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

DANIEL J. MACPHEE

Department of Zoology

Thesis submitted in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

Faculty of Graduate Studies
The University of Western Ontario
London, Ontario
August, 1996

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ISBN 0-612-15066-6
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 reaction (RT-PCR), whether all α (α1, α2, α3, α4) and β (β1 and β2) isoforms of the sodium pump were expressed during preimplantation development. Previous work by Watson and Kidder (1988) had shown that the α subunit was localized to the basolateral domains of mural trophectoderm surrounding the blastocoel. The spatial localization of α1 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.

α3 and β2 isoform transcripts were detected throughout mouse preimplantation stages. α2 transcripts were only detected in oocytes while α4 transcripts were not detected in any embryo stages. None of the α3, α2, or β2 isoform subunits were detected by immunocytochemistry, in membranes of preimplantation stages. α3 subunits were detected in morulae/blastoct total protein on a western blot.

α1 subunits were found in membranes of all preimplantation stages while β1 subunits were only detectable from the morulae stage onwards. α1 transcripts were detected throughout the cytoplasm of mouse morulae and blastocysts correlating with the presence of α1 subunits in membranes. These results provide evidence that α1 and β1 subunits are the most probable subunit isoforms of the sodium pump, to mediate fluid accumulation in mouse morulae and blastocysts.

Using antisense oligodeoxynucleotides (ODNs), β1 subunit mRNA was targeted in the mouse preimplantation embryo to help determine the role of the β1 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 β1 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. α1 and β1 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
ACKNOWLEDGEMENTS

First and foremost, I have to thank Dr. Gerry Kidder for all his guidance and friendship during my tenure. His professionalism, continuous scientific curiosity, and respect for graduate students and graduate student issues does not go unnoticed. I have always felt that a happy, productive experience in a laboratory is the direct result of a great leader running the group, and I thank him for six great years. I know our paths will continually cross in the future.

Over a number of years, I have had the pleasure to meet a lot of good people. The closest inevitably are the ones you work with every day: Dr. Gunnar Valdimarsson, Dr. Paul De Sousa, Dr. Andy Watson, Dr. Ashley Garrill, Dr. Subhash Juneja, Kevin Barr, Tyler Davies, Holly Jones, Daguang and Ying Zhu, Dean Betts and Quinton Winger are not only colleagues but good friends. We will always be in touch.

Many thanks go out to people in the department of Zoology who have been vital in getting this thesis completed. Ian Craig for help with photography, Harry Leung and Rick Harris for help with confocal microscopy, and of course Mary Martin who is the best friend a graduate student can have in the department. I do not know what students would do without her.

Finally, I wish to dedicate this thesis to my family. My parents always instilled in me scientific curiosity, stubbornness (to never give up or give in), and above all else, the will to be the best I can be. Many thanks go out to my two best friends, Ian and Chantelle, who put up with my constant complaining, nagging, and bad temper during the completion of this thesis.
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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 = α1 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 I
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 $10^3$ 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.
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 α and β (for reviews see Lingrel, 1992, Mercer, 1993). Both α and β 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 al., 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, α3, 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 α-subunit is responsible for the physiological role of the enzyme (Jorgensen, 1986), while the β-subunit may facilitate the processing and insertion of the α-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 (Jaiser et al., 1992, Lutsenko and Kaplan, 1992).

The α subunit is a catalytic protein of about 1000 amino acids and approximately 100–115 kDa, containing binding sites for ATP and cardiac glycosides. In rodents the α1 subunit is resistant to ouabain, a cardiac glycoside and specific inhibitor of the sodium pump, while α2 and α3 isoforms are ouabain sensitive (Vasilets and Schwarz, 1994) The α-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 Ca2+ -ATPase and H/K-ATPase. When the enzyme is an E1 form, the amino terminus of the α-subunit can be rapidly cleaved by trypsin, altering equilibrium between E1 and E2 conformations. The trypic 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 Ca2+ -ATPase SERCA1 on the luminal 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 (Chibalin 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 β1 subunits, do not greatly change the structure of the protein (Mercer, 1993).

The β2 subunit of the mouse has 42% amino acid sequence identity with mouse β1 (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 β1 (Martin-Vasallo et al., 1989). These sites can be found at amino acid positions 159, 193 and 250 of β2 and at the corresponding positions of 158, 193 and 266 of the rat β1. Hydropathy profile suggests that the β2-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 β1 and β2 subunits appear to be virtually identical. A β3 subunit is also known to exist but it appears to be restricted to the nervous system of *Xenopus laevis* (Good et al., 1990).

