEBS Lett  269(1) 105-108

Ahn, K Y, Madsen, K.M, Tisher, C.C, Kone, B C  1993  Differential expression and cellular distribution of mRNAs encoding α- and ß-isoforms of Na .K -ATPase in rat kidney  Am J Physiol  265  F792-F801

Albers, R W  1967  Biochemical aspects of active transport  Ann  Rev  Biochem  36  727-756

Antolovic, R , Bruller, H.J., Bunk, S , Linder, D , Schoner, W  1991  Epitope mapping by amino acid sequence specific antibodies reveals that both ends of the alpha subunit of Na/K-ATPase are located on the cytoplasmic side of the membrane  Eur  J  Biochem  199 195-202

Antonicek, H., Persohn, E., Schachner, M  1987  Biochemical and functional characterization of a novel neuron-glia adhesion molecule that is involved in neuronal migration. J. Cell Biol. 104: 1587-1595

Ao, A., Erickson, R.P., Bevilacqua, A., Karolyi, J  1991  Antisense inhibition of ß-glucuronidase expression in preimplantation mouse embryos  a comparison of transgenes and oligodeoxynucleotides. Antisense Res  Dev  1  1-10

Ao, A., Erickson, R.P  1992  Injection of antisense RNA specific for E-cadherin demonstrates that E-cadherin facilitates compaction, the first differentiative step of the mammalian embryo. Antisense Res  Dev  2  153-163

Becker, D.L., Davies, C.S  1995  Role of gap junctions in the development of the preimplantaton mouse embryo  Microsc. Res. Tech  31. 364-374

Beguin, P., Beggah, A.T., Chibalin, A V., Burgener-Kairuz, P , Jaisser, F , Mathews, P.M , Rossier, B C., Cotecchia, S., Geering, K  1994. Phosphorylation of the Na,K-ATPase α-subunit by protein kinase A and C in vitro and in intact cells  J  Biol  Chem 269(39)  24437-24445.

Bennett, F. C , Chiang, M -Y., Chan, H , Shoemaker, J.E E , Mirabelli, C K  1992 Cationic lipids enhance cellular uptake and activity of phophorothioate antisense oligonucleotides. Mol  Pharm  41  1023-1033

Benos, D.J., and Biggers, J.D  1981  Blastocyst fluid formation  In  Fertilization and Embryonic Development In Vitro. (L  Mastroianni and J D  Biggers, eds), pp  283-297 Plenum Press, New York, NY.

Benos, D.J. Balaban, R S 1990 Current topic Transport mechanisms in preimplantation mammalian embryos Placenta 11.373-380.

Berterello, A M , Aperia, A., Walaas, S I , Nairn, A.C , Greengard, P 1991 Phosphorylation of the catalytic subunit of Na,K-ATPase inhibits the activity of the enzyme Proc Natl Acad Sci USA 88 11359-11362

Betts, D H , MacPhee, D J , Kidder, G M , Watson, A.' 1996 Ouabain sensitivity and expression of Na,K-ATPase α and ß subunit isoform genes during bovine early. development Mol Reprod Dev in press

Bevilacqua, A , Erickson, R P , Hieber, V 1988 Antisense RNA inhibits endogenous gene expression in mouse preimplantation embryos Lack of double-stranded RNA "melting" activity Proc Natl Acad Sci USA 85 831-835

Bevilacqua, A , Loch-Caruso, R., Erickson, R.P. 1989. Abnormal development and dye coupling produced by antisense RNA to gap junction protein in mouse preimplantation embryos Proc Natl Acad Sci USA 86 5444-5448

Biggers, J D 1972 Mammalian blastocyst and amnion formation. In. The water metabolism of the fetus (A C., Barnes, A E , Seeds, eds) p⁻ 3-31 Charles C Thomas. New York, NY

Biggers, J D, Bell, J E, Benos, D J 1988 Mammalian blastocyst Transport functions in a developing epithelium Am J Physiol 255 C419-C432

Biggers, J D , Lawitts, J A , Lechene, C.P 1993 The protective action of betaine on the deleterious effects of NaCl on preimplantation mouse embryos in vitro Mol Reprod Dev 34 380-390

Blanco, G , Koster, J C , Mercer, R.W 1994 The α subunit of the Na,K-ATPase specifically and stably associates into oligomers Proc Natl Acad Sci USA 91 8542-8546

Blanco, G , Sanchez, G , Mercer, R W 1995a Comparison of the enzymatic properties of the Na,K-ATPase α3ß1 and α3ß2 isozymes Biochemistry 34: 9897-9903.

