AN ABSTRACT OF THE THESIS OF

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Formation of the blastocoel is an important event during preimplantation mammalian development and is dependent upon Na+/K+ ATPase. What factor(s) triggers blastocoel formation is not known; however, the literature suggests that the Na+/K+ ATPase gene is expressed shortly before the appearance of the blastocoel. Our hypothesis is that Na+/K+ ATPase and Na+/K+ ATPase mRNA content increase before blastocoel formation and during blastocyst expansion. The objectives of this study were to measure changes in Na+/K+ ATPase and Na+/K+ ATPase mRNA content during preimplantation mouse and rabbit embryo development.

Analysis of total RNA samples from mouse and rabbit embryos was conducted using northern and slot blots hybridized with random primer
labeled cDNA for Na⁺/K⁺ ATPase α subunit from sheep kidney. Western blots, using polyclonal antiserum against guinea pig Na⁺/K⁺ ATPase, were used to detect changes in Na⁺/K⁺ ATPase α and β subunit content in mouse and rabbit embryos. Northern blots exhibited a single mRNA band with molecular weight of 3.65 kb in sheep and mouse kidneys and mouse and rabbit embryos. Although Na⁺/K⁺ ATPase α subunit mRNA content of mouse embryos increased 45-fold between Days 1 and 4 of development, Na⁺/K⁺ ATPase α subunit content remained constant and β subunit content increased 9-fold. In rabbit embryos, Na⁺/K⁺ ATPase α subunit mRNA content increased 35-fold between Days 4 and 6 of development, whereas α subunit content increased 22-fold.

The results suggest that in mouse embryos blastocoel formation is not triggered by an increase in Na⁺/K⁺ ATPase α subunit content. Changes in β subunit content may be important in regulation of Na⁺/K⁺ ATPase activity and blastocoel formation. The Na⁺/K⁺ ATPase α subunit and α subunit mRNA content did not increase in a similar manner. It is possible that α subunit and α subunit mRNA content are consistent with total protein and total mRNA of the early mouse embryo. The similar increase in Na⁺/K⁺ ATPase α subunit mRNA and α subunit content in rabbit embryos suggests that Na⁺/K⁺ ATPase is partly regulated at the mRNA level during blastocyst expansion.
Na⁺/K⁺ ATPase α Subunit and β Subunit mRNA Levels in Preimplantation Mouse and Rabbit Embryos

by

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Na⁺/K⁺ ATPase α Subunit and α Subunit mRNA Levels in Preimplantation Mouse and Rabbit Embryos

Introduction

The fertilized egg is a single cell that can generate all the cells of a complete organism. However, by the blastocyst stage the embryonic cells have lost this totipotency and differentiated into two distinct cell lineages, the inner cell mass and the trophectoderm. These two cell lineages continue through a series of differentiations to form all the tissues of the embryo and the extraembryonic or placental membranes.

During preimplantation development in mammals, the embryo undergoes a series of morphological and biochemical changes. In the mouse, development from the one- to eight- cell stage is characterized by cleavage divisions resulting in similar cells, or blastomeres, one-eighth their original size. By the 16-cell stage the mouse embryo has undergone its first morphological change and is known as a compacted morula. Because intercellular flattening has occurred at this stage, the individual cells are no longer distinguishable. Intercellular flattening maximizes cell contacts, is dependent on cell adhesiveness and coincides with the formation of tight junctions between blastomeres. Cells of the compacted morula are still totipotent, but two distinct cell types have developed; smaller, apolar inner cells and larger, polar outer cells.
The morphological stage of development after the morula is called the blastocyst. The blastocyst is characterized by a fluid filled-pocket, or blastocoel, and the differentiation of outer cells into trophectoderm and inner cells into inner cell mass. The blastocoel is produced by the trophectodermal cells moving fluid into the intercellular spaces. Tight junctions that formed at the morula stage prevent the fluid from escaping. Continued fluid accumulation by the trophectoderm results in expansion of the blastocoel which causes stretching of the zona pellucida, the extra-cellular matrix surrounding the embryo. Stretching of the zona pellucida by the blastocoel facilitates embryonic hatching, or escape from the zona pellucida. After hatching, the embryo is free to implant in the uterus and continue development.

Several reasons merit studying the differentiation of the blastocyst. First, the blastocyst is a useful system to research the biology of differentiation because it marks the first determinative and differentiative event in mammalian development. Chromatin of the cleavage stage mammalian embryo is in its least programmed, and possibly simplest, form. Differentiation is regulated both by cell contact-induced changes that influence trophectodermal and inner cell mass development and by a biological clock that controls the timing of compaction and blastocoel formation. Studies are necessary to elucidate how positional signals in the embryo cause differential programming of the chromatin in the two cell types and how the timing of the expression of proteins required for compaction and blastocoel
formation is controlled. Second, because of the invasive nature of trophectoderm and other similarities between embryonic differentiation and cancer metastasis, studying early development may aid in understanding aspects of cancer. A third reason to investigate blastocyst differentiation at the molecular level is because it represents a critical period in development that may influence early embryonic death. Early embryonic death accounts for a 20 to 30% loss in fertility in livestock species and a higher loss in the human. The timing of embryonic death encompasses the period of blastocyst formation and hatching. Perhaps if the processes occurring during this time were understood, it may eventually be possible to manipulate the embryo or its environment and improve fertility. Also, it may be possible to more accurately predict embryo viability and thereby improve the success of techniques such as in vitro fertilization and embryo transfer. A final reason to study early embryonic differentiation would be to develop new techniques to increase reproductive efficiency through embryo manipulation. For example, if the differentiation of the two cell types could be delayed it might be feasible to produce a large culture of totipotent embryonic cells that could subsequently be used to generate individual embryos. This would allow the production of a limitless number of animals with identical genomes.

One way to investigate the processes of determination and differentiation in the early embryo is to follow the regulation of a stage or tissue specific gene. One such gene may be $\text{Na}^+/\text{K}^+$ ATPase,
which appears to be expressed in both a stage and tissue specific manner. This protein is first detected at the morula stage, immediately before the formation of the blastocoel. After blastocoel formation, $\text{Na}^+\text{K}^+$ ATPase is only detected on the trophectoderm. To gain some knowledge of the molecular regulation of this gene, research was undertaken to measure and compare $\text{Na}^+\text{K}^+$ ATPase $\alpha$ subunit and $\text{Na}^+\text{K}^+$ ATPase $\alpha$ subunit mRNA levels during preimplantation development of the mouse and rabbit embryo.
Literature Review

Several reviews (Denker, 1976; Johnson, 1979; Johnson, 1981) summarize the fundamental aspects of early embryonic development. Differentiation in the context of this review refers to the process of a cell or group of cells acquiring a physiological, morphological or biochemical change compared with other cells of the same genotype. Determination occurs prior to differentiation and refers to the commitment of a cell to differentiate into a certain cell type. Once a particular cell has undergone determination, it is committed to a certain and limited fate. Differentiation follows determination in a series of steps, with each step resulting in a narrower set of choices and a more specialized cell type. This review will be divided into four sections beginning with cellular aspects of embryonic differentiation. The second section will deal with the molecular aspects of embryonic differentiation. The third section will cover aspects of Na\(^{+}/K^{+}\) ATPase in the early embryo. Finally, there will be a review of current knowledge concerning Na\(^{+}/K^{+}\) ATPase gene regulation in other systems.

**Cellular aspects of embryonic differentiation**

Trophectoderm and inner cell mass are considered to be distinct
differentiated tissues because of differences in certain characteristics. When isolated from 3.5 day mouse blastocysts, only trophectodermal cells induced a decidual reaction in the pseudopregnant uterus. If the trophectoderm and inner cell mass are separated in vitro and allowed to develop independently, trophectodermal cells form a hollow fluid-filled ball, resembling a blastocyst; whereas inner mass cells form a solid cell cluster. When inner mass cells are injected into the cavity of another blastocyst they will integrate into the embryo, while trophectodermal cells will not. There are differences between the two cell types in cytoplasmic density, number and structure of organelles, enzyme activity levels and the presence of junctional complexes. The rate of mitosis is higher in the inner mass cells. Finally, trophectoderm and inner cell mass differ in their susceptibility to cytotoxic antisera and viruses.

Two theories were proposed to explain the cause of determination of the two cell types. One theory stated that determination of a cell to become either trophectoderm or inner cell mass is dependent upon its position in the embryo. According to this theory, embryonic cells are identical until the eight-cell stage; after this stage those cells on the inside of the embryo are programmed to become inner mass cells and those on the outside are programmed to become trophectoderm. This determination occurs because of differences in cell contacts, inside cells being completely surrounded by other cells and outside cells having a free surface. Evidence to support
this theory includes labeling of inside or outside cells with various techniques and following their fate.

The second theory suggests that determination depends on a polar distribution of morphogenetic factors within the egg cytoplasm, which when cleaved asymmetrically, results in two distinct cell types. These two cells subsequently give rise to the inner cell mass and the trophectoderm. This theory is supported by experiments in which the destruction of three blastomeres of a four cell embryo resulted in formation of embryos lacking some cell types. Further experimentation on cleavage patterns and fates of individual blastomeres after rearrangement, led to wide acceptance of the first theory as the main determinative event in blastocyst differentiation. Determination of cells to become either inner cell mass or trophectoderm is dependent upon positional signals.

A number of morphological changes and cellular interactions precede the formation of trophectoderm and inner cell mass. The first morphological change observed occurs at the eight-cell stage in the compacted mouse morula. During compaction, blastomeres undergo intercellular flattening, change from a spherical to an epithelial morphology and form tight junctions. Microvilli and various ligand binding sites become located on the free cell surface. There is a redistribution of cellular organelles and enzymes, such that the cells become polarized. Polarization of the blastomeres may be separated from the event of compaction because polarization precedes compaction. It was shown that polarity is induced by cell contacts
because isolated individual blastomeres do not polarize. The ability of a blastomere to become polarized first develops at the eight-cell stage. When two blastomeres of an eight-cell embryo are aggregated, they will induce polarity in each other. Polarity of the eight-cell blastomere is stable through cell division. Depending on the orientation of the cleavage plane in relation to the axis of polarity, the cells of the eight-cell embryo divide to form either two polar cells or one apolar and one polar cell. At the 16-cell stage the blastomeres are arranged into inside, apolar and outside, polar cell types. Outside cells continue to have asymmetric cell contacts and become mature trophectoderm, eventually pumping fluid. The timing of these morphological changes in a variety of species is listed in Table 1 and diagrammed for the mouse in Figure 1. In conclusion, the signals for differentiation of the trophectoderm are cell-contact mediated and begin at the eight-cell stage when the individual blastomeres become polarized.

Numerous recent studies have been conducted to elucidate the mechanisms of cellular interactions that result in the formation of the two distinct cell types in the morula; the inside, apolar and outside, polar cells. Blastomeres of the early eight-cell embryo are not polarized. These cells are spherical, have an even distribution of microvilli and surface receptors, and have a homogeneous dispersion of golgi apparati, endosomes, clathrin-coated vesicles and cytoplasmic actin. By the late eight-cell stage each blastomere is radically reorganized into clear apical and basolateral domains
(Reeve and Ziomek, 1981). There is a redistribution of mitochondria and acidic organelles from the diffuse pattern in uncompacted eight-cell blastomeres to a cortical pattern in compacted eight-cell blastomeres and finally to a perinuclear pattern in trophectoderm (Batten et al., 1987). Ability to polarize is first present at the eight-cell stage and is dependent upon the age of the blastomere in a contact independent manner (Ziomek, 1987). Blastomeres of the eight-cell embryo are capable of polarization but only polarize when induced by asymmetric cell contacts.