The β2 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 β 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 kDa (Mercer et al., 1993) and peptide sequencing has established that it is not a breakdown product of α or β (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 α3 isoforms. 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 substitutions. 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 of 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-isofrom 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 30 kb species predominates in kidney, whereas the 24 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 β2 cDNA was originally isolated from a human fetal liver λgt11 cDNA library (Martin-Vasallo et al., 1989) by screening with a β1 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 β2 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 β2 cDNA exhibited 53% and 55% homology with the corresponding rat and Torpedo californica B1 cDNA (Mercer et al., 1986, Young et al., 1987, Martin-Vasallo et al., 1989). On the other hand, the 3′-untranslated region of rat β2 showed virtually no sequence similarity with the 3′-untranslated region of rat or Torpedo B1. The protein encoded by the human β2 clones exhibited 98% identity with the rat β2 amino acid sequence indicating the two cDNAs isolated encoded almost identical proteins (Martin-Vasallo et al., 1989).

Using the rat β2 cDNA as a probe, northern blots of rat tissue RNA were analyzed to determine tissue specific expression of β2 mRNA (Martin-Vasallo et al., 1989). A single β2 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 β2 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 (T3) 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 α and β subunits. This response presumably involves interaction of aldosterone-receptor complexes with specific hormone responsive elements in the promoters of sodium pump α and β subunit genes (Ewart and Klip, 1995) T₃ is a major determinant of steady state sodium pump activity in heart, skeletal muscle, liver and kidney (Ismail-Beigi, 1993) T₃ stimulates pump activity by increasing the number of pump molecules

T₃ was found to cause a dose dependent effect on α1, α2, β1, and β2 isoforn 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 T₃ administration, in α and β-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 α1 promoter has found an α1 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 α2 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, Ser145, on the α1-subunit is the phosphorylation site for PKA while Thr15 and Ser16 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⁺, Ca²⁺, and Mg²⁺ 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 $1 \times 10^{-4}$ M ouabain, the embryos fail to reexpand (DiZio and Tasca, 1977; Betts et al., 1990, 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 $\alpha$ 1-subunit of the Na,K-ATPase in all stages of mouse preimplantation development (Watson et al., 1990a; Gardiner et al., 1990b). The $\alpha$1-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). $\beta$1-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, $\beta$1-transcripts have been detected in early cleavage stages of the mouse using the more sensitive reverse transcriptase-polymerase chain reaction (RT-PCR) technique (Kidder, 1993b). Northern blot analysis failed to detect other isoform mRNAs ($\alpha$2, $\alpha$3, $\beta$2) 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 $\alpha$ subunit synthesis during preimplantation development may be imposed downstream of transcription (MacPhee et al., 1994). According to immunofluorescence analysis on sectioned embryos, the $\alpha$ 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 $\alpha$1 mRNA in the 2-cell stage.
in fact, the α1 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 α1 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 α1 mRNA located during preimplantation development and is the α1 subunit present in the inner cell mass and polar trophectoderm of blastocyst stage mouse embryos?

2. Where and when are α2, α3, and β2 subunits expressed in early cleavage stages of mouse development?

3. Are α1 and β1 subunits expressed in horse conceptuses during fluid accumulation and blastocoel expansion?

4. Is the β1 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 CB6F1/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 Ca²⁺- and Mg²⁺-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 MgCl₂, 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 (ddH₂O).
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 μL of 50 mM Tris-HCl, 0.1 M EDTA, pH 8.5 and 1 μg/μL 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 α1 cDNA was ligated to 1 μg of dephosphorylated pGEM4 with 10 Units of T4 DNA ligase (Life Technologies) in 20 μL of 50 mM Tris-HCl (pH 7.6), 10 mM MgCl2, 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, 1 mM EDTA, pH 8.0) and 1 μL of 0.5 M EDTA was added to each sample. To twenty microlitres of the mixture was added 100 μL 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 μL 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 μg/mL ampicillin (amp). The remaining volume of sample was gently centrifuged and the resulting pellet resuspended in 100 μL 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 μg/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 α1-subunit-containing pGEM4 (pGEM4α1), 1 μg of each plasmid DNA clone was incubated with the restriction endonuclease KpnI in 30 μL of 20 mM Tris-HCl, pH 7.4, 5 mM MgCl2, 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 μg/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 80 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 μL of 3 M sodium acetate, transferred to 15 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 0.1 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