Blanco, G , Koster, J C , Sanchez, G , Mercer, R W 1995b. Kinetic properties of the α2ß1 and α2ß2 isozymes of the Na,K-ATPase Biochemistry 34 319-325.

Borland, R M , Biggers, J D , Lechene, C.P 1976 Kinetic aspects of rabbit blastocoele fluid accumulation An application of electron probe microanalysis. Dev Biol 50 201-211

Borland, R M , Hazra, S , Biggers, J D , Lechene, C P 1977 The elemental composition of the environments of the gametes and preimplantation embryo during the initiation of pregnancy Biol Reprod 16 147-157

Brice, E C , Wu, J -X , Muraro, R , Adamson, E , Wiley, L M 1993 Modulation of mouse preimplantation development by epidermal growth factor receptor antibodies, antisense RNA, and deoxyoligonucleotides Dev Genet 14(3) 174-184

Cabrera, C V , Alonso, M C , Johnston, P , Phillips, R G , Lawrence, P A 1987 Phenocopies induced with antisense RNA identify the wingless gene Cell 50 659-663

Cameron, R , Klein, L , Shyjan, A W , Rakic, P , Levenson, R 1994 Neurons and astroglia express distinct subsets of Na,K-ATPase α and β subunits Mol Brain Res 21 333-343

Campbell, J M , Bacon, T A , Wickstrom, E 1990 Oligodeoxynucleotide phosphorothioate stability in subcellular extracts, culture media, sera, and cerebrospinal fluid J Biochem Biophys Methods 20 259-267

Chatot, C L , Lewis, J L , Torres, I , Ziomek, C A 1990 Development of 1-cell embryos from different strains of mice in CZB medium Biol Reprod 42 432-440

Chiang, M -Y , Chan, H , Zounes, M A , Freier, S M , Lima, W F , Bennett, C F 1991 Antisense oligonucleotides inhibit intercellular adhesion molecule 1 expression by two distinct mechanisms J Biol Chem 266 18162-18171

Chibalin, A V., Vasilets, L A , Henekes, H , Pralong, D , Geering, K 1992 Phosphorylation of Na,K-ATPase α-subunits in microsomes and in homogenates of Xenopus oocytes resulting from the stimulation of protein kinase A and protein kinase C J Biol Chem 267 22378-22384

Chomczynski, P , Sacchi, N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction Anal Biochem 162 156-159

Clapp, W L, Bowman, P , Shaw, G S , Patel, P , Kone, B C 1994 Segmental localization of mRNAs encoding Na -K -ATPase α- and β-subunit isoforms in rat kidney using RT-PCR Kidney Int 46 627-638

Clarke, D M , Loo, T W , Inesi, G , MacLennan, D H 1990 The epitope for monoclonal antibody A20 (amino acids 870-890) is located on the lumenal surface of the Ca -ATPase of sarcoplasmic reticulum J Biol Chem 265 17405-17408

123

Cohen, S M , Nauber, U , Schuh, R , Seifert, E , Jackle, H 1988 Phenocopies produced by antisense RNA identify genes requried for pattern formation inthe *Drosophila* embryo In Melton, D A (ed) Antisense RNA and DNA Cold Spring Harbor, NY Cold Spring Harbor Laboratory, pp 65-69

Colige, A , Sokolov, B P , Nugent, P , Baserga, R , Prockop, D J 1993 Use of an antisense oligonucleotide to inhibit expression of a mutated human procollagen gene (COL1A1) in transfected mouse 3T3 cells Biochemistry **32** 7-11

Collins, J H , Leszyk, J 1987 The γ subunit of Na,K-ATPase a small, amphiphilic protein with a unique amino acid sequence Biochemistry **26** 8665-8669

Colman, A 1990 Antisense strategies in cell and developmental biology J Cell Sci **97** 399-409

Conlon, R A and Rossant, J 1992 Exogenous retinoic acid rapidly induces anterior ectopic expression of murine *Hox-2* genes in vivo Development **116:** 357-368

Con. esy-Theulaz, I , Merillat, A -M , Honegger, P , and Rossier, B C 1991 Differential regulation of Na-K-ATPase isoform gene expression by T₃ during rat brain development Am J Physiol **261:** C124-C131

De Sousa, P A. Valdimarsson, G , Nicholson, B J , Kidder, G M 1993 Connexin trafficking and the control of gap junction assembly in mouse preimplantation embryos Development **117** 1355-1367

Diamond, J M , Bossert, W H 1967 Standing-gradient osmotic flow J Gen Physiol **50** 2061-2083