The plane of cell division of eight- and sixteen-cell blastomeres is influenced by cell interactions, and leads to the production of two distinct cell types (Johnson and Ziomek, 1981). Continuing action of asymmetric or symmetric cell contacts on the 16-cell blastomeres are necessary to maintain their polar or apolar phenotype. An apolar 16-cell blastomere will remain apolar if it continues to have symmetric cell contacts, that is if it is surrounded on all surfaces by other cells (Johnson and Ziomek, 1983).

Ability to induce polarity in single blastomeres from eight-cell embryos is maintained by the cells of the morula and inner mass cells, but is lost in the trophectoderm (Adler and Ziomek, 1986). A further differentiated cell type is less capable of inducing polarity in single blastomeres from an eight-cell embryo, with decreasing inducing ability proceeding from morula to inner mass to polar trophectodermal to mural trophectodermal cells (Ziomek, 1987). In support of the correlation between polarity inducing ability and
degree of differentiation, it was shown that the teratocarcinoma cell line F-9 induces polarity, but this inducing ability is decreased when the F-9 cells are caused to differentiate by exposure to retinoic acid (Ziomek, 1987).

By the 16- to 32-cell stage, tight junctions are localized on polarized outer blastomeres. These tight junctions separate the free cell surfaces that have relatively nonadhesive microvilli from the more adhesive surfaces found on apposed cell membranes (Ducibella et al., 1975; Magnuson et al., 1977). The junctions form a permeability seal at the outside surface of the embryo and the fluid pumping action of the blastomeres causes the formation of the blastocoel (McLaren and Smith, 1977; Wiley and Eglitis, 1981).

In efforts to understand the mechanism of polarity induction, a number of metabolic inhibitors and manipulations have been performed on early embryos in attempts to block polarization. Polarization is resistant to agents that disrupt gap junction formation (Goodall and Johnson, 1982; McLachlin et al., 1983), cell flattening (Johnson et al., 1979; Pratt et al., 1982) and glycosylation (Surani et al., 1981; Pratt et al., 1982; Sutherland and Calarco-Gillam, 1983). Colcemid and nocodazole, which cause the breakdown of microtubules to monomeric tubulin, and taxol, which causes uncontrolled polymerization of microtubules, inhibit intracellular polarization but not surface polarization (Ducibella, 1982; Maro and Pickering, 1984). Immunofluorescence and immunoelectron microscopy were used to
demonstrate a reorganization of microtubules and pericentriolar material during the eight-cell stage (Houlston, 1987).

Evidence suggests that gap junctions are not involved in polarity induction because junctions do not form between four- and eight-cell blastomeres, yet this interaction induces polarity in the eight-cell blastomere (Goodall and Johnson, 1982). The organization of actin and myosin is involved with cell flattening and polarity (Ducibella and Anderson, 1975; Sobel, 1983; Johnson and Maro, 1984) and polarization of myosin is dependent on cell contacts (Sobel, 1983). Differences in cell surface adhesiveness between inside and outside cells of the morula participate in maintaining the position and shape of the two cell types; outside cells are less adhesive and spread over inside cells (Kimber et al., 1982; Randle, 1982; Surani and Barton, 1984; Sato and Muramatsu, 1987). Transcellular ion currents that may be involved in polarization have been detected in blastomeres of the morula (Nuccitelli and Wiley, 1985). Use of the sterol binding antibiotic, filipin, with freeze fracture and electron microscopy demonstrated an asymmetric distribution of membrane molecules prior to the appearance of overt surface polarity (Pratt, 1985). Formation and stabilization of endocytotic polarity and maintenance of polarized membrane domains are dependent on intact microtubules and microfilaments (Fleming et al., 1986; Fleming and Goodall, 1986).

Because cell adhesiveness and intercellular communication may play an important role in the control of differentiation, much
research has been conducted with cell adhesion molecules, surface glycoproteins, cell flattening and gap junctions in the early embryo. A number of glycoproteins and lipids play a role in embryonic cell interaction. Treatment of embryos with tunicamycin inhibits intercellular flattening and alters the type of glycosylated molecules found on the embryonic surface (Surani, 1979; Magnuson and Epstein, 1981; Surani et al., 1981). Laminin is found extracellularly on apposed cell surfaces of the eight- and sixteen-cell embryo, especially at sites of developing intercellular junctions (Leivo et al., 1980; Wu et al., 1983). Aggregation of lectin-coated agarose beads with single blastomeres from eight-cell embryos causes blastomeres to undergo a process of flattening and engulfment (Kimber and Surani, 1982).

When the cell adhesion molecule, uvomorulin/cadherin is removed from blastomeres by trypsinization and low calcium treatment, intercellular flattening is blocked (Bilozur and Powers, 1983). However, inhibition of uvomorulin/cadherin at the 16- to 32-cell stage with monoclonal antibodies does not completely block cell flattening or tight junction formation (Shirayoshi et al., 1983). Two-cell embryos cultured with uvomorulin antibodies develop into loosely attached cell clusters, which after removal of the antibody, compact and form blastocysts. Also, uvomorulin/cadherin is detected on all cell stages from the one-cell on, and hence, is not stage specific (Vestweber et al., 1987).
Gap junctions first appear in mouse embryos at the eight-cell stage about three hours after the third cleavage (McLachlin and Kidder, 1986). A monoclonal antibody to the cell adhesion system does not prevent cellular communication (Goodall, 1986). Culture of embryos in low levels of calcium or with an embryonal carcinoma antiserum blocks cell flattening and intercellular communication, but does not affect development of cell polarity (Goodall, 1986). Timing of gap junction assembly does not appear to be linked to cell flattening, cytokinesis, cell number, intact microtubules or intact microfilaments (Kidder et al., 1987). Antibodies to gap junctional proteins inhibit cell-to-cell communication, compaction, and subsequently blastocyst formation in the early mouse embryo (Lee et al., 1987).

Embryonic compaction is inhibited with culture in calcium-free medium and cytochalasin B (Ducibella and Anderson, 1975). Tunicamycin, cytochalasin B and colcemid blocks or reverses compaction and decreases microvilli polarization (Sutherland and Calarco-Gillam, 1983). Taxol inhibits compaction (Maro and Pickering, 1984). Culture of early embryos with antibody to cell adhesion molecule 120/80 inhibits compaction (Richa et al., 1985). The use of embryo reactive sera alters the morphology of 16- to 64-cell stage blastomeres (Morgan and Edidin, 1986). These embryos first form monolayers and subsequently form blastocysts of normal morphology 48 h later than control embryos.
Cell interactions and direction of the plane of cleavage lead to two morphologically distinct cell types at the 16-cell stage. Without interference, the outside cells form trophectoderm and the inside cells form inner cell mass. However, these two cell types are not irreversibly differentiated. If the positions of the inside and outside cells are rearranged, each cell type is induced to develop into both inner cell mass and trophectoderm, although the timing of appearance of characteristics differs (Ziomek et al., 1982). By labeling inside and outside cells of the 16-cell embryo, it was shown that 99% of the trophectoderm are derived from outside cells whereas an average of 75% of inner mass cells are derived from inside cells (Fleming, 1987). At the 16-cell stage the blastomeres are still totipotent. It is also believed that the inner mass cells retain totipotency until the midblastocyst stage, but that the late expanded blastocyst is composed of two irreversibly differentiated cell types.

Cells that remain on the outside of the embryo at the 32-cell stage are committed to becoming trophectoderm (Pedersen et al., 1986). During the early blastocyst stage the inner mass cells irreversibly differentiate. Pedersen and Spindle (1980) demonstrated that inner cell mass determination is cell contact dependent and not due to diffusible components. Totipotent cells exposed to blastocoelic fluid form entire normal blastocysts, but those in contact with the blastocyst's inside surface form compact cell clusters similar to inner cell mass. Use of electron microscopy has shown this contact
mechanism involves the formation of trophectodermal processes that surround the inner cell mass (Fleming et al., 1984).

**Molecular aspects of embryonic differentiation**

The molecular mechanisms responsible for early embryonic differentiation are an intense subject of research. There has been a long standing interest in how the embryo maintains a biological clock for morphological differentiation and the control of gene expression. Also, there is interest in elucidating the mechanisms whereby a positional signal is converted into a heritable cellular phenotype.

Early mammalian embryo development is controlled by the maternal genome. In the ovary, mRNAs transcribed from the maternal genome are stored in the developing oocyte. These stored mRNAs serve as templates for embryonic protein production to the two-cell stage. The use of RNA polymerase II inhibitors demonstrated that the increased synthesis of polypeptides of molecular weight 35 kD at the early two-cell stage is dependent upon post-transcriptional regulation of stored mRNAs (Braude et al., 1979). When mRNAs extracted from unfertilized mouse oocytes are used in an mRNA-dependent, cell-free translation system, high levels of these 35 kD proteins are synthesized (Cascio and Wassarman, 1982). These results indicate that the unfertilized oocyte contains mRNAs coding for the 35 kD proteins but translation of these mRNAs is only stimulated after fertilization. One-cell mouse embryos can cytoplasmically add
poly (A) sequences to pre-existing poly (A) containing RNA tracts (Young and Sweeney, 1979; Clegg and Piko, 1982; Clegg and Piko, 1983). The mRNA for tissue-type plasminogen activator is cytoplasmically polyadenylated during meiotic maturation of the oocyte and coincides with its translation into protein (Huart et al., 1987). Furthermore, injection of antisense RNA specific for the 3' noncoding region of the tissue plasminogen activator mRNA into mouse oocytes results in cleavage of the 3' sequences, loss of regulation and translation of t-PA mRNA, and stabilization of the 5' segment (Strickland et al., 1988). This indicates the 3' sequence of stored maternal mRNAs may be important in translational regulation and there exists in mouse oocytes a double stranded ribonuclease activity. Cytoplasmic adenylation of pre-existing mRNAs is proposed to be one mechanism to selectively translate subsets of mRNAs. This could explain the appearance of stage specific proteins in the embryo in the absence of gene transcription.

Transcription of the embryonic genome is first detectable in the mouse at the late two-cell stage and coincides with the degradation of most maternal mRNAs stored in the egg (Flach et al., 1982). Tritiated uridine incorporation indicated the rate of RNA synthesis per cell per two hours in the mouse increases from 2.5 pg in the two- to four-cell, to 5 pg in the eight-cell and 10 pg in the morula-early blastocyst (Clegg and Piko, 1977). Using an RNA polymerase II inhibitor it was shown that during the two-cell stage, embryonic gene transcription occurs at 18 - 21h and 26 - 29h post-insemination. The
first phase of transcription is correlated with the synthesis of a complex of polypeptides of molecular weight 67 kD, whereas the second phase is correlated with a major change in synthetic profile involving many polypeptides (Flach et al., 1982). Use of an in vitro translation system confirmed the presence of the two phases of transcription in the two-cell mouse embryo (Bolton et al., 1984). This study also demonstrated that DNA replication occurs between the two transcriptional phases and that this DNA replication is not necessary for the subsequent transcriptional events. Use of tritiated uridine incorporation and detection of argentophilic nucleolus organizer regions suggest embryonic transcription of mRNA and rRNA is first occurring at the eight-cell stage in the bovine embryo (Camous et al., 1986; King et al., 1988). Sheep embryos also appear to initiate transcription at the eight-cell stage (Crosby et al., 1988). In the pig, embryonic RNA synthesis begins at the four-cell stage (Freitag et al., 1988). Transcription of the embryonic genome in the human embryo is detected at the four-cell stage and rRNA synthesis is detected at the six- to eight-cell stage (Tesarik et al., 1986).