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>cDNA Length</th>
<th>Linearize with</th>
<th>RNA Polymerase for Transcription</th>
<th>cDNA Orientation</th>
</tr>
</thead>
<tbody>
<tr>
<td>pGEM4α!a</td>
<td>3.2 Kb</td>
<td><em>Sma</em>I</td>
<td>T7</td>
<td>Sense</td>
</tr>
<tr>
<td>pGEM4α1b</td>
<td>3.2 Kb</td>
<td><em>Sma</em>I</td>
<td>T7</td>
<td>Antisense</td>
</tr>
<tr>
<td>pSP64U1b</td>
<td>0.4 Kb</td>
<td><em>Eco</em>RI</td>
<td>SP6</td>
<td>Sense</td>
</tr>
<tr>
<td>pSP65U1b</td>
<td>0.4 Kb</td>
<td><em>Hind</em>III</td>
<td>SP6</td>
<td>Antisense</td>
</tr>
</tbody>
</table>
Boehringer-Mannheim Canada (Laval, Québec) One microlitre of template plasmid DNA, linearized with the appropriate restriction endonuclease (Table 2.1), was transcribed at 37°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 MgCl₂, 10 mM dithioerythritol, 2 mM spermidine, 10 mM NaCl, 1 Unit of RNase inhibitor and containing 10 μCi of 10.5 nmol/mL [³⁵S]-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°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 ddH₂O, 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 × 14 cm were soaked in diethylpyrocarbonate-treated (DEPC) ddH₂O, then soaked in 3 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 NaH₂PO₄, 5 mM EDTA, pH 7.4 (5X SSPE) then prehybridized for 4 hours at 42°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-Cl 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 onto GF/F filters (Whatman, VWR Canlab) for determination of total and trichloroacetic acid (TCA) precipitable 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™ 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-Cl 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-Cl 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 30) until the color reaction had occurred. Color reactions were stopped with a 5 minute soak in 10 mM Tris-HCl, 1 mM EDTA (Fig 22)

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, 1 mM 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 T1 (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 (Testorg, Chicago, Ill.) 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 is associated with spatially restricted α1 mRNA accumulation, a wholomount 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 control 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 α1 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 α1 and U1b sense riboprobes did not hybridize to target mRNAs (data not shown)
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 α1 riboprobes, respectively. E,F- labeled and unlabeled control RNAs, respectively.
FIGURE 2.3  Distribution of J1b RNA in the morula and early blastocyst stages as revealed by wholamount 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 trophoderm demonstrates that transcription of the α1 gene is
not limited to the mural trophoderm.

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 trophoderm, 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 α1 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 β 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 β subunits in functional expression of α subunits
(reviewed by Geering, 1990b, 1991b) and the fact that, although β1 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 β 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/β 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 isoforms in addition to α1β1 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 β subunit
isoforms. 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 β subunit gene.

The expression of Na,K-ATPase α and β 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, α1 mRNAs are much more abundant than β1 mRNAs during early stages of fetal lung development, with the abundance of β1 mRNAs increasing to approach that of α1 mRNAs just before birth (O'Brodovich et al., 1993). In general, α subunit mRNA abundance exceeds that of β subunit mRNA in developing and adult organs such that β subunit mRNA content appears to determine the total Na,K-ATPase activity (Gick et al., 1993). Interestingly, post-transcriptional regulation of α subunit expression has been demonstrated during brain development (Corthesy-Theulaz et al., 1991).
CHAPTER 3

EXPRESSION OF Na,K-ATPASE α AND β 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. Waechli (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. Waechli 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 MgCl₂, 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 $\alpha$- and $\beta$-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 $\mu$M of sequence-specific primers (4 $\mu$M for $\beta_2$ 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 $\alpha_2$, $\alpha_3$, and $\alpha_4$ isoforms were amplified for 40, 34, and 40 cycles respectively while $\beta_2$ was amplified for 38 cycles. Horse $\alpha_1$ and $\beta_1$ isoforms were amplified for 40 cycles. PCR products (11 $\mu$l) 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., 1987, Martin-Vasallo et al., 1989, Kano et al., 1989, Gloe et al., 1990) and the sizes of the expected PCR products are shown in Table 31. 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 31). To confirm identity, each DNA product was subjected to cleavage with an appropriate diagnostic restriction enzyme (Table 31) 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