DiZio, S M , Tasca, R J 1977 Sodium-dependent amino acid transport in preimplantation mouse embryos III Na,K-ATPase linked mechanism in blastocysts Dev Biol **59** 198-205

Enders, A C , Schlafke, S , Lantz, K C , Liu, I K M 1993 Endoderm cells of the equine yolk sac from day 7 until formation of the definitive yolk sac placenta Equine vet J Suppl **15** 3-9

Erickson, R P 1993 The use of antisense approaches to study development Dev Gen **14** 251-257

Ewart, H S , Klip, A 1995 Hormonal regulation of the Na⁺-K⁺-ATPase mechanisms underlying rapid and sustained changes in pump activity Am J Physiol **269** C295-C311

Fambrough, D M 1988 The sodium pump becomes a family Trends Neurosci **11** 325-328

Fambrough, D , Lemas, M V , Takeyasu, K , Renaud, K J , Inman, E M 1994 Structural requirements for subunit assembly of the Na,K-ATPase Curr Topics Membr **41** 45-69

Fisone, G , Cheng, S X -J , Nairn, A C , Czernik, A J , Hemmings, Jr H C , Hoog, J -O , Bertorello, A M , Kaiser, R , Bergman, T , Jornvall, H , Aperia , A , Greengard, P 1994 Identification of the phosphorylation site for cAMP-dependent protein kinase on Na ,K - ATPase and effects of site-directed mutagenesis J Biol Chem **269**(12) 9368-9373

Fleming, T P , Johnson, M H 1988 From egg to epithelium Ann Rev Cell Biol **4** 459-485

Fleming, T P , McConnell, J , Johnson, M H , Stevenson, B R 1989 Development of tight junctions de novo in the mouse early embryo Control of assembly of the tight junction-specific protein ZO-1 J Cell Biol **108** 1407-1418

Fleming, T P , Hay, M J 1991 Tissue specific control of expression of the tight junction polypeptide ZO-1 in the mouse early embryo Development **113** 295-304

Gardiner, C S, Grobner, M A , Menino, A.R., Jr 1990a Sodium/potassium adenosine triphosphatase α-subunit mRNA levels in early rabbit embryos Biol Reprod **42** 539-544

Gardiner, C S, Williams, J S , Menino, A.R 1990b Sodium/Potassium adenosine triphosphatase α- and ß-subunit and α-subunit mRNA levels during mouse embryo development in vitro Biol Reprod **43** 788-794

Gardner, D K , Sakkas, D 1993 Mouse embryo cleavage, metabolism, and viability role of medium composition Hum Reprod **8** 288-295

Geering, K , Theulaz, F , Verrey, M , Hauptle, T , Rossier, B C 1989 A role for the ß-subunit in the expression of functional Na,K-ATPase in *Xenopus* oocytes Am J Physiol **257** C851-C858

Geering, K 1990 Subunit assembly and functional maturation of Na,K-ATPase J Membrane Biol. **115:** 109-121

Geering, K 1991a Posttranslational modifications and intracellular transport of sodium pumps Importance of subunit assembly Soc Gen Physiol Ser **46** 31-43

Geering, K 1991b. The functional role of the beta subunit in the maturation and intracellular transport of Na/K-ATPase FEBS Lett **285** 189-193

Ghosh, M , Cohen, J S 1992 Oligodeoxynucleotides as antisense inhibitors of gene expression Prog Nucleic Acids Res Molec Biol **42** 79-126

Gick. G G . Ismail-Beigi. F , Edelman. I S. 1988 Thyroidal regulation of rat renal and hepatic Na,K-ATPase gene expression J Biol Chem **263** 16610-16618

Gick. G G. Hatala. M A, Chon, D . Ismail-Beigi. F 1993 Na,K-ATPase in several tissues of the rat Tissue-specific expression of subunit mRNAs and enzyme activity J Membrane Biol **131** 229-236

Ginther, O J 1986 Ultrasonic imaging and reproductive events in the mare Equiservices, Cross Plains, Wisconsin, USA

Gloor. S . Antonicek. H . Sweadner, K J , Pagliusi, S , Frank. R . Moos. M . Schachner. M 1990 The adhesion molecule on glia (AMOG) is a homologue of the ß subunit of the Na,K-ATPase J Cell Biol **110** 165-174

Good, P J . Richter, K . Dawid. I B 1990 A nervous system specific isotype of the ß subunit of Na,K-ATPase expressed during early development of *Xenopus laevis* Proc Natl Acad Sci USA **87** 9088-9092