Another method for detecting the start of embryonic transcription involves the use of isozymes. If maternal and paternal alleles for an enzyme differ, then the types of isozymes present in the embryo indicate whether the enzyme is a maternal or embryonic gene product. This technique was used in the mouse to demonstrate the expression of the embryonic gene for glucose phosphate isomerase
by the morula stage (Duboule and Burki, 1985). Translation of maternally derived message for glucose phosphate isomerase is not detected in mouse morula (Duboule and Burki, 1985). Using pronuclear transplantation Gilbert and Solter (1985) detected production of glucose phosphate isomerase encoded by the embryonic genome by Day 4 of development, coincident with the loss of stored mRNA for this gene. Subsequently, West et al. (1986) demonstrated that oocyte-coded glucose phosphate isomerase is stable until 2.5 days and is depleted by 6 days post-insemination, whereas embryo-coded enzyme first appears at 2.5 days. These results are consistent with the idea that there is a mechanism for the removal of oocyte-coded gene products at about Day 2.5 post-insemination and subsequent protein synthesis is dependent on embryonic transcription.

To further our understanding of the control of protein synthesis in the early embryo, the amount of total RNA, total poly (A)$^+$ RNA and ribosomes in the mouse embryo from the one-cell to the blastocyst stage were determined (Piko and Clegg, 1982). Number of ribosomes accounts for 60 to 70% of the total RNA content at all stages studied. Total RNA, ribosomal RNA and poly (A)$^+$ RNA levels of the mouse embryo are depicted in Table 2. Table 3 compares total RNA content in eggs of mice, rabbits and frogs. Clegg and Piko (1983) determined the average length of mouse embryo poly (A)$^+$ tracts and subsequently estimated the number of poly (A)$^+$ RNA to be $1.7 \times 10^7$ per ovulated egg, $2.4 \times 10^7$ per zygote, $0.7 \times 10^7$ per late two-cell, $1.3 \times 10^7$ per eight-cell, and $3.4 \times 10^7$ per blastocyst. Kidder and
Pedersen (1982) investigated turnover of total embryonic mRNA in preimplantation mouse embryos. They found the mRNA decay curve to be monophasic in the morula with an average half-life of 9.5 h and biphasic in the blastocyst with a greater overall average half-life. Distribution of cytoplasmic poly (A)$^+$ RNA differs between morula and blastocyst stage embryos (Kidder and Conlon, 1985). In the morula, poly (A)$^+$ RNA is evenly distributed between ribosomal and subribosomal fractions. However, poly (A)$^+$ RNA is almost entirely found in the active translating ribosomal fraction at the blastocyst stage.

It is of interest to compare the levels and distribution of the different types of RNA to the levels of protein found in the early mouse embryo. Several investigators have determined protein content through blastocyst formation in the mouse and their results are summarized in Table 4 (Brinster, 1967; Schiffner and Spielmann, 1976; Sellens et al., 1981). During development from the two-cell to the blastocyst stage, protein content decreases whereas total RNA and poly (A)$^+$ RNA content increases five- to six-fold. The rates of protein synthesis and degradation increase dramatically between the two-cell and blastocyst stage (Brinster et al., 1976). Average half-life for newly synthesized proteins decreases from 17.2 h in the fertilized egg to 12.2 h in the two-cell, 13.8 h in the eight-cell and 13.3 h in the blastocyst (Merz et al., 1981). Although the rate of synthesis of total protein increases 1.5-fold during development from fertilization to the compacted eight-cell stage in the mouse,
the rate of synthesis of ribosomal proteins increases 11.3-fold during the same time period (LaMarca and Wassarman, 1979).

Levels of RNA in the rabbit embryo have been reviewed by Schultz and Tucker (1979) and are listed in Table 5. Poly (A)$^+$ RNA synthesis is detected during cleavage of the rabbit embryo and is associated with polysomes by the 16-cell stage. Ribosomal RNA synthesis begins by Day 3 in the rabbit embryo. The rate of poly (A)$^+$ RNA synthesis per embryo increases about 100-fold between Days 2 and 6. However, on a cellular basis this represents a 50-fold decline in mRNA levels whereas protein synthesis per cell during the same time period remains constant. This increase in protein synthesis without a similar increase in poly (A)$^+$ RNA synthesis may be accounted for by an increase in the number of ribosomes or other factors required for translation. The major qualitative changes in the pattern of protein synthesis occurs between the one-cell and morula stage in both the rabbit and the mouse embryo (Schultz and Tucker, 1977).

Metabolic inhibitors have been used to begin to understand the regulation of the morphological changes observed at the morula and blastocyst stages. Use of the RNA polymerase II inhibitor α-amanitin on mouse morulae blocks blastocyst formation and the associated change in polypeptide profile (Braude, 1979a). This work suggests that newly transcribed stage specific proteins are essential for morphological and biochemical changes in the mouse embryo. When α-amanitin is added to embryo cultures before 77 h post-hCG, blastocyst formation, the associated increase in amino acid incorporation and
the synthesis of new polypeptides is blocked (Braude, 1979b). However, embryos that had completed their fifth division before culture with α-amanitin formed blastocysts and exhibited normal quantitative and qualitative patterns of protein synthesis (Braude, 1979b). These observations indicate that a critical transcriptional event associated with blastocyst formation is occurring around 80 h post-hCG. Culturing rabbit one- to two-cell embryos with α-amanitin results in continued cleavage and development for two to three cycles in the absence of RNA synthesis (Schultz and Tucker, 1977). Culturing early mouse embryos with tunicamycin blocks blastocyst formation and the production of blastocyst-characteristic proteins although cell divisions proceed normally (Iwakura and Nozaki, 1985). These results indicate a role for glycoproteins or large polysaccharides in morphological development and expression of stage specific proteins in the early mouse embryo.

The timing of transcription and protein synthesis required for the morphological changes of compaction, cavitation and hatching have been further investigated with the use of inhibitors (Kidder and McLachlin, 1985). Transcription necessary for compaction and hatching occur well in advance. However, the transcriptional and translational events required for blastocoel formation occur within a few hours of the start of this process. Continuous exposure to protein synthesis inhibitors from as early as the late two-cell or early four-cell stage does not prevent cell flattening or surface polarization of mouse embryos but does block cell coupling and
cytoplasmic polarization (Levy et al., 1986).

The functional mechanisms of the biological clock that controls the timing of stage specific proteins and morphological changes has been an intriguing area of research. The timing of blastocoel formation is not controlled by chronological age, cell number or number of cell divisions (Smith and McLaren, 1977). In a review article, Satoh (1982) suggests that the biological clock for cellular differentiation is based on the number of DNA replications that have occurred. According to this model, demethylation of specific genes occurs at each DNA replication and this subsequently leads to the expression of these proteins. The methylation pattern would be heritable and would thus lead to the production of differentiated cell types.

Dean and Rossant (1984) used aphidicolin, which delays DNA replication, and observed that blastocoel formation in the mouse embryo was not delayed. However, their aphidicolin treatment may have missed the DNA replication cycle critical for the programming of the biological clock for blastocoel formation. Treatment of fertilized mouse eggs with cytochalasin B prevents cleavage but not karyokinesis. Petzoldt (1984) found that this treatment results in normal embryonic progression of stage specific protein synthesis. However, treatment of fertilized mouse eggs with aphidicolin maintains protein synthesis patterns at the fertilized egg level (Petzoldt, 1984).
Inhibition of polyamine biosynthesis with methylglyoxal-
bis(guanylylhydrazone; MGBG), in cleaving mouse embryos reversibly
blocks DNA replication (Alexandre and Geuskens, 1984). Transient
treatment of mouse embryos with MGBG results in a delay of blastocoel
formation and a similar delay in stage specific RNA synthesis
(Alexandre and Geuskens, 1984). However, these delayed nascent
blastocysts have a lower cell number, indicating a desynchronization
between number of chromosome replications and morphological
differentiation (Alexandre and Geuskens, 1984). When aphidicolin
treatment is initiated at the late four-cell stage, blastocyst
formation is blocked (Spindle et al., 1985). This research suggests
the first half of the fourth DNA replication cycle is critical for
subsequent blastocyst formation. Aphidicolin treatment of two-cell
embryos results in blockage of cell flattening and polarization that
normally occurs at the morula stage (Smith and Johnson, 1985). The
MGBG-induced inhibition of blastocyst formation is reversed by the
addition of spermine and/or spermidine to the culture medium
(Zwierzchowski et al., 1986).

It appears that either the number of rounds of DNA replication
or the nuclear/cytoplasmic ratio may be serving as the biological
clock in mouse embryo development. To further understand which of
these two factors is the critical mechanism, nuclear transplantation
experiments have been conducted. When enucleated zygotes receive
pronuclei, 95% develop to the morula-blastocyst stages whereas only
19% develop if the donor nuclei is from a two-cell stage embryo
Furthermore, the use of an 8- to 16-cell stage nuclei results in only 3% of embryos developing to the 8-cell, morula and blastocyst stages (McGrath and Solter, 1986). Normalization of the nuclear/cytoplasmic ratio of haploid embryos improves development to the morula-blastocyst stages (McGrath and Solter, 1986). Bisection of pronuclear zygotes often results in both pronuclei in an egg of one-half the original size (Petzoldt and Muggleton-Harris, 1987). The resulting egg has a doubled nucleo-cytoplasmic ratio. Protein synthesis patterns and stage-specific surface antigen production of bisected embryos with doubled nucleo-cytoplasmic ratios, bisected embryos with normal nucleo-cytoplasmic ratios and control embryos are equivalent (Petzoldt and Muggleton-Harris, 1987). These results suggest that the number of DNA replications and not the nucleo-cytoplasmic ratio may be serving as the biological clock mechanism.

As mentioned earlier, DNA methylation may be a mechanism for reinforcing or stabilizing the activity or inactivity of genes in the chromatin. The degree of overall methylation and methylation of specific sequences have been determined in the mouse oocyte, sperm and early embryo (Monk et al., 1987; Sanford et al., 1987). The egg genome is undermethylated, whereas the sperm genome is relatively more methylated. Eight-cell stage embryonic DNA is more methylated than oocyte DNA but blastocyst DNA has a very low level of methylation. Embryonic DNA from 7.5 day conceptus is highly methylated whereas extraembryonic DNA is less methylated. Level of
DNA methylation in cleavage stage embryos is intermediate when compared with the level in the sperm or the egg. De novo methylation does not appear to occur in the cleavage stage embryo (Sanford et al., 1987). The differences in methylation level of the male and female gamete appear to be maintained in the early embryo. It has also been demonstrated that the methylation patterns of exogenous DNA sequences in transgenic mice can be varied by switching their gamete of origin (Sapienza et al., 1987). All of these data lend support to the hypothesis that DNA methylation is functioning as a means of imprinting the maternal and paternal genome, and results in differential regulation of these genomes in the early embryo. Obviously the biological clock and the mechanism for programming the chromatin of the early embryo are not completely understood but interesting aspects are beginning to be revealed.