<table>
<thead>
<tr>
<th>Subunit isoform</th>
<th>Primer sequences</th>
<th>Amplicon position and size (bp)</th>
<th>Genomic DNA size (bp)</th>
<th>Diagnostic enzyme</th>
<th>Fragment Sizes (bp)</th>
</tr>
</thead>
</table>
| α1              | 5’ primer= ACCTGCTGGGACTCCGCGTG
               | 3’ primer= AGGGGAAGGCACAGAACCACCA | Exons 18-22 or horse cDNA = 336 | 4246 | Nsp I | 220 416 |
| α2              | 5’ primer= GGCTGCTTGGGATCCGCTTGA
               | 3’ primer= AGGGGAAGGCACAGAACCACCA | 2731-3066 of rat cDNA = 335 | 2946 | Ava I | 267 68 |
| α3              | 5’ primer= ACCTGCTGGGACTCCGCGTG
               | 3’ primer= AGGGGAAGGCACAGAACCACCA | 2755-3091 of rat cDNA = 336 | 2946 | Bcl I | 179 157 |
| α4              | 5’ primer= GGAGTTGAAGAAAGGAAGTGG
               | 3’ primer= AGCCAGTGATGATGACCACG | 114-452 of rat cDNA = 338 | 3478 | Hae III | 242 96 |
| β1              | 5’ primer= TTCAGCCTCAAGAGGACGACATG
               | 3’ primer= AGGGAAAGCCGTAGTATCCGCCCA | 378 | 6346 | Hmd III | 266 112 |
| β2              | 5’ primer= GCCTTACAACGACTCCATCCA
               | 3’ primer= CCGCATTCTACATTACCTCC | 805-1246 of rat cDNA = 441 | 1664 | Hae III | 291 150 |


* 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**

*Dihydroxyethylene Glycol Disterate (DGD) Embedding*

Embryos were fixed in 4% paraformaldehyde-0 2% glutaraldehyde or 4% paraformaldehyde in PHEM buffer (60 mM Pipes, 25 mM Hapes, 10 mM EGTA, 1 mM MgCl₂, 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°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°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°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%, 70%, and 50% ethanol by immersion for 3 minutes each During the 70% ethanol soak, a second 70% ethanol soak containing 1 mg/mL NaBH₄ 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°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 Swedner (Massachusetts General Hospital, Boston, Massachusetts, USA). All primary
Table 3.2
A Summary of the Antibodies Used in Immunocytochemistry (IC) and Western Blotting

<table>
<thead>
<tr>
<th>Name</th>
<th>Type</th>
<th>Recognition Site</th>
<th>Used for Western blotting</th>
<th>Used for Immunocytochemistry</th>
<th>Recommended Dilutions for IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>UBI α1</td>
<td>Pc</td>
<td>Rat a a 338-513</td>
<td>✓(X)³</td>
<td>✓(X)¹</td>
<td>1 200</td>
</tr>
<tr>
<td>UBI α2</td>
<td>Pc</td>
<td>Rat a a 335-519</td>
<td>✓(X)</td>
<td></td>
<td>1 200</td>
</tr>
<tr>
<td>UBI α3</td>
<td>Pc</td>
<td>Rat a a 320-514</td>
<td>✓(X)</td>
<td>✓(X)</td>
<td>1 200</td>
</tr>
<tr>
<td>UBI B1</td>
<td>Pc</td>
<td>Rat a a 152-304</td>
<td>✓(X)</td>
<td>✓(X)</td>
<td>1 200</td>
</tr>
<tr>
<td>UBI B2</td>
<td>Pc</td>
<td>Rat a a 63-285</td>
<td>✓(X)</td>
<td>✓</td>
<td>1 200</td>
</tr>
<tr>
<td>NASE α1</td>
<td>Pc</td>
<td>Rat a a 489-499</td>
<td>✓</td>
<td>✓(X)</td>
<td>1 100</td>
</tr>
<tr>
<td>HERED α2</td>
<td>Pc</td>
<td>Rat a a 489-500</td>
<td>✓</td>
<td>✓(X)</td>
<td>1 100</td>
</tr>
<tr>
<td>TED α3</td>
<td>Pc</td>
<td>Rat a a 489-500</td>
<td>✓(X)</td>
<td>✓(X)</td>
<td>1 100</td>
</tr>
<tr>
<td>ASKARI B1</td>
<td>Pc</td>
<td>Dog whole B1</td>
<td>✓</td>
<td>✓</td>
<td>1 100</td>
</tr>
<tr>
<td>CAPLAN α1</td>
<td>Mc²</td>
<td>Dog a a 3-16</td>
<td>✓</td>
<td>✓</td>
<td>1 100</td>
</tr>
<tr>
<td>CAPLAN α3</td>
<td>Pc</td>
<td>Rat a a 2-14 ³</td>
<td>✓</td>
<td>✓</td>
<td>1 50</td>
</tr>
<tr>
<td>CAPLAN B1</td>
<td>Mc</td>
<td>Dog B1 subunit</td>
<td>✓</td>
<td></td>
<td>1 50</td>
</tr>
<tr>
<td>McB2 α2</td>
<td>Mc</td>
<td>Rat N-termminus</td>
<td>✓</td>
<td></td>
<td>1 4</td>
</tr>
<tr>
<td>RDI α3</td>
<td>Mc</td>
<td>Dog α3 subunit</td>
<td>✓</td>
<td></td>
<td>1 100</td>
</tr>
<tr>
<td>McTub</td>
<td>Mc</td>
<td>Sea Urchin α-</td>
<td>✓</td>
<td></td>
<td>1 100</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tubulin</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