Gottardi. C J . Caplan, M J 1993 Molecular requirements for the cell-surface expression of multisubunit ion-transporting ATPases J Biol Chem **268**(19) 14342-14347

Herrera. V L M . Emanuel. J R . Ruiz-Opazo, N . Levenson, R . and Nadal-Ginard. B 1987 Three differentially expressed Na,K-ATPase α subunit isoforms Structural and functional implications J Cell Biol **105**: 1855-1865

Herrera,V L M , Cova, T , Sassoon, D , Ruiz-Opazo, N 1994 Developmental cell-specific regulation of Na'-K'-ATPase α1-, α2-, α3- isoform gene expression Am J Physiol **266** C1301-C1312

Hogan. B . Constantini. F , Lacy, E 1986 Manipulating the mouse embryo A laboratory manual Cold Spring Harbor. NY Cold Spring Harbor Laboratory Press

Holt. J T . Redner, R L . Nienhuis, A W 1988 An oligomer complementary to c-myc mRNA inhibits proliferation of HL-60 promyelocytic cells and induces differentiation Mol Cell Biol **8** 963-973

Horowitz. B . Hensley. C B . Quintero. M . Azuma, K K . Putnam, D . McDonough, A A 1990 Differential regulation of Na/K-ATPase α1, α2. and ß-subunit mRNA and protein levels by thyroid hormone J Biol Chem **265** 14308-14314

Ikeda. K . Nagano. K . Kawakami, K 1994 Sp1 and an E-box binding protein regulate Na /K -ATPase α2-subunit gene In The sodium pump Structure, mechanism, hormonal control and its role in disease (E . Bamberg. W , Schoner, eds). pp 1-10 Springer-Verlag. New York, NY

Jaisser, F , Horisberger, J -D., Rossier, B C 1992 The ß-subunit modulates potassium activation of the Na-K pump In Ion-Motive ATPases Structure, function and regulation (A , Scarpa, E , Carafoli, S , Papa, eds) Ann N Y Acad Sci **671** 113-119

Jewell, E A, Lingrel, J B 1991 Comparison of the substrate dependence properties of the rat Na,K-ATPase α1, α2, α3 isoforms expressed in HeLa cells J Biol Chem **266**(25) 16925-16930

Jorgensen, P L 1982 Mechanisms of the Na/K-pump protein structure and conformations of the pure Na/K-ATPase Biochim Biophys Acta **649** 27-68

Jorgensen, P L 1986 Structure, function and regulation of the Na/K-ATPase in the kidney Kidney Int **29** 10-20

Jorgensen, P L , Collins, J H 1986 Tryptic and chymotryptic cleavage sites in sequence of α-subunit of Na,K-ATPase from outer medulla of mammalian kidney Biochim Biophys Acta **860** 570-576

Kano, I , Nagai, F , Satoh, K., Ushiyama, K , Nakao, T , and Kano, K 1989 Structure of the α1 subunit of horse Na,K-ATPase gene FEBS Letters **250**: 91-98

Kawakami, K , Noguchi, S., Noda, M , Takahashi, H , Ohta, T , Kawamura, M , Nojima, H , Nagano, K , Hirose, T , Inayama, S , Hayashida, H , Miyata, T , Numa, S 1985 Primary structure of the α-subunit of Torpedo californica Na,K-ATPase deduced from cDNA sequence Nature **316** 733-736

Kawakami K , Nagano K , Nojima H., Ohto T 1986 Molecular-cloning and sequence-analysis of human Na/K-ATPase beta subunit Nucleic Acids Res **14** 2833-2844

Kawakami K , Suzuki-Yagawa Y , Watanabe Y , Ikeda K , Nagano K 1994 Transcription factors regulating the Na ,K -ATPase genes In The sodium pump structure mechanism, hormonal control and its role in disease (E , Bamberg, W , Schoner, eds) pp 1-10 Springer, New York, NY

Kent R B , Fallows D A , Geissler E., Glaser T , Emanuel J R , Lalley P A , Levenson R , Housman R 1987 Genes encoding α and ß-subunits of Na/K-ATPase are located on three different chromosomes in the mouse Proc Natl Acad Sci USA **84** 5369-5373

Khidir, M A , Stachecki, J J , Krawetz, S A , Armant, D R 1995 Rapid inhibition of mRNA synthesis during preimplantation embryo development vital permeabilization by lysolecithin potentiates the action of α-amanitin Exp Cell Res **219** 619-625

Kidder, G M., McLachlin, J R 1985 Timing of transcription and protein synthesis underlying morphogenesis in preimplantation mouse embryos Dev Biol **112** 265-275