Differences in DNA methylation patterns between the sperm and the egg are indicative of the differential programming of the maternal and paternal chromosomes in the early embryo. The idea of differential programming of the parental genomes has long been supported by data that demonstrate that only the maternal X chromosome is active in extraembryonic membranes of mouse embryos and several mutations have different actions when inherited on the maternal or paternal chromosomes (reviewed by Magnuson and Epstein, 1987). Other evidence has been derived from data on the development of androgenetic and gynogenetic embryos. By microsurgically removing the male or female pronuclei from a fertilized egg and replacing it
with a pronuclei of the opposite sex, diploid biparental embryos with two male pronuclei (androgenetic) or two female pronuclei (gynogenetic) can be made. These androgenetic and gynogenetic embryos do not complete normal embryogenesis when compared with control manipulated embryos (McGrath and Solter, 1984). Additionally there are differences between the development of gynogenetic versus androgenetic embryos. Gynogenetic embryos achieve dramatically more advanced embryo development (as far as the 25-somite stage), however, the extraembryonic tissues are deficient (Barton et al., 1984). In contrast, the androgenetic embryos develop normal extraembryonic tissues but retarded embryos (5 somites)(Barton et al., 1984). These results suggest that the paternally programmed chromatin is critical for normal development of extraembryonic tissues whereas the maternal chromatin is critical for embryogenesis. Subsequently, reconstituted blastocysts have been microsurgically constructed with inner cell mass from a gynogenetic embryo and trophectoderm from a normal embryo, and vice versa (Barton et al., 1985). When the trophectoderm is of normal genetic makeup the gynogenetic embryo develops as far as the 40 somite stage. However, these embryos do not develop to term and have deficient yolk sacs. Chimeric embryos derived from aggregation of gynogenetic embryonic cells with normal embryonic cells develop to term and become fertile adult mice in which the gynogenetic cells contribute to all tissues, including germ cells (Stevens, 1978). A similar experiment was conducted by aggregating four-cell stage gynogenetic and androgenetic embryos to form chimeras
(Surani et al., 1987). These embryos do not develop to term. Embryo development in these chimeras is similar to development of gynogenetic embryos and the trophoblast is similar to androgenetic embryos. Furthermore the trophoblast is composed of the androgenetic cells, whereas the embryo is composed of the gynogenetic cells. The yolk sac contains both cell types. This distribution of cells is not due to differences in the proportion of late and early dividing cells in androgenetic versus gynogenetic embryos. The distribution of cells may be related to differences in cell surface properties, which control cellular interactions, a function of the chromosomal programming. Clearly differential parental programming of the chromatin occurs during gametogenesis. The mechanisms involved and its role in embryo development and placental immunology are not understood.

Because metabolic inhibitors have side effects that may lead to faulty data and conclusions, the control of gene expression in the early embryo has recently been investigated with the use of specific gene probes. Brulet and Jacob (1982) synthesized a cDNA to trophoblastoma intermediate filament protein. This protein is believed to be present on trophectoderm cells but not inner mass cells. This cDNA detected specific mRNA in trophoblastoma but not embryonal carcinoma cells (Brulet and Jacob, 1982). Brulet and coworkers (1983) also detected a RNA coding for a transposon-like or retrovirus-like element (ETn) present in undifferentiated embryonal carcinoma cells but not in a number of differentiated tissues. Using
in situ hybridization ETn transcripts have been detected in the pluripotent cell lineage of the 3.5 to 7.5 day mouse embryo (Brulet et al., 1985). However, some extra-embryonic ectoderm derivatives also show a high level of ETn transcription and older embryos have a uniform low level of ETn transcripts (Brulet et al., 1985). Thus ETn is not a tissue specific marker exclusive for the inner cell mass and its physiological role, if any, is unknown. The structure of the long terminal repeat (LTR) bordering the ETn sequences has been investigated and is similar to retroviral LTRs (Kaghad et al., 1985). The ETn gene has 200–400 scattered copies per mouse haploid genome (Brulet et al., 1985). Nucleotide sequencing of two ETn elements did not result in detection of any long open reading frame or significant homology to retroviral proteins (Sonigo et al., 1987). Two genes coding for the intermediate filament protein (Endo A or cytokeratin A) have been detected in the mouse genome and their structure analyzed (Vasseur et al., 1985). Endo A is encoded by a 7.5 kb gene, α1, with seven introns and a 1.65 kb pseudogene, α2, without introns. The 5' flanking sequence and the third intron of α1 contain potential cis-acting regulatory sequences. Nuclease S1 mapping has been used to measure the amount of Endo A transcripts in RNA extracted from two-cell to 7.5 day mouse embryos (Duprey et al., 1985). Endo A mRNA is detectable in eight-cell embryos. The amount of the transcript increases at the blastocyst stage, suggesting this gene is at least partially regulated at the mRNA level during blastocyst formation. In situ hybridization reveals Endo A mRNA is detectable in the
trophectoderm but not the inner cell mass (Duprey et al., 1985). The increase in endo A mRNA from the eight-cell to the blastocyst stage is about 10-fold (Brulet et al., 1985). The activity of the endo A promoter was analyzed by transfection experiments using the chloramphenicol acetylase (CAT) system (Brulet et al., 1985). The Endo A promoter is efficient in all cell lines tested, even lines that normally do not express the Endo A gene. This may be an indication that these transient expression assays do not reflect the normal in vivo gene expression, perhaps due to the suppression of other cis-acting factors. Further research demonstrated no tissue-specific enhancers in the vicinity of the Endo A promoter (Brulet et al., 1985). Possibly ETn and Endo A may be used as markers to study how cellular interactions and biological clock mechanisms are programming the chromatin of the early embryo. However, these genes are not expressed in a completely tissue or stage specific manner and the encoded proteins are of unknown function.

Heat shock gene expression has also been investigated during early development of the mouse embryo (Wittig et al., 1983). Heat shock genes are not expressed in two- to eight-cell embryos but are expressed in morula/blastocyst stage embryos. Similarly heat shock genes are not expressed in undifferentiated teratocarcinoma stem cells, but are expressed in teratocarcinoma-derived differentiated tissue (Wittig et al., 1983). However, the heat shock response is not detected in teratocarcinoma cells differentiated in vitro (Wittig et al., 1983).
Heat and other stress increased the synthesis of a mRNA for a 70 kD heat-shock protein in the Day 6 rabbit blastocyst (Heikkila and Schultz, 1984). The stressful treatments did not affect the level of actin mRNA in these embryos but decreased overall protein synthesis. The physiological roles of heat shock proteins are not known but development from early cleavage to implantation is the period most susceptible to thermal stress (Heikkila and Schultz, 1984). Also, there is a correlation between thermotolerance and heat shock protein synthesis during development (Heikkila et al., 1985). Heat shock gene expression is also increased in the Day 6 rabbit blastocyst due to collapsing the blastocoel by puncturing it with a needle (Heikkila and Schultz, 1984). The increase in heat shock protein synthesis is correlated to a similar increase in the level of the mRNA, indicating heat shock protein synthesis is at least partially regulated at the mRNA level in the rabbit embryo (Heikkila and Schultz, 1984). Synthesis of metallothionein (MT) mRNAs and protein is induced by exposure of Day 4 and 6 rabbit embryos to zinc ions (Andrews et al., 1987). In contrast, cadmium induced a dramatic increase in MT mRNA but not MT protein. Cadmium is toxic to Day 6 rabbit blastocysts and induces heat shock protein gene expression (Andrews et al., 1987). Heat shock protein synthesis is not inducible in the eight-cell mouse embryo but is inducible by the blastocyst stage (Heikkila et al., 1985). The unfertilized mouse egg does not synthesize any heat shock proteins (Hahnel et al., 1986). At the two- to eight-cell stage the mouse embryo constitutively expresses two heat shock proteins (Mr=74
kD and Mr=70 kD) which are not stress-induced (Hahnel et al., 1986). By the blastocyst stage the mouse embryo begins to synthesize a stress-inducible protein (Mr=68 kD) (Hahnel et al., 1986). The timing and level of synthesis of these proteins are correlated to the levels of the mRNAs for the proteins (Hahnel et al., 1986). Similar to undifferentiated embryos, two mouse embryonal carcinoma cell lines do not possess an inducible 68 kD heat shock protein (Morange et al., 1984). Treatment of these cells with retinoic acid causes differentiation and subsequently inducible synthesis of the 68 kD heat shock protein (Morange et al., 1984).

Two types of intracisternal A-type particles (IAPs) are present in the mouse embryo (Piko et al., 1984). The amount of IAP RNA in the mouse blastocyst is 100-fold higher than in the mouse zygote (Piko et al., 1984). The role of these retrovirus like particles in early mouse embryo development is unknown but may be involved in cell surface antigen expression (Piko et al., 1984).

In efforts to understand the transition from dependence on maternal to embryonic transcripts in the two-cell stage mouse embryo, a cDNA library was made of late two-cell embryos (Taylor and Piko, 1987). About one-half of the transcripts present at the two-cell stage were not present in the egg. This indicates that a major qualitative shift in mRNA population occurs at the two-cell stage in the mouse embryo (Taylor and Piko, 1987).

Changes in the level of histone and actin mRNA in the preimplantation mouse embryo have been investigated using Northern
blots (Giebelhaus et al., 1983). The maternal store of histone and actin mRNA decreases about 10-fold by the mid-two-cell stage. By the early blastocyst stage the histone mRNA content is about equal to that in the egg. The actin mRNA content of the early blastocyst is about double the amount found in the egg (Giebelhaus et al., 1983). The amounts of particular histone mRNAs change between development from the egg to the blastocyst stage (Graves et al., 1985). After the four-cell stage the embryo has a constant amount of histone mRNA per cell (about 20,000 molecules) (Graves et al., 1985). Approximately 3% of the mRNA in mouse eggs is histone mRNA whereas 2% of the protein is histone protein (Graves et al., 1985). Actin mRNA content of the late blastocyst is about six times the level found in the egg (Giebelhaus et al., 1985). The level of actin mRNA per embryo is proportional to the cell number from the eight-cell to the blastocyst stage (Table 6) (Giebelhaus et al., 1985). Actin protein synthesis represents about 0.25% of total protein synthesis in the two-cell stage mouse embryo, 2% in the eight cell stage and 5.7% in the early blastocyst (Giebelhaus et al., 1985).

Amounts of mitochondrial DNA (mtDNA), 12 and 16S mitochondrial rRNAs and the mRNAs for cytochrome c oxidase subunits I and II were determined by dot blots in the one-cell to blastocyst stage mouse embryo (Piko and Taylor, 1987). The mtDNA level was similar throughout this time at 2.13 pg or 119,000 mtDNA molecules per embryo. The amount of rRNAs and mRNAs increased 25- to 50-fold between the two-cell stage and the early blastocyst. Mitochondrial mRNA molecules
constitute 23% of the total embryo poly(A)$^+$ RNA at the early blastocyst stage (Piko and Taylor, 1987). Similarly, Southern blot analysis has shown that the amount of mtDNA does not change during development of the mouse embryo to the egg cylinder stage (Ebert et al., 1988).

The potentially powerful technique of antisense RNA injection has been successfully used in the mouse embryo (Bevilacqua et al., 1988). Injection of B-glucuronidase antisense RNA into each blastomere of the four-cell mouse embryo yielded a 75% inhibition of enzyme activity at the blastocyst stage. Injection of the sense or unrelated RNA did not alter enzyme activity. No RNA-duplex "melting" activity is detectable in the early mouse embryo.