¹Polyclonal Antiserum
²Monoclonal Antiserum
³Check mark indicates that the antiserum was used for the described experiments

If an X is present in brackets this signifies that no fluorescent signal (immunocytochemistry) 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 (Uryuama 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, 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. Sweedner, personal communication), centrifuged 10 minutes at 10,000 rpm at 4°C and the supernatant recovered. 30 μg of mouse kidney, 30 μg of mouse brain and liver total protein, total protein from 650 mouse embryos, and 25 μL of horse embryo protein were loaded onto 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% polyacrylamide-0.1% SDS resolving gel (12% polyacrylamide was used for analyzing β1 subunits in horse samples). The samples were then electrophoresed at 100 V.

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% Coomassie 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% 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% Boehringer 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% 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 β-subunit isoform transcripts in murine preimplantation embryos. Each lane represents the α- and β-subunit RT-PCR products derived from total RNA from the equivalent of 5 embryos. Transcripts encoding Na/K-ATPase α2, α3, α4, and β2-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 β2-subunits and mouse testis cDNA for the α4-subunit. Transcripts encoding Na/K-ATPase α3 and β2-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.
<table>
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its derivation from both maternal and embryonic gene transcription. The identity of the \( \alpha3 \) amplicon was confirmed with restriction endonuclease digestion analysis. For example, the restriction enzyme \( BclI \) cut the amplicon into the predicted 178 and 158 bp fragments (Table 3, Fig 3.2). Transcripts of the \( \alpha4 \)-isoform gene were not detected in any of the preimplantation stages examined (Fig 3.1). However, an \( \alpha4 \)-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 \( \beta2 \) amplicon was also detected in RNA samples throughout murine preimplantation development (Fig 3.1). Again, the apparent increase in \( \beta2 \) mRNA after the 4-cell stage indicated that the gene may be embryonically transcribed. Amplicon identity was confirmed with the restriction enzyme \( HaeIII \) (Table 3, Fig 3.2). \( \alpha3 \) and \( \beta2 \) 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 \( \alpha1 \) and \( \beta1 \)-subunit mRNAs during horse development**

Both \( \alpha1 \) and \( \beta1 \) 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. \( NspI \) cut the \( \alpha1 \) amplicon into the predicted 220, 116 bp pieces, while \( HindIII \) cut the \( \beta1 \) 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 \( \alpha1 \) amplicon was found to be 97.6% identical with the corresponding region, exons 19-22, of the published horse \( \alpha1 \) sequence (Kano et al. 1989) and the \( \beta1 \) amplicon was found to be 83.5% identical with the corresponding region of the rat \( \beta1 \) 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.
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.
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 SspI producing two bands of 237.99 bp. Right panel β1 isoform UC = uncut amplicon, C = β1 amplicon digested with HinfI III producing two bands of 266, 112 bp. L = 1 kb Ladder.
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.
FIGURE 3.7 Verification of horse sodium pump β1 isoform identity by direct sequencing. The amplicon (1-324 bases) was compared to the published rat β1 sequence (801-1197 bases; Genbank Accession # J02701) with the computer program GAP. The amplicon had 83% sequence identity with the rat sequence.
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).