Kidder, G M and Watson, A J 1990 Gene expression required for blastocoel formation in the mouse In Early embryo development and paracrine relationships (S Heyner, L M , Wiley, eds) pp 97-107 Wiley-Liss, Inc , New York, NY

Kidder, G M 1992 The genetic program for preimplantation development Dev Genet 13: 319-325

Kidder, G M 1993a Genetic information in the preimplantation embryo In Meiosis II Contemporary Approaches to the Study of Meiosis (F P , Haseltine, S , Heyner, eds) pp 187-199 AAAS Press,Washington, D C

Kidder, G M 1993b Genes involved in cleavage, compaction, and blastocyst formation In Genes in Mammalian Reproduction (R B L , Gwatkin, ed) pp 45-71 Wiley, New York, NY

Koster, J C , Blanco, G , Mercer, R W 1995 A cytoplasmic region of the Na,K-ATPase α-subunit is necessary for specific α/α association J Biol Chem 270(24) 14332-14339

Koster, J C , Blanco, G , Mills, P B , Mercer, R W 1996 Substitutions of glutamate 781 in the Na,K-ATPase α subunit demonstrate reduced cation selectivity and an increased affinity for ATP J Biol Chem 271(5) 2413-2421

Krieg, A M 1992 Applications of antisense oligodeoxynucleotides in immunology and autoimmunity research Immunomethods 1 191-202

Lane, L K , Shull, M M , Whitmer, K R , Lingrel, J B 1989 Characterization of two genes for the human Na,K-ATPase β subunit Genomics 5 445-453

Lappalainen, K , Urtti, A , Soderling, E , Jaaskelainen, I , Syrjanen, K , Syrjanen, S 1994 Cationic liposomes improve stability and intracellular delivery of antisense oligonucleotides into CaSki cells Biochim Biophys Acta 1196 201-208

Lauger, P 1991 Electrogenic Ion Pumps Sinauer and Associates, Inc Sunderland, Massachussetts, USA pp 168-225

Lewis, P R , Lutwak-Mann, C 1954 The content of sodium, potassium and chloride in rabbit blastocysts Biochim Biophys Acta 14 589-590

Lingrel, J B 1992 Na,K-ATPase Isoform structure, function, and expression J Bioenerg Biomembr 24(3) 263-270

Lobo, S M , Marzluff, W F , Seufert, A C , Dean, W L , Schultz, G A , Simerly, C , and Schatten, G 1988 Localization and expression of U1 RNA in early mouse embryo development Dev Biol 127 349-361

Lutsenko, S , Kaplan, J H 1992 Evidence of a role for the Na,K-ATPase ß-subunit in active cation transport In Ion-motive ATPase Structure, function and regulation (A , Scarpa, E , Carafoli, S , Papa, Eds) Ann N Y Acad Sci 671 113-119

MacPhee, D J , Barr, K J , De Sousa, P A , Todd, S D L , Kidder, G M 1994 Regulation of Na/K-ATPase α-subunit gene expression during mouse preimplantation development Dev Biol 162 259-266

Magyar, J P , Schachner, M 1990 Genomic structure of the adhesion molecule on glia (AMOG, Na/K-ATPase beta 2 subunit) Nucleic Acids Res 18 6695-6696

Magyar, J P , Bartsch, U , Wang, Z -Q , Howells, N , Aguzzi, A , Wagner, E F , Schachner, M 1994 Degeneration of neural cells in the central nervous system of mice deficient in the gene for the adhesion molecule on glia, the b₂subunit of murine Na/K-ATPase J Cell Biol 127(3) 835-845

Malo, D , Schurr, E , Levenson, R , and Gros, P 1990 Assignment of Na,K-ATPase ß2-subunit gene (Atpb-2) to mouse chromosome 11 Genomics 6: 697-699

Manejwala, F M , Cragoe, E J Jr , and Schultz, R M 1989 Blastocoel expansion in the preimplantation mouse embryo role of extracellular sodium and chloride and possible apical routes of their entry. Dev Biol 133 210-220

Maniatis, T and Reed, R 1987 The role of small nuclear ribonucleoprotein particles in pre-mRNA splicing Nature 325 673-678

Martin-Vasallo, P., Levenson, R., Emanuel, J R., Dackowski, W 1989 Identification of a putative isoform of the Na/K-ATPase beta-subunit-primary structure and tissue-specific expression J Biol Chem 264 4613-4618

Matthews, I , Sharma, R.P., Lee, A G., East, J.M 1990 Transmembranous organization of (Ca²-Mg² )-ATPase from sarcoplasmic reticulum Evidence for lumenal location of residues 877-888 J Biol Chem 265 18737-18740