The role of the nuclear lamina in gene regulation is not clear but there is evidence which suggests that its organization is dramatically different in the early mammalian embryo (Steart and Burke, 1987). Early mouse embryos and undifferentiated embryonal carcinoma cells only express lamin B. In adult mammalian somatic cells the nuclear lamina is composed of lamin A, B and C. Lamin A and C are detected in fertilized eggs, disappear during early cleavage divisions and reappear by Day 8 in post-implantation embryos. The structural organization of the nucleus may be undergoing dramatic changes during early development.
Aspects of Na⁺/K⁺ ATPase during early embryo development

Expression of several genes in the early embryo have now begun to be investigated and certainly much more work is in progress. However, none of these genes have both a known physiological function and tissue or stage specific expression. One gene expressed in the early embryo that may meet these criteria is that for Na⁺/K⁺ ATPase. Several excellent reviews exist on the general function and structure of Na⁺/K⁺ ATPase (Sweeney et al., 1981; Jorgensen, 1982; Glyn, 1985). This enzyme is found in most animal cell membranes and has several functions including: maintenance of K⁺ levels for intracellular enzyme function, osmotic pressures and ionic gradients for excitable cells, transport of salt and water, maintenance of ionic gradients used to transport other substances, and generation of heat. Na⁺/K⁺ ATPase is found in the plasma membrane and is composed of an α and β subunit and possibly a small γ subunit. The β subunit is a glycoprotein of approximately 40 kD whose function is unknown. The α subunit, a protein of approximately 110 kD, is the catalytic subunit. The active enzyme appears to be composed of 2α and 2β subunits. Na⁺K⁺ ATPase pumps three sodium ions out of the cell for every two potassium ions it pumps into the cell, thus producing an ionic gradient. The transporting epithelium of the kidney is the most concentrated source of Na⁺/K⁺ ATPase and the enzyme functions to regulate fluid excretion. Other transporting epithelia also contain high levels of Na⁺/K⁺ ATPase. In these cells the enzyme is only
detectable on the basolateral cell surfaces. Sorting and designation of Na⁺/K⁺ ATPase as a basolateral plasma membrane protein appears to occur prior to insertion into the membrane (Caplan et al., 1986). The next highest level of the enzyme is found in excitable tissues such as the brain and muscle, where Na⁺/K⁺ ATPase serves as an electrogenic pump to maintain action potentials.

Because embryo trophectoderm is the first transporting epithelium to develop in the mammal, it is reasonable to expect to find high Na⁺/K⁺ ATPase levels in the preimplantation embryo. Ultrastructural cytochemistry has been used to detect Na⁺/K⁺ ATPase levels in early mouse embryos (Vorbrodt et al., 1977). This procedure visualizes enzyme activity in fixed embryos by converting strontium phosphate precipitates into lead phosphate. Na⁺/K⁺ ATPase is first detected at the morula stage, immediately before blastocoel formation. Similar to other transporting epithelia, the enzyme is localized to the basolateral or apposed cell surfaces of the morula and is not found on the free or apical cell surfaces. Ouabain inhibits fluid accumulation or blastocoel expansion in mouse embryos (DiZio and Tasca, 1977). Ouabain also inhibits sodium-dependent alanine transport in blastocysts, indicative of an epithelial type of mechanism for amino acid transport (DiZio and Tasca, 1977). Ouabain binding has been used to determine the level of Na⁺/K⁺ ATPase in the Day 4 to 7 rabbit blastocyst (Benos, 1981). Total number of ouabain binding sites per trophoblast cell increases about 7.5-fold between Day 4 and 5 and subsequently remains constant. Ouabain inhibited
radioactive sodium influx increased about 50% between Day 5 and 6. Levels of $\text{Na}^+/\text{K}^+$ ATPase in the rabbit blastocyst are similar to the levels found in the kidney. Level of oxygen consumption before and after the addition of ouabain has been used in the rabbit blastocyst as an indication of the metabolic activity of $\text{Na}^+/\text{K}^+$ ATPase (Benos and Balaban, 1980). Ouabain inhibits oxygen consumption by 43% in Day 4 blastocysts, 62% in Day 5 blastocysts, 70% in Day 6 blastocysts and 16% in Day 7 blastocysts. This level of oxygen consumption by $\text{Na}^+/\text{K}^+$ ATPase is as high as that found in the kidney. More recently transepithelial $\text{Na}^+$ transport in the rabbit blastocyst has been further investigated (Benos and Balaban, 1983). This experiment demonstrated that the ouabain-sensitive transepithelial $\text{Na}^+$ transport accounts for only 6% of the total ATP output of the rabbit blastocyst. This discrepancy may be explained by the fact that ouabain inhibits other processes than active $\text{Na}^+$ transport. Certainly ouabain can alter intracellular concentrations of $\text{Na}^+$, $\text{K}^+$ and $\text{Ca}^{++}$ secondary to $\text{Na}^+/\text{K}^+$ ATPase inhibition. These changes in intracellular ion concentrations could subsequently inhibit other enzyme systems that utilize ATP, such as protein synthesis. The use of ouabain and varying levels of potassium demonstrated that $\text{Na}^+/\text{K}^+$ ATPase is involved in localization of organelles to apposed cell borders, the production of blastocoel fluid and cavitation in the mouse embryo (Wiley, 1984). Further determination of ouabain binding sites in the rabbit blastocyst demonstrated a 50-fold increase between Day 4 and 5, no change between Day 5 and 6, and a 5-fold
increase between Day 6 and 7 (Benos et al., 1985). The surface area of the embryo does not increase in the same biphasic pattern (Table 7). Between Day 6 and 7 the rabbit blastocyst acquires an amiloride-sensitive component to transepithelial Na\(^+\) influx, indicative of Na\(^+\) channel regulation on the apical cell surface. Radioactive methionine incorporation into proteins of the blastocyst was used to determine the level of synthesis of Na\(^+\)/K\(^+\) ATPase in the rabbit blastocyst (Benos et al., 1985). Purified Na\(^+\)/K\(^+\) ATPase was used as one of the protein markers. Coelectrophoresis and immunoblotting techniques demonstrated a 90-fold increase in the synthetic rate of Na\(^+\)/K\(^+\) ATPase between Day 4 and 6 of development. This increase coincides with maximal blastocyst expansion. During the same time period total protein synthesis increases 20-fold in the rabbit blastocyst.

Immunofluorescence has been used to localize Na\(^+\)/K\(^+\) ATPase in the preimplantation mouse embryo (Watson and Kidder, 1986; Watson and Kidder, 1988). The enzyme is first detected at the late morula stage and is localized to the cytoplasm. Once a blastocoel is present, Na\(^+\)/K\(^+\) ATPase is localized to a ring encircling the blastocoel. On the blastocyst cells exhibiting Na\(^+\)/K\(^+\) ATPase, the enzyme is localized to the basolateral surface. Blocking cleavage at the eight-cell stage does not affect the timing of appearance of Na\(^+\)/K\(^+\) ATPase. At the blastocyst stage the enzyme is localized to the inner surface of the mural trophectoderm and the surface of the blastocoel adjacent to the inner cell mass. Because it has been demonstrated
that extensions of the mural trophectoderm cover the inner cell mass at this stage (Fleming et al., 1984), it appears that Na\(^+/K^+\) ATPase is a tissue specific marker for mural trophectoderm. Furthermore it appears that tight junctions are required to maintain the basolateral distribution of Na\(^+/K^+\) ATPase (Watson and Kidder, 1986).

Ouabain binding and sodium influx levels have been measured in the pig blastocyst (Overstrom, 1987). Sodium is actively transported in the Day 7 to 10 pig blastocyst. Influx rates show little change between Day 7 and 9 while area (0.68 to 1.79 cm\(^2\)) and volume (0.005 to 0.23 ml) increase slightly. On Day 10 dramatic increases in blastocyst area (13-fold) and volume (46-fold) occur concomitant with a rise in Na\(^+\) influx.

**Regulation of Na\(^+/K^+\) ATPase gene expression in other systems**

The first Na\(^+/K^+\) ATPase catalytic subunit cDNA to be isolated and characterized was from the sheep kidney (Shull et al., 1985). Sequencing of this complementary DNA demonstrated an abundance of CpG dinucleotides in the 5'-untranslated region, a 3,063-base open reading frame, the translation initiation site, the phosphorylation site and the ATP-binding site. The protein is 1,016 amino acids in length, has eight transmembrane domains and is located mainly on the cytoplasmic side of the membrane. Subsequently the cDNA of the rat brain \(\alpha\)-subunit was cloned (Schneider et al., 1985). This cDNA was used to determine the relative abundance of Na\(^+/K^+\) ATPase mRNA in
different tissues. The abundance of the 27S mRNA is highest in kidney, lowest in liver and intermediate in brain.

It was further shown that rat kidney contained a single class of α subunit mRNA whereas rat brain contained three different α subunit mRNAs (Shull et al., 1986). The most abundant brain form (α+) is 5.1 kilobases (kb). The second most abundant brain form (α) is 3.65 kb and identical to the kidney form. The least abundant brain form (αIII) is 3.55 kb. The sequence of the rat kidney α form, which is ouabain-resistant, was compared to the sequence of the sheep kidney α form which is ouabain-sensitive. Several amino acid sequence variations may explain the tissue and species differences in ouabain sensitivity. Subsequently, other research characterizing the α subunit mRNAs in rat tissues produced different results (Herrera et al., 1987). Again cDNAs for three α subunit isoforms were cloned. The α1 isoform, 4.5 kb, is expressed in all tissues examined. The α2 isoform, 4.5 kb, is expressed in the brain. The α3 cDNA detected two mRNAs, 4.5 and 6.0 kb, present in a variety of tissues.

Tissue distribution of the mRNAs for the α subunit isoforms and the β subunit have been investigated (Young and Lingrel, 1987; Herrera et al., 1987; Emanuel et al., 1987; Chehab et al., 1987; Schneider et al., 1988). The α1 (or α+) isoform and the β subunit are found in all tissues tested, including human placenta (Chehab et al., 1987). The α3 isoform is found predominantly in the brain. Again there is disagreement on the distribution of the α2 (α+) isoform but it is abundant in brain.
Screening a human genomic library with the sheep $\alpha$ and $\alpha^+$ isoforms of Na$^+$/K$^+$ ATPase revealed four genes (Shull and Lingrel, 1987). Two of these genes encode for the $\alpha$ and $\alpha^+$ isoform while the other two, which have sequence homology to Na$^+$/K$^+$ ATPase, do not code for any known isoforms. Other researchers detected five Na$^+$/K$^+$ ATPase genes in the human genome (Sverdlov et al., 1987). In the mouse the $\alpha_1$, $\alpha_2$ and $\alpha_3$ isoforms are located on three different chromosomes, whereas the $\beta$ subunit is found on chromosome 1, the same as $\alpha_3$ (Kent et al., 1987). In the human the $\alpha_1$ isoform is found on chromosome 1 (Chehab et al., 1987).