UBIα1 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 β1 subunits in DGD sections of mouse blastocysts with Askari β1 A blastocyst B morulae C blastocyst incubated with secondary goat antiserum raised against rabbit IgG  Scale Bar= 25 μm
Wholernount 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. UBIα1 and β1 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. α 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 α isoforms or β1 subunits were detected in methanol fixed blastocysts or morulae with NASEα1, HEREDα2, TEDα3 or Askariβ1 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 α-tubulin was detectable in microtubules of mouse embryos examined. The lack of detectable α1 subunits was surprising because Becker and Davies (1994), using the identical immuno-cytochemical procedure and NASEα1, observed general cytoplasmic localization but no membrane staining of α1 subunits throughout mouse blastocysts. Higher concentrations of NASE and Askari antisera were tested (1:20) but no α1 or β1 subunits were detected in blastocysts.

With the localization of α-tubulin throughout the mouse embryo after extraction with 0.01% Triton X-100, future experiments utilized the same detergent concentrations. Utilizing CAPLAN α1, α1 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 RD1α3, 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 B1, B1 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 B1, B1 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™ 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 β2 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.
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 β2 subunits is unknown UBIβ2 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 β1 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 β1 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 trophoblast (Watson and Kidder, 1988). In contrast, Gardiner et al. (1990b) reported the presence of Na,K-ATPase α- and β-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 β 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\textsuperscript+-pump isoform is predominantly confined to the testes (Shamraj and Lingrel, 1994). Due to the absence of an antibody against the α4 subunit, it was not possible to determine the presence or absence of the protein in preimplantation embryos. α2 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 α1 and α2 isoform transcripts in the 9.5-10.5d post-coital mouse embryo. By 15.5-16.5 days post-coitus, differential expression of α1, α2, and α3-transcripts becomes evident in developing organs. The results in the preimplantation embryo raise the possibility that two Na,K-ATPase α subunit isoforms (α3, α1) are co-expressed in the early mammalian embryo prior to tissue specific differentiation.

Northern blot analysis has revealed that transcripts encoding the mouse B1-isoform are first expressed in the late morula with the level increasing by the blastocyst stage (Watson et al., 1990a). The B1-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 B1-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 B2 isoform mRNA was detected throughout preimplantation development (Fig. 3.1), the mRNAs encoding the B2-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 B2 protein was detected and high background was observed with this antiserum. This analysis, therefore, requires a new antiserum raised against the B2 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 B2 null mutant mouse appears to suffer no ill effects during preimplantation development (Magyar et al., 1994). Therefore, the B1-isoform is still the most reasonable
candidate for regulating the expression of functional enzyme units in the trophoderm
cordinate with the onset of cavitation. Gardiner et al. (1990b) detected a 2 fold increase
in β1 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 β1 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). α1, α2, α3,
and β2 transcripts were detected from the 1-cell zygote to blastocyst stages. However, β1
transcripts were only detectable from the morula stage onward. This expression pattern is
similar to that in the mouse, where β1 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 β1 subunit is closely coordinated with the onset of cavitation.
The two species are different, however, because unlike the mouse, α2 transcripts were
detected throughout bovine preattachment stages and the α3 subunit was detected in
apical membrane domains of the trophoderm (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 α-subunit
mRNA exceeds that of β-subunit mRNA suggesting that β-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, α1 subunit mRNA is more abundantly
expressed than β1 mRNA (O’Brodovich et al., 1993). The presence of multiple Na-pump
isoforms in adult rodent and human tissues is well documented. α1, α2, and α3 isoforms
are co-expressed in organs such as brain, eye, heart, and skeletal muscle (Rose and
Valdes. 1994) while β1 and β2 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 α and β 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 α1- and α2-containing sodium pump molecules, expressed in HeLa cells, have similar apparent affinities for Na⁺, but that α3-containing enzyme has a 2-3 fold lower affinity for this ion. McDonough et al. (1992) found that low K⁺ concentrations decrease α2 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 β-subunit may be involved in stabilizing the correct transmembrane folding of the α-subunit (Geering, 1991, McDonough et al., 1993) rather than being involved directly in the ion transport process. However, different β-isoforms (β1 or β2) combined with various α-isoforms may produce modified membrane conformations and therefore influence enzyme kinetic properties. For example, when the mouse β2 isoform was expressed with α1 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 factors 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 (Gintner, 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 \( \beta \)-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, 1993iv) 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 \textit{in vivo}. 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 \textit{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 targeting 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 the *ugless* 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 IGF-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 Oligodeoxyribonucleotides (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′-GGGGCGAGTGGCCT-3′
Set #2 Sense 5'-GGAGCAGCCACCATG-3'
Antisense 5'-CATGGTGCTGCTCC-3'
Random 5'-CAGTTCGCGCGTCGCT-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 annealing 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 ddH₂O 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 ddH₂O 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, Burlington, Ontario, Canada) were added to 2 μL of ddH₂O 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.