McDonough, A A., Azuma, K.K., Lescale-Matys, L , Tang, M , Nakhoul, F , Hensley, C.B , Komatsu, Y 1992 Physiologic rationale for multiple sodium pump isoforms Differential regulation of α1 vs α2 by ionic stimuli In Ion-motive ATPases Structure, function, and regulation (A , Scarpa, E , Carafoli, S , Papa, eds) Ann NY Acad Sci 671 156-169

McDonough, A.A , Geering, K., Farley, R A 1990 The sodium pump needs its ß subunit FASEB 4 1598-1605

McLaren, A 1982 The embryo In Reproduction in Mammals 2 Embryonic and Fetal Development (C R , Austin, R V , Short, eds) pp 1-26 Cambridge University Press, Cambridge

McLachlin, J R , Kidder, G M 1986 Intercellular junctional coupling in preimplantation mouse embryos effect of blocking transcription or translation Dev Biol 117 146-155

Mercer, R W Schneider, J W , Savitz, A , Emanuel, J , Benz, E.J Jr., Levenson, R 1986 Rat-brain Na,K-ATPase ß-chain gene primary structure, tissue-specific expression, and amplification in ouabain-resistant HeLa C cells Mol Cell Biol 6(11). 3884-3890

Mercer, R W , Biemesderfer, D , Bliss, D P Jr , Collins, J.H., Forbush III, B 1993 Molecular cloning and immunological characterization of the γ polypeptide, a small protein associated with the Na,K-ATPase J Cell Biol 121(3) 579-586

Munroe, S 1988 Antisense RNA inhibits splicing of pre-mRNA in vitro EMBO J 7 2523-2532

Munzer, J S , Daly, S E , Jewell-Motz, E A , Lingrel, J B , Blostein, R 1994 Tissue- and isoform-specific kinetic behaviour of the Na,K-ATPase J Biol Chem 269(24) 16668-16676

O'Brodovich, H , Staub, O , Rossier, B C , Geering, K , Kraehenbuhl, J -P 1993 Ontogeny of α1 and ß1-isoforms of Na , K -ATPase in fetal distal rat lung epithelium Am J Physiol 264 C1137-C1143

Orlowski, J , Lingrel, J B 1990 Thyroid and glucocorticoid hormones regulate the expression of multiple Na/K-ATPase genes in cultured neonatal rat cardiac myocytes J Biol Chem 265 3462-3470

Ovchinnikov, Y A , Modyanov, N N , Broude, N E , Petrukhin, K E., Grishin, A V , Arzamazova, N M , Aldanova, N.A., Monastyrskaya, G S , Sverdlov, E D 1986 Pig kidney Na,K-ATPase primary structure and spatial organization FEBS Lett 201(2) 237-245

Ovchinnikov, Y A , Monastyrskaya, G S., Broude, N E , Ushkaryov, A M., Melkov, A M , Smirnov, Y V , Malyshev, I V , Allikmets, R.L., Kostina, M B., Dulubova, I E , Kiyatkin, N I , Grishin, A V , Modyanov, N N., and Sverdlov, E D 1988 Family of human Na ,K -ATPase genes Structure of the gene for the catalytic subunit (αIII-form) and its relationship with structural features of the protein. FEBS Letters 233 87-94

Overstrom, E W 1987 In vitro assessment of blastocyst differentiation In: The Mammalian Preimplantation Embryo Regulation of Growth and Differentiation In Vitro (B D , Bavister, ed) pp 95-116 Plenum Press, New York, NY

Pampfer, S , Vanderheyden, I., Vesela, J , De Hertogh, R 1995 Neutralization of tumor necrosis factor α (TNF α) action on cell proliferation in rat blastocysts by antisense oligodeoxyribonucleotides directed against TNF α p60 receptor Biol Reprod **52** 1316-1326

Paria, B D , Dey, S K , Andrews, G K 1992 Antisense c-myc effects on preimplantation embryo development Proc Natl Acad Sci USA **89** 10051-10055

Pietrini, G , Matteoli, M , Banker, G., Caplan, M J 1992 Isoforms of the Na,K-ATPase are present in both axons and dendrites of hippocampal neurons in culture Proc Natl Acad Sci USA **89** 8414-8418

Pietrini, G , Suh, Y J , Edelmann, L , Rudnick, G , Caplan, M 1994 The axonal γ-aminobutyric acid transporter GAT-1 is sorted to the apical membranes of polarized epithelial cells J Biol Chem **269**(6) 4668-4674