The $\beta$ subunit Na$^+$/K$^+$ ATPase cDNA has also been cloned from the sheep kidney (Shull et al., 1986) and the human HeLa cell (Kawakami et al., 1986). The two forms of $\beta$ subunit mRNA found in HeLa cells were shown to be from one gene with two different poly-adenylation signals (Kawakami et al., 1986). Subsequently it was shown that the multiple $\beta$ subunit mRNAs present in other tissues also code only for a single protein and the size differences observed are due to the use of multiple transcription initiation sites (Young et al., 1987).

In the brine shrimp cyst, Na$^+$/K$^+$ ATPase activity is not detectable in the first 6 h of development (Fisher et al., 1986). Radioimmunoassay for detection of the $\alpha$ and $\beta$ subunit and cell free translation of extracted mRNAs demonstrated that developmental regulation of Na$^+$/K$^+$ ATPase is partially controlled at the mRNA and partially controlled at the translational level in the brine shrimp (Fisher et al., 1986).
Regulation of Na⁺/K⁺ ATPase by potassium levels has been investigated in cultured canine kidney cells (Bowen and McDonough, 1987). Culturing the cells in the presence of decreased potassium results in a 1.7-fold increase in α and β subunit levels. The rate of synthesis of the α and β subunit increases approximately 2-fold. Also, the mRNA levels for the α and β subunit increases 1.9 and 2.3-fold.

Gene amplification appears to be a method of regulation of Na⁺/K⁺ ATPase in ouabain-resistant HeLa C¹ cells (Emanuel et al., 1986; Mercer et al., 1986). In these cells the α subunit gene is amplified approximately 100-fold and the mRNA level is increased (Emanuel et al., 1986). In addition these cells express a mRNA not found in normal HeLa cells. Similarly the β subunit is amplified approximately 20-fold in these cells and the β subunit mRNA level is also increased (Mercer et al., 1986).

Aldosterone regulation of Na⁺/K⁺ ATPase in cultured Xenopus laevis kidney cells has been investigated (Verrey et al., 1987). Sodium transport, protein synthesis of the α and β subunits and mRNA levels for α and β subunits were determined after incubation with hormone. Relative rate of synthesis of the α and β subunit increases 1.65 and 2.0-fold after 6h of hormone incubation and two to three-fold after 96h. Sodium transport increases 3.5-fold after 6h and 8.7-fold after 96h. Finally, α and β subunit mRNA increases 2 to 4-fold at 6h and no further increase is detected at 96h.

Thyroid hormone has been found to regulate Na⁺/K⁺ ATPase in
kidney cortex and myocardium but not cerebrum (Chaudhury et al., 1987). Injection of thyroid hormone to hypothyroid rats results in an increase in Na\(^+\)/K\(^+\) ATPase activity and a 2.1 to 2.5-fold increase in \(\alpha\) subunit mRNA in kidney cortex and myocardium. No effect on either enzyme activity or mRNA level is observed in the cerebrum.
Table 1. Timing of embryonic development in various species

<table>
<thead>
<tr>
<th>Stage and days of development</th>
<th>Rabbit</th>
<th>Cow</th>
<th>Mare</th>
<th>Ewe</th>
<th>Sow</th>
<th>Mouse</th>
<th>Woman</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-cell</td>
<td>.6</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>.6-.8</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Four-cell</td>
<td>----</td>
<td>1.5</td>
<td>1.5</td>
<td>1.3</td>
<td>1</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Eight-cell</td>
<td>----</td>
<td>3</td>
<td>3</td>
<td>1.5</td>
<td>2.5</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Morula</td>
<td>2-3</td>
<td>6</td>
<td>4</td>
<td>4</td>
<td>3-4</td>
<td>2.5-3.5</td>
<td>4</td>
</tr>
<tr>
<td>Blastocyst</td>
<td>3-4</td>
<td>7-9</td>
<td>6</td>
<td>6-7</td>
<td>5-6</td>
<td>3-4</td>
<td>5-8</td>
</tr>
<tr>
<td>Hatching</td>
<td>----</td>
<td>9-11</td>
<td>8</td>
<td>7-8</td>
<td>6</td>
<td>4-5</td>
<td>----</td>
</tr>
<tr>
<td>Transport to uterus</td>
<td>3-4</td>
<td>3-4</td>
<td>4-6</td>
<td>2-4</td>
<td>2-4</td>
<td>3-4</td>
<td>3</td>
</tr>
</tbody>
</table>

(Compiled from Cole and Cupps, 1977; Hafez, 1980)
Table 2. Total RNA, poly (A)$^+$ RNA and ribosomal RNA content of early mouse embryos

<table>
<thead>
<tr>
<th>Hours after hCG</th>
<th>Developmental Stage</th>
<th>Total RNA ng/embryo</th>
<th>Poly(A)$^+$ RNA pg/embryo</th>
<th>% of total RNA</th>
<th>rRNA ng/embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>unfertilized</td>
<td>.35</td>
<td>.70</td>
<td>.20</td>
<td>---</td>
</tr>
<tr>
<td>24</td>
<td>zygote</td>
<td>---</td>
<td>.83</td>
<td>.24</td>
<td>.22</td>
</tr>
<tr>
<td>48</td>
<td>late 2-cell</td>
<td>.24</td>
<td>.26</td>
<td>.11</td>
<td>.17</td>
</tr>
<tr>
<td>72</td>
<td>8- to 16-cell</td>
<td>.69</td>
<td>.44</td>
<td>.06</td>
<td>.40</td>
</tr>
<tr>
<td>94</td>
<td>early (32-cell)</td>
<td>1.47</td>
<td>1.42</td>
<td>.10</td>
<td>1.0</td>
</tr>
</tbody>
</table>

(From Piko and Clegg, 1982)
Table 3. Total RNA content in the eggs of various animal species

<table>
<thead>
<tr>
<th>Species</th>
<th>Mouse</th>
<th>Rabbit</th>
<th>Xenopus laevis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Egg diameter (um)</td>
<td>70</td>
<td>125</td>
<td>1100</td>
</tr>
<tr>
<td>Egg volume (um³)</td>
<td>(1.8 \times 10^5)</td>
<td>(1.0 \times 10^6)</td>
<td>(7.0 \times 10^8)</td>
</tr>
<tr>
<td>RNA content per egg (ng)</td>
<td>0.35</td>
<td>6.0</td>
<td>4000</td>
</tr>
<tr>
<td>RNA content per um³ (fg)</td>
<td>2.0</td>
<td>6.0</td>
<td>5.7</td>
</tr>
</tbody>
</table>

(From Piko and Clegg, 1982)
Table 4. Protein content of preimplantation mouse embryos

<table>
<thead>
<tr>
<th>Stage of Development</th>
<th>ng protein / embryo, values obtained by:</th>
<th>(Sellens et al., 1981)</th>
<th>(Brinster, 1967)</th>
<th>(Schiffner &amp; Spielmann, 1976)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized</td>
<td></td>
<td>29.9</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Zygote</td>
<td></td>
<td>28.3</td>
<td>27.8</td>
<td>24.5</td>
</tr>
<tr>
<td>Two-cell</td>
<td></td>
<td>27.5</td>
<td>26.1</td>
<td>26.2</td>
</tr>
<tr>
<td>Four-cell</td>
<td></td>
<td>26.1</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Eight-cell</td>
<td></td>
<td>27.0</td>
<td>23.4</td>
<td>22.0</td>
</tr>
<tr>
<td>Morula</td>
<td></td>
<td>25.2</td>
<td>20.6</td>
<td>20.9</td>
</tr>
<tr>
<td>Early Blastocyst</td>
<td></td>
<td>25.3</td>
<td>23.9</td>
<td>24.8</td>
</tr>
<tr>
<td>Mid Blastocyst</td>
<td></td>
<td>24.2</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Mid Blastocyst (w/o zona)</td>
<td></td>
<td>20.3</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Late Blastocyst (unhatched)</td>
<td></td>
<td>24.0</td>
<td>----</td>
<td>----</td>
</tr>
<tr>
<td>Late Blastocyst (hatched)</td>
<td></td>
<td>25.2</td>
<td>20.0</td>
<td>20.8</td>
</tr>
</tbody>
</table>
Table 5. RNA content of the early rabbit embryo

<table>
<thead>
<tr>
<th>Day</th>
<th>RNA content (ug/embryo)</th>
<th>Cell number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.020</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>0.028</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>0.034</td>
<td>16</td>
</tr>
<tr>
<td>3</td>
<td>0.069</td>
<td>128</td>
</tr>
<tr>
<td>4</td>
<td>0.123</td>
<td>1024</td>
</tr>
<tr>
<td>5</td>
<td>0.414</td>
<td>9000</td>
</tr>
<tr>
<td>6</td>
<td>2.800</td>
<td>80,000</td>
</tr>
</tbody>
</table>

(From Schultz and Tucker, 1977)
Table 6. Actin mRNA content in mouse embryos

<table>
<thead>
<tr>
<th>Stage and age</th>
<th># of embryos per assay</th>
<th>Actin mRNA content per embryo (fg)</th>
<th>Actin mRNA content per cell (fg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unfertilized egg</td>
<td>500 to 1000</td>
<td>431</td>
<td>431.0</td>
</tr>
<tr>
<td>14 - 16 h post hCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Two-cell</td>
<td>750 to 1000</td>
<td>35</td>
<td>17.5</td>
</tr>
<tr>
<td>42 - 44 h post hCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eight-cell</td>
<td>500 to 750</td>
<td>182</td>
<td>22.8</td>
</tr>
<tr>
<td>66 - 68 h post hCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early blastocyst</td>
<td>150 to 300</td>
<td>854</td>
<td>26.7</td>
</tr>
<tr>
<td>(32-cell)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>92 - 94 h post hCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Late blastocyst</td>
<td>75 to 150</td>
<td>2417</td>
<td>22.0</td>
</tr>
<tr>
<td>106 - 108 h post hCG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(From Giebelhaus et al., 1985)
Table 7. Fluid accumulation, Na\(^+\) influx, and ouabain binding sites in rabbit blastocysts

<table>
<thead>
<tr>
<th>Days post-coitum</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluid accumulation rate (ul/cm(^2)h)</td>
<td>7.57</td>
<td>26.05</td>
<td>32.26</td>
<td>70.98</td>
</tr>
<tr>
<td>Net Na(^+) accumulation rate (umol/cm(^2)h)</td>
<td>0.098</td>
<td>0.342</td>
<td>0.452</td>
<td>0.820</td>
</tr>
<tr>
<td>Ouabain-sensitive Na(^+) influx (umol/cm(^2)h)</td>
<td>------</td>
<td>0.20</td>
<td>0.19</td>
<td>0.09</td>
</tr>
<tr>
<td>Ouabain-binding sites (billion/cm(^2))</td>
<td>27.2</td>
<td>229</td>
<td>91.3</td>
<td>170</td>
</tr>
<tr>
<td>Turnover number (ion/second/site)</td>
<td>------</td>
<td>146</td>
<td>348</td>
<td>91</td>
</tr>
</tbody>
</table>

(From Benos et al., 1985)
Figure 1. Diagram of early mouse embryo development. Total hours of development (h) and total cell number (C) are indicated by arabic numerals. Roman numerals indicate cell cycle.
Materials and Methods