<table>
<thead>
<tr>
<th></th>
<th>SECM</th>
<th>CZB</th>
<th>MTF</th>
<th>KSOM</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl 84.10</td>
<td>81.62</td>
<td></td>
<td>114.19</td>
<td>95.00</td>
</tr>
<tr>
<td>KCl 47.8</td>
<td>48.3</td>
<td></td>
<td>47.8</td>
<td>25.0</td>
</tr>
<tr>
<td>KH₂PO₄ 1.19</td>
<td>1.18</td>
<td></td>
<td>1.19</td>
<td>0.35</td>
</tr>
<tr>
<td>CaCl₂·2H₂O 0.00</td>
<td>1.70</td>
<td></td>
<td>1.71</td>
<td>1.71</td>
</tr>
<tr>
<td>MgSO₄·7H₂O 1.19</td>
<td>1.18</td>
<td></td>
<td>1.19</td>
<td>0.20</td>
</tr>
<tr>
<td>NaHCO₃ 22.50</td>
<td>25.12</td>
<td></td>
<td>25.00</td>
<td>25.00</td>
</tr>
<tr>
<td>Na-lactate 19.71</td>
<td>31.30</td>
<td></td>
<td>4.79</td>
<td>10.00</td>
</tr>
<tr>
<td>Na-pyruvate 0.25</td>
<td>0.27</td>
<td></td>
<td>0.37</td>
<td>0.20</td>
</tr>
<tr>
<td>α-lactate 1.71</td>
<td>0.00</td>
<td></td>
<td>0.00</td>
<td>0.00</td>
</tr>
<tr>
<td>glucose 0.00</td>
<td>5.55</td>
<td></td>
<td>3.40</td>
<td>0.20</td>
</tr>
<tr>
<td>Na-EDTA 0.00</td>
<td>0.11</td>
<td></td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>glutamine 0.00</td>
<td>1.00</td>
<td></td>
<td>0.00</td>
<td>1.00</td>
</tr>
<tr>
<td>BSA (mg/mL) 3.00</td>
<td>5.00</td>
<td></td>
<td>4.00</td>
<td>1.00</td>
</tr>
<tr>
<td>phenol red 10.00</td>
<td>10.00</td>
<td></td>
<td>10.00</td>
<td>10.00</td>
</tr>
<tr>
<td>(μg/mL) penicillin 100.00</td>
<td>100.00</td>
<td>100.00</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>(units/mL) streptomycin 50.00</td>
<td>50.00</td>
<td>50.00</td>
<td>50.00</td>
<td></td>
</tr>
</tbody>
</table>
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% CO₂ 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% CO₂ 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 variability 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.

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

\(^1\)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 43)

**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 < 0.05) than control embryos at 102 hrs post hCG of culture (Fig. 43). 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. 44). 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.

<table>
<thead>
<tr>
<th>ODN Concentration</th>
<th>Sense</th>
<th>Antisense</th>
<th>Random</th>
<th>Control (no ODN)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tested</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 μM</td>
<td>102.7 (1.5)</td>
<td>101.3 (1.5)</td>
<td>103.0 (1.2)</td>
<td>103.0 (2.9)</td>
</tr>
<tr>
<td>3 μM</td>
<td>100.7 (0.6)</td>
<td>100.7 (1.5)</td>
<td>100.0 (0)</td>
<td>102.0 (2.5)</td>
</tr>
<tr>
<td>4 μM</td>
<td>100.3 (2.3)</td>
<td>100.3 (1.2)</td>
<td>101.0 (0.6)</td>
<td>99.0 (1.5)</td>
</tr>
<tr>
<td>5 μM(^1)</td>
<td>100.0, 101.0</td>
<td>98.0, 100.0</td>
<td>n/d</td>
<td>102.0, 99.0</td>
</tr>
<tr>
<td>10 μM(^1)</td>
<td>100.0, 99.0</td>
<td>101.0, 99.0</td>
<td>n/d</td>
<td>102.0, 99.0</td>
</tr>
<tr>
<td>15 μM(^1)</td>
<td>100.0, 101.0</td>
<td>102.0, 100.0</td>
<td>n/d</td>
<td>102.0, 99.0</td>
</tr>
<tr>
<td>20 μM(^2)</td>
<td>100.0, 101.0</td>
<td>100.0, 98.0</td>
<td>n/d</td>
<td>98.0, 101.0</td>
</tr>
<tr>
<td></td>
<td>100.0 (0.5)</td>
<td>100.6 (0.6)</td>
<td>101.0 (1.0)</td>
<td>100.5 (0.9)</td>
</tr>
<tr>
<td></td>
<td>n/d</td>
<td>96.0, 96.0</td>
<td>97.0, 96.0</td>
<td>96.0, 97.5</td>
</tr>
<tr>
<td>40 μM(^2)</td>
<td>99.0, 103.0</td>
<td>102.0, 102.0</td>
<td>n/d</td>
<td>98.0, 101.0</td>
</tr>
<tr>
<td></td>
<td>n/d</td>
<td>96.0, 96.0</td>
<td>94.0, 95.0</td>
<td>96.0, 97.5</td>
</tr>
</tbody>
</table>