Powers, R D, Tupper, J T 1975 Ion transport and permeability in the mouse egg Exp Cell Res **91** 413-421

Powers, R.D, Tupper, J T 1977 Developmental changes in membrane transport and permeability in the early mouse embryo Dev Biol **56** 306-315

Pressley, T A 1992 Phylogenetic conservation of isoform-specific regions within α-subunit of Na,K-ATPase Am J Physiol **262** C743-C751

Quattrone, A , Papucci, L , Schiavone, N , Mini, E , Capaccioli, S 1994 Intracellular enhancement of intact antisense oligonucleotide steady state levels by cationic lipids Anti-Cancer Drug Design **9** 549-553

Rappolee, D A , Wang, A., Mark, D , and Werb, Z 1989 Novel method for studying mRNA phenotypes in single or small numbers of cells J Cell Biochem **39** 1-11

Rose, A M , Valdes, Jr , R 1994 Understanding the sodium pump and its relevance to disease Clin Chem **40**(9) 1674-1685

Rosenberg, U B , Preiss, A , Seifert, E , Jackle, H , Knipple, D C 1985 Production of phenocopies by *Kruppel* antisense RNA injection into *Drosophila* embryos Nature **313** 703-706

Rossier, B D , Geering, K , Kraehenbuhl, J P 1987 Regulation of the sodium pump How and why? Trends Biochem Sci **12** 483-487

Sambrook, J , Fritsch, E F , Maniatis, T 1989 Molecular cloning a laboratory manual 2nd ed , Cold Spring Harbor Laboratory Press, New York

Schmalzing, G , Kroner, S , Schachner, M , Gloor, S 1992 The adhesion molecule on glia (AMOG/ß2) and α1-subunits assemble to form functional sodium pumps in *Xenopus* oocytes J Biol Chem **267** 2212-2216

Schmalzing, G , Gloor, S 1994 Na+/K+-pump beta subunits structure and function Cell Physiol Biochem **4** 96-114

Schneider, J W , Mercer, R W , Caplan, M , Emanuel, J R , Sweadner, K J Benz, E J Jr , Levenson, R 1985 Molecular cloning of rat brain Na,K-ATPase α-subunit cDNA Proc Natl Acad Sci USA **82** 6357-6363

Shahedi, M , Laborde, K , Bussieres, L , Sachs, C 1993. Acute and early effects of aldosterone on Na,K-ATPase activity in Madin-Darby canine kidney epithelial cells Am J Physiol **264** F1021-F1026

Shamraj, O I , Lingrel, J B 1994 A putative fourth Na/K-ATPase α-subunit gene is expressed in testis Proc Natl Acad Sci USA **91** 12952-12956

Shull, G E , Schwartz, A , Lingrel, J B 1985 Amino-acid sequence of the catalytic subunit of the (Na + K ) ATPase deduced from a complementary DNA Nature **316** 691-695

Shull, G E , Greeb, J , Lingrel, J B 1986 Molecular-cloning of 3 distinct forms of the Na+,K+-ATPase alpha-subunit from rat-brain Biochemistry **25** 8125-8132

Shull, G E , Greeb, J 1988. Molecular cloning of two isoforms of the plasma membrane Ca2+-transporting ATPases Structural and functional domains exhibit similarity to Na,K- and other cation transport ATPases J Biol Chem **263** 8646-8657

Shull , M M , Pugh, D G , and Lingrel, J B 1989 Characterization of the human Na,K-ATPase α2 gene and identification of intragenic restriction fragment length polymorphisms J Biol Chem **264** 17532-17543

Shyjan, A W , Levenson, R 1989 Antisera specific for the α1, α2, α3, and ß subunits of the Na,K-ATPase Differential expression of α and ß subunits in rat tissue membranes Biochemistry **28** 4531-4535

Shyjan, A W , Gottardi, C , Levenson, R 1990 The Na,K-ATPase ß2 subunit is expressed in rat brain and copurifies with Na,K-ATPase activity J Biol Chem **265**(9) 5166-5169

Slavkin, H C 1995 Antisense oligonucleotides an experimental strategy to advance a causal analysis of development Int J Dev Biol **39** 123-126

Spindle, A 1980 An improved culture medium for mouse blastocysts In Vitro 16 669-674

Ueno, S., Takeda, K., Noguchi, S., Izumi, F, Kawamura, M 1995 The expression of Na/K-ATPase ß-subunit cRNA injected into *Xenopus* oocytes is affected by coinjection with α-subunit cRNA 148 51-56