Mouse embryo collection and culture

Prepubertal Swiss-Webster female mice (11-13g) were synchronized and superovulated by intraperitoneal injection of 5 IU of pregnant mare serum gonadotropin (PMSG) and 44 to 48 h later with 5 IU of human chorionic gonadotropin (hCG). Pubertal CFI female mice (23 - 25g) were similarly injected, except for an increased dosage of PMSG (10 IU). Following hCG injection, females were caged with proven breeder male Swiss-Webster mice. The following morning female mice were inspected for the presence of vaginal plugs, as an indication of copulation. Bred females were sacrificed by cervical dislocation approximately 36 h after ovulation (see Table 8). Oviducts were excised and flushed with Whitten's medium (WM; Whitten and Biggers, 1968). Embryos of normal morphology (Lindner and Wright, 1983) were removed from flushing medium and washed by passing through several drops of culture medium, consisting of WM supplemented with 10 mg/ml bovine serum albumin (BSA). Embryos were either immediately frozen for Western blot analysis, extracted for RNA or placed in culture for later use. Embryos were cultured for 24 (Day 2), 48 (Day 3) or 72 h (Day 4) in microdrops of culture medium under paraffin oil at 37 C in a humidified atmosphere of 5% CO2 in air. At the end of culture the percentage of embryos exhibiting a blastocoel was recorded.
**Rabbit embryo collection**

New Zealand White does were superovulated by subcutaneous injection of 0.5 mg porcine follicle stimulating hormone twice daily for three days. On the fourth day does were bred and injected (i.p.) with 100 IU of hCG. Embryos were surgically collected from does 2, 3, 4, 5 and 6 days after mating. For embryo collection, does were anaesthetized by i.m. injection of 60 mg Ketamine and 10 mg Rompun per kg of body weight. A ventral midline laparotomy was performed, the reproductive tract was exteriorized, and the uterus or oviducts (see Table 9) flushed with Ham's F-12 supplemented with 25mM Hepes. Embryos exhibiting normal morphology were washed in Ham's F-12 supplemented with 25mM Hepes and 15 mg/ml BSA. For Western blot analysis embryos were immediately placed on ice and subsequently frozen. Embryos to be used for Northern blot analysis were immediately transported to the laboratory for nucleic acid extraction.

**Nucleic acid extraction from embryos**

Groups of rabbit or mouse embryos were collected in the smallest volume of medium possible (e.g., 5 ul) and placed into 500 ul microcentrifuge tubes. Immediately, tRNA (10 ug), 10X TES [10ul; 10X TES= 100mM Tris pH 7.5 (trishydroxymethylaminomethane), 50mM EDTA
(ethylenediaminetetraacetic acid), 10% SDS (sodium dodecyl sulfate)] and proteinase K (20 ug) were added to the tubes containing embryos. The total volume was adjusted to 100 ul with glass distilled autoclaved water (GDAW). The tubes were vortexed for one minute and incubated in a 55 C water bath for one to two hours. After incubation, 5 ul of 5M NaCl and 100 ul of phenol were added to the tubes. Samples were vortexed for one minute and centrifuged at 13,000 x g at room temperature for three minutes. The aqueous phase was removed with a sterile Pasteur pipet (previously drawn to a fine tip) and transferred to a new microcentrifuge tube. Subsequently, 50 ul of phenol were added and samples were vortexed one minute. Chloroform (50 ul) was added and samples were again vortexed one minute and centrifuged at 13,000 x g for three minutes. The aqueous phase was again transferred and 100 ul of chloroform were added. Samples were vortexed for one minute, centrifuged for one minute, and the aqueous phase transferred to a clean microcentrifuge tube. Ethanol (250 ul) was added and the sample was allowed to precipitate at -20 C overnight or longer. After precipitation, samples were centrifuged at 13,000 x g at 4 C for 30 minutes. Ethanol was removed with a sterile fine tipped Pasteur pipet and the nucleic acid pellet was resuspended in GDAW.

RNA extraction from kidneys (see Appendix for further details)

Ribonucleic acid was extracted from fresh mouse and sheep
kidneys by the guanidinium/cesium chloride method. Quantification of RNA utilized spectrophotometry at 260 and 280 nm.

Northern blotting (see Appendix for further details)

Nucleic acid samples were separated on denaturing horizontal 1.1% agarose gels. After electrophoresis the lane containing molecular weight markers was removed, stained with ethidium bromide and photographed under ultraviolet light. Alternatively the entire gel was stained with acridine orange prior to northern blotting. The size separated RNA was transferred from the agarose gel to nitrocellulose by capillary blotting. The resulting nitrocellulose (northern blot) was baked at 80°C under vacuum and the gel was stained with ethidium bromide to determine completeness of the transfer. Northern blots were probed with random primer labeled cDNA of sheep kidney Na\(^+\)/K\(^+\) ATPase \(\alpha\)-subunit (Shull et al., 1985). The northern blots with the bound radioactive cDNA were exposed to x-ray film. Intensity of the bands on the resulting autoradiogram was used as an indication of the relative amount of Na\(^+\)/K\(^+\) ATPase mRNA in the sample. Densitometric scanning was used to determine the level of Na\(^+\)/K\(^+\) ATPase mRNA in the samples.

Slot blot preparation (see Appendix for further details)

Denatured RNA samples were directly applied to nitrocellulose by
the use of a hybri-slot apparatus and vacuum pressure. Subsequently the nitrocellulose was handled as in northern blotting.

**Preparation of radioactive Na⁺/K⁺ ATPase cDNA probe**
(see Appendix for further details)

Purified plasmid DNA was prepared by the alkaline lysis method (Maniatis et al., 1982). Plasmid DNA was digested with restriction endonucleases PVU II and Bgl II and fragments were separated by electrophoresis. The appropriate fragment was removed from the agarose gel by HAP recovery. The cDNA was labeled using 32P labeled dCTP, a random primer labeling kit and a spun column.

**Western Blotting (see Appendix for further details)**

Mouse embryos of normal morphology at Days 1, 2, 3 and 4 of development were passed through two microdrops of Whitten's medium supplemented with 0.1 mg/ml BSA. Subsequently, the embryos were placed in microcentrifuge tubes in the smallest volume possible and frozen at -20 C. Rabbit embryos of normal morphology were washed in Ham's F-12 medium supplemented with 25mM Hepes, placed in a microcentrifuge tube in the smallest volume possible and immediately frozen at -20 C.

Mouse and rabbit kidney microsomes were used as a standard source of Na⁺/K⁺ ATPase and were prepared by homogenization and
differential centrifugation (see appendix) using the method of Jorgensen (1974) and the homogenization buffer of McDonough and Schmitt (1985). The resulting membrane fractions were assayed for total protein content (BioRad) and frozen at -20 C.

Mouse and rabbit embryos and kidney microsomes were thawed in sample buffer and separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). After electrophoresis the size separated proteins were transferred from the gel to nitrocellulose paper by electrophoretic blotting. The resulting western blot was incubated with first antibody (anti-guinea pig Na⁺/K⁺ ATPase rabbit serum) and subsequently second antibody (goat-anti rabbit IgG conjugated to alkaline phosphatase). The addition of alkaline phosphatase substrate to the blot resulted in a color reaction and the appearance of bands wherever the first antibody was bound.

Densitometric scanning of photographic transparencies of western blots were used to determine relative changes in Na⁺/K⁺ ATPase α and β subunits during early rabbit and mouse embryo development.

**Statistical analysis**

Differences in blastocoel formation rate were analyzed by Chi-square analysis. Linearity of film responses for northern and western blots was validated by determining correlation coefficients for standard curves of kidney total RNA and kidney microsome protein.
Differences in α subunit, β subunit and α subunit mRNA levels were detected by analysis of variance and Fisher's least significant differences procedures and the pooled t-test.
Table 8. Schedule for embryo collection from pubertal CF1 mice housed with a dark cycle from 7:00 PM to 7:00 AM

<table>
<thead>
<tr>
<th>Day</th>
<th>Event and timing</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3</td>
<td>PMSG (10 IU) injection between 3 PM and 6 PM</td>
</tr>
<tr>
<td>-1</td>
<td>hCG (5 IU) injection between 2 PM and 4 PM</td>
</tr>
<tr>
<td>0</td>
<td>Ovulation and fertilization 3 AM, plug detection AM</td>
</tr>
<tr>
<td>1</td>
<td>Collect late 2-cell embryos between 1 PM and 5 PM, embryos harvested (at the 2-cell stage) approximately 3 PM</td>
</tr>
<tr>
<td>2</td>
<td>Embryos harvested from culture (mostly at the 4-cell stage) 3 PM</td>
</tr>
<tr>
<td>3</td>
<td>Embryos harvested from culture (mostly at the morula stage) 3 PM</td>
</tr>
<tr>
<td>4</td>
<td>Embryos harvested from culture (all blastocysts) 3 PM</td>
</tr>
</tbody>
</table>
Table 9. Schedule for embryo collection from New Zealand White does

<table>
<thead>
<tr>
<th>Day</th>
<th>Event and timing</th>
</tr>
</thead>
<tbody>
<tr>
<td>-3</td>
<td>pFSH injection (.5 mg) twice daily</td>
</tr>
<tr>
<td>-2</td>
<td>pFSH injection (.5 mg) twice daily</td>
</tr>
<tr>
<td>-1</td>
<td>pFSH injection (.5 mg) twice daily</td>
</tr>
<tr>
<td>0</td>
<td>Does mated and administered hCG injection (100 IU) between 8 AM and 9 AM, ovulation approximately 7 PM</td>
</tr>
<tr>
<td>2</td>
<td>Collect embryos from oviducts 8 AM to 1 PM (8-cell to morula)</td>
</tr>
<tr>
<td>3</td>
<td>Collect embryos from oviducts and uterus 8 AM to 1 PM (mostly morula)</td>
</tr>
<tr>
<td>4</td>
<td>Collect embryos from uterus 8 AM to 1 PM (blastocysts)</td>
</tr>
<tr>
<td>5</td>
<td>Collect embryos from uterus 8 AM to 1 PM (blastocysts)</td>
</tr>
<tr>
<td>6</td>
<td>Collect embryos from uterus 8 AM to 1 PM (blastocysts)</td>
</tr>
</tbody>
</table>
Results

Na⁺/K⁺ ATPase α subunit mRNA levels in early mouse embryos

Because the cDNA used in these experiments was for the α subunit of Na⁺/K⁺ ATPase from sheep kidney (Shull et al., 1985) and the RNA source was mouse embryos, preliminary experiments were performed to determine cross-hybridization of sheep kidney Na⁺/K⁺ ATPase α₁ subunit cDNA (NKA-1) to RNA extracted from mouse kidneys. Northern blots exhibited bands of similar intensity with molecular weights of 3.65 kb for Na⁺/K⁺ ATPase α₁ subunit mRNA from both sheep and mouse kidneys (Figure 2). As a test of hybridization specificity for NKA-1 with mRNA, the intensity of autoradiographic bands of poly (A)⁺ RNA and total RNA were compared. Poly (A)⁺ selection of total mouse or sheep kidney RNA resulted in a dramatic increase in band intensity (Figure 2). In our laboratory, northern blot analysis permitted detection of Na⁺/K⁺ ATPase α₁ subunit mRNA in as little as 400 ng of total RNA from mouse kidneys.