\(^1\)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 10 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 significantly 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).
Number of Embryos Cavitating

Culture Time (hrs phCG)

- Lipofectin
- Antisense
- Random
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 β1 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 whether significant amounts of ODNs be degraded by nucleases during the ODN treatment period, thereby preventing significant targeting of β1 mRNA? It is possible that β1 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 mRNA. As 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 medium 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 amount of 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 affected.

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 amide 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 trophoderm 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 proc1 (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-Menten kinetics.

In an experiment testing 10 μM 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, 10 μM 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 β1 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 β1 subunit is required for cavitation during mouse preimplantation development (Kidder, 1993b, Watson et al., 1990a). However, several changes should be made to improve the targeting of the β1 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 β1 subunit present in antisense treated embryos. If future antisense experiments are unable to magnify the effect on cavitation, a second method to target β1 expression may be utilized. The β2 mRNA coding region could be “knocked-in” to the β1 mRNA coding region to replace it during mouse preimplantation development. This strategy could address two questions: The potential role of the β2 subunit in the preimplantation embryo and the effect of removing β1 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 α1 mRNA is distributed throughout mouse morulae and blastocysts.

2. Na,K-ATPase α1 subunits are present in membranes of all preimplantation stages of mouse development while β1 subunits are detectable in membranes beginning at the morulae stage.

3. Na,K-ATPase α3 and β2 isoform mRNAs are present in preimplantation stages while α4 is not detectable. α2 isoform mRNA is only detectable in oocytes.

4. Na,K-ATPase α3, α2 and β2 subunits are not detectable by immunofluorescence in membranes of mouse preimplantation embryos.

5. The sodium pump α3 subunit can be detected in total protein from 650 blastocysts and morulae, unlike the α2 subunit. Results for β2 are inconclusive and future research will require a new antiserum directed against this subunit.

6. As a result of a collaboration, sodium pump α1 and β1 mRNAs and subunit proteins were found to be detectable in horse conceptuses during blastocoel expansion.

7. Initial results from experiments targeting the sodium pump β1 subunit with antisense ODNs suggest that this subunit could be important for initiation of cavitation in the preimplantation mouse embryo.

The detection of α1 mRNA throughout mouse morulae and blastocysts correlates with the detection of the α1 subunit in membranes of morulae and membranes of mural.
and polar trophectoderm and inner cell mass. The β1 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 β1 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 β1 mRNA until the morula stage when the transcript level surges. This result strengthens the hypothesis that the sudden increase in the amount of the β1 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 β2 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 α3 isoform has 2-3 fold lower affinity for sodium ions than the other α isoforms (McDonough *et al*., 1992), but whether this will be significant during mouse blastocyst formation is unknown and should be investigated.

Targeting the β1 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 β1 subunit may also shed some light on the functional potential of the β2 subunit during mouse preimplantation development. A “knock-in” of the β2 subunit coding region into the β1 coding region may be productive, helping to determine more specifically the role of the β1 subunit and determining whether another isoform (β2) 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 α1 and β1 isoforms are the predominant isoforms during this developmental period and that the β1 isoform may be the critical subunit for initiation of cavitation. This study has also indicated that the α3 isoform may have a role during preimplantation development, although not likely as a mediator of cavitation.
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