Uruyama, O, Shutt, H, Sweadner, K J 1989 Identification of three isozyme proteins of the catalytic subunit of the Na,K-ATPase in rat brain J Biol Chem 264(14) 8271-8280

Valdimarsson, G, Huebner, E 1989 Diethylene glycol distearate as an embedding medium for immunofluorescence microscopy Biochem Cell Biol 67 242-245

Valdimarsson, G, DeSousa, P A, Beyer, E C, Paul, D L, Kidder, G M 1991 Zygotic expression of the connexin 43 gene supplies subunits for gap junction assembly during mouse preimplantation development Mol Reprod Dev 30 18-26

Valdimarsson, G, De Sousa, P A, Kidder, G M 1993 Co-expression of gap junction proteins in the cumulus-oocyte complex Mol Reprod Dev 36 7-15

Van Why, S K, Mann, A S, Ardito, T, Siegel, N J, Kashgarian, M 1994 Expression and molecular regulation of Na -K -ATPase after renal ischemia Am J Physiol 267 F75-F85

Van Winkle, L J and Campione, A L 1991 Ouabain-sensitive Rb uptake in mouse eggs and preimplantation conceptuses Dev Biol 146 158-166

Vasilets, L A, Schwarz, W 1994 The Na/K pump structure and function of the alpha subunit Cell Physiol Biochem 4 81-95

Verrey, F, Kraehenbuhl, J P, Rossier, B C 1989 Aldosterone induces a rapid increase in the rate of Na,K-ATPase gene transcription in cultured kidney cells Mol Endocrinol 3 1369-1376

Waelchli, R O, Gerber, D., Volkmann, D H, Betteridge, K J 1996 Changes in the osmolality of equine blastocyst fluid between days 11 and 25 of pregnancy Theriogenology 45 290

Watanabe, Y, Kawakami, K, Hirayama, Y, Nagano, K 1993 Transcription factors positively and negatively regulating the Na,K-ATPase α1 subunit gene J Biochem 114 849-855

Watson, A J, Kidder, G M 1988 Immunofluorescence assessment of the timing of appearance and cellular distribution of Na/K-ATPase during mouse embryogenesis Dev Biol 126 80-90

Watson A J , Pape, C , Emanuel, J R , Levenson, R , Kidder, G M 1990a Expression of
Na/K-ATPase α and β subunit genes during preimplantation development of the mouse
Dev Genet 11 41-48

Watson, A J , Damsky, C H , Kidder, G M 1990b Differentiation of an epithelium
Factors affecting the polarized distribution of Na/K-ATPase in mouse trophectoderm
Dev Biol 141 104-114

Watson, A J 1992 The cell biology of blastocyst development Mol Reprod Dev 33
492-504

Wiley, L M 1988 Trophectoderm The first epithelium to develop in the mammalian
embryo Scan Microsc 417-426

Wiley, L M , Kidder, G M , Watson, A J 1990 Cell polarity and development of the first
epithelium Bioessays 12 67-73

Woolf, T M , Melton, D A., Jennings, C G B 1992 Specificity of antisense
oligonucleotides in vivo Proc Natl Acad Sci USA 89 7305-7309

Yakubov, L A , Deeva, E A , Zarytova, V F , Ivanova, E M , Ryte, A S , Yurchenko,
L V , Vlassov, V V 1989 Mechanism of oligonucleotide uptake by cells involvement of
specific receptors Proc Natl Acad Sci USA 86 6454-6458

Yaswen, P , Stampfer, M R , Ghosh, K., Cohen, J S 1992 Effects of thioated
oligonucleotides on cultured human mammary eptithelial cells Antisense Res Devel
in press

Young, R M , Shull, G E , Lingrel, J B 1987 Multiple mRNAs from rat kidney and brain
encode a single Na', K -ATPase beta subunit protein J Biol Chem 262 4905-4910

Zamecnik, P , Stephenson, M 1978 Inhibition of rous sarcoma virus replication and cell
transformation by a specific oligodeoxynucleotide Proc Natl Acad Sci USA 75 280-
284

Ziomek, C A 1987 Cell polarity in the preimplantation mouse embryo In Bavister, B D
(ed ) The mammalian preimplantation embryo Regulation of growth and differentiation in
vitro NewYork Plenum Publishing pp 23-41

Zlokovic, B V , Mackic, J B , Wang, L , McComb, J G , McDonough, A 1993.
Differential expression of Na,K-ATPase α and β subunit isoforms at the blood-brain
barrier and the choroid plexus J Biol Chem 268(11) 8019-8025