Northern blots for Na⁺/K⁺ ATPase α₁ subunit mRNA were performed on nucleic acids extracted from Swiss Webster mouse embryos on Days 1, 2, 3 and 4 of development. Na⁺/K⁺ ATPase mRNA was detected in Day 2, 3 and 4 mouse embryos and was of the same molecular weight as that detected in mouse and sheep kidneys (Figure 3). No mRNA band was detected in Day 1 mouse embryos; however, this may have been due to
the limited sensitivity of the northern blots. Because of their
greater sensitivity, slot blots were used to further quantify the
relative increase in Na⁺/K⁺ ATPase α subunit mRNA in the early mouse
embryo. Slot blot hybridization conditions were identical to
hybridization conditions for northern blots in which no non-specific
hybridization was observed. In addition, tRNA and RNase-treated
nucleic acid samples from embryos were analyzed with slot blots and
neither displayed hybridization. Slot blot analyses detected Na⁺/K⁺
ATPase α subunit mRNA in Day 1 to 4 Swiss Webster mouse embryos.
Densitometric scanning demonstrated Na⁺/K⁺ ATPase α subunit mRNA
content increased 45-fold between Days 1 and 4 in the mouse embryo.
Figure 4 depicts the relative increase in Na⁺/K⁺ ATPase α subunit
mRNA levels compared with blastocyst formation rate during the first
4 days of mouse embryo development.

The Na⁺/K⁺ ATPase α subunit mRNA content of mouse embryos was
also determined in Day 1 to 4 embryos collected from CF1 female mice
bred to Swiss Webster males. The CF1 x Swiss Webster embryos formed
fewer blastocysts (P<.005) in vitro and exhibited retarded
development compared to Swiss Webster embryos (Table 10). Because
blastocoel formation is dependent on Na⁺/K⁺ ATPase, we were
interested in whether the Na⁺/K⁺ ATPase gene was being regulated
differently in the two strains. Two-way analysis of variance
revealed no significant differences (P>.05) in changes in α subunit
mRNA content due to strain of embryo; however, the increase in Na⁺/K⁺
ATPase mRNA content between Days 2 and 3 was greater than the
increase between Days 3 and 4 (P<.05). Changes in Na⁺/K⁺ ATPase α subunit mRNA content between Days 2 and 4 in Swiss Webster and CF1 x Swiss Webster embryos as determined by slot and northern blot analysis are listed in Table 11.

**Na⁺/K⁺ ATPase α and δ subunit levels in early mouse embryos**

Western blots were used to identify and quantify the relative change in Na⁺/K⁺ ATPase α and δ subunit content of early mouse embryos. Rabbit anti-guinea pig Na⁺/K⁺ ATPase antiserum was used as the first antibody. This antiserum has been characterized by McDonough et al., (1982). The antiserum binds to both the α and δ subunit, does not cross-react with Ca⁺⁺ ATPase and has been extensively used in western blot analysis (McDonough et al., 1982; McDonough and Schmitt, 1985; Farley et al., 1986; Schmitt and McDonough, 1986; McDonough and Schmitt, 1987; Bowen and McDonough, 1987). The antibody cross-reacts with human, bovine, dog, rabbit, rat, mouse, turtle and toad α subunit, but is less cross-reactive with δ subunit of other species. It also cross-reacts with the α+ subunit. It has also been reported that this antibody cross-reacts with ovalbumin as did other Na⁺/K⁺ ATPase polyclonal antisera (Farley et al., 1986). Microsomes prepared from mouse kidneys were used as a control source of Na⁺/K⁺ ATPase α and δ subunit on the western blots. Preliminary analyses of mouse embryos on western blots revealed a
band comigrating with BSA. Bovine serum albumin is a component of the embryo culture medium and therefore is present in the embryo samples. To verify the identity of this band, mouse kidney microsomes and mouse kidney microsomes with added BSA were compared on western blots (Figure 5). As a test of non-specific binding, mouse kidney microsomes and embryo samples were run on western blots with normal rabbit serum as the first antibody. Neither the α or the β subunit bands were visible on these normal rabbit serum western blots. In addition to the BSA band, mouse kidney microsomes supplemented with BSA exhibited two bands on western blots. Based on their electrophoretic mobility, the slowest migrating band (102 kD) is the α subunit and the fastest migrating band (43 kD) is the β subunit (Figure 5). In contrast, the mouse embryo samples displayed three bands which migrated similar to the α subunit (108, 104 and 92 kD) and one band comigrating with the β subunit (43 kD; Figure 8).

On a per embryo or per mg of protein basis, the relative amount of the α subunit protein (108 kD) was not significantly different between Days 1 to 4 of mouse embryo development (Table 12). However, the relative amount of the β subunit protein increased dramatically (9 fold) over this same time period (Table 12).

\[ \text{Na}^+/\text{K}^+ \text{ ATPase} \, \alpha \text{ subunit mRNA levels in early rabbit embryos} \]

Rabbit embryos collected on Days 2, 3, 4, 5 and 6 of development were analyzed for Na\(^+/\)K\(^+\) ATPase α subunit mRNA content by northern
blot hybridization. Only one band was apparent in Days 4, 5 and 6 rabbit embryo samples and it comigrated with the $\alpha$ subunit of sheep and mouse kidney total RNA (3.65 kb; Figure 7). No $\text{Na}^+/\text{K}^+$ ATPase $\alpha$ subunit mRNA band was detected in Days 2 and 3 rabbit embryos. Densitometric scanning revealed the relative amount of $\text{Na}^+/\text{K}^+$ ATPase $\alpha$ subunit mRNA increased approximately 35-fold between Days 4 and 6 of rabbit embryo development (Table 13).

$\text{Na}^+/\text{K}^+$ ATPase $\alpha$ and $\beta$ subunit levels in early rabbit embryos

Rabbit embryos collected on Days 3, 4, 5 and 6 of development were analyzed by western blotting. Rabbit kidney microsomes supplemented with BSA were used as a control source of $\text{Na}^+/\text{K}^+$ ATPase and displayed three bands on western blots (Figure 5). Based on its electrophoretic mobility, the slowest migrating band, the $\alpha$ subunit of $\text{Na}^+/\text{K}^+$ ATPase, is approximately 96 kD. The middle band is serum albumin. Based on its electrophoretic mobility, the fastest migrating band, the $\beta$ subunit, was only observed when large amounts of protein were loaded on the gel and was approximately 42 kD. Three bands of similar mobility were seen on western blots of Day 6 rabbit embryos (Figure 8). Rabbit embryos collected on Days 3, 4 and 5 only exhibited $\alpha$ subunit and serum albumin bands. Densitometric scanning revealed the relative amount of $\alpha$ subunit increased 85 fold between Days 3 and 6 of rabbit embryo development (Table 14).
Table 10. Blastocyst formation rate of Swiss Webster and CF1 x Swiss Webster mouse embryos

<table>
<thead>
<tr>
<th>Type of embryo</th>
<th>No. of embryos with a blastocoel/ Total No. of embryos on (%)</th>
<th>Day 1</th>
<th>Day 2</th>
<th>Day 3</th>
<th>Day 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swiss Webster</td>
<td></td>
<td>0/2770 (0)ᵃ</td>
<td>0/1006 (0)ᵃ</td>
<td>248/292 (85)ᵃ</td>
<td>2134/2203 (97)ᵃ</td>
</tr>
<tr>
<td>CF1 x Swiss Webster</td>
<td></td>
<td>0/4608 (0)ᵃ</td>
<td>0/4603 (0)ᵃ</td>
<td>200/2418 (8)ᵇ</td>
<td>2881/4138 (70)ᵇ</td>
</tr>
</tbody>
</table>

ᵃ,ᵇ Values in the same column without common superscripts are different (P<.005)
Table 11. Changes in Na\textsuperscript{+}/K\textsuperscript{+} ATPase α subunit mRNA content of mouse embryos from Days 2 to 4 of development

<table>
<thead>
<tr>
<th>Strain</th>
<th>Days of development:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>CF1 x SW</td>
<td>8.8±2.8</td>
<td>16.5±4.9</td>
<td></td>
</tr>
<tr>
<td>SW x SW</td>
<td>5.9±1.7</td>
<td>7.4±3.0</td>
<td></td>
</tr>
</tbody>
</table>

Values reported are means± standard errors and are expressed as units/embryo, where 1 unit is equal to the autoradiographic band intensity of Na\textsuperscript{+}/K\textsuperscript{+} ATPase α subunit mRNA in Day 2 embryos.
Table 12. Changes in Na⁺/K⁺ ATPase α and β subunit content of mouse embryos from Days 1 to 4 of development

<table>
<thead>
<tr>
<th>Subunit</th>
<th>Days of development</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>α</td>
<td>1.1±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.9±0.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.5±0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>β</td>
<td>2.1±0.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.7±0.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.2±3.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Values reported are means± standard errors and are expressed as units/embryo, where 1 unit is equal to the autoradiographic band intensity of the Na⁺/K⁺ ATPase α or β subunit in Day 1 embryos. <sup>a</sup>,<sup>b</sup> Values in the same row without common superscripts are different (P<0.10).
Table 13. Changes in Na⁺/K⁺ ATPase α subunit mRNA content of rabbit embryos from Days 4 to 6 of development

<table>
<thead>
<tr>
<th>Days of development:</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>20.4±0.6</td>
<td>34.8±11.2</td>
<td></td>
</tr>
</tbody>
</table>

Values reported are means ± standard errors and are expressed as units/embryo, where 1 unit is equal to the autoradiographic band intensity of the Na⁺/K⁺ ATPase α subunit mRNA in Day 4 embryos.
Table 14. Changes in Na\textsuperscript{+}/K\textsuperscript{+} ATPase $\alpha$ subunit content of rabbit embryos from Days 3 to 6 of development

<table>
<thead>
<tr>
<th>Days of development</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.9±0.6\textsuperscript{a}</td>
<td>45.2±17.6\textsuperscript{b}</td>
<td>85.3±9.3\textsuperscript{c}</td>
</tr>
</tbody>
</table>

Values reported are means± standard errors and are expressed as units/embryo, where 1 unit is equal to the autoradiographic band intensity of the Na\textsuperscript{+}/K\textsuperscript{+} ATPase $\alpha$ subunit in Day 3 embryos. \textsuperscript{a,b,c} Values without common superscripts are different (P<0.10).
Figure 2. Northern blot of mouse and sheep kidney total RNA and poly (A)$^+$ RNA. Lane 1, 5 ug mouse kidney total RNA; lane 2, 5 ug mouse kidney poly (A)$^+$ RNA; lane 3, 1 ug mouse kidney poly (A)$^+$ RNA; lane 4, 1 ug mouse kidney total RNA; lane 5, 1 ug sheep kidney total RNA; lane 6, 1 ug sheep kidney poly (A)$^+$ RNA; lane 7, DNA molecular weight markers.
Figure 3. Northern blot of RNA extracted from Swiss Webster mouse embryos on Days 1 through 4 of development. Lane 1, total RNA from 1328 Day 1 mouse embryos; lane 2, total RNA from 1392 Day 2 mouse embryos; lane 3, total RNA from 1182 Day 3 mouse embryos; lane 4, total RNA from 1067 Day 4 mouse embryos; lane 5, 0.4 ug sheep kidney total RNA; lane 6, 2 ug sheep kidney total RNA; lane 7, 9 ug sheep kidney total RNA.
Figure 4. Graph of Na⁺/K⁺ ATPase α subunit mRNA content and blastocyst formation rate of Swiss Webster mouse embryos from Days 1 through 4 of development. The Na⁺/K⁺ ATPase α subunit mRNA content is expressed as units of autoradiographic band intensity from slot blot analysis.
Figure 5. Western blot of mouse and rabbit kidney microsomes and mouse and rabbit kidney microsomes supplemented with BSA. Lane 1, 12 ug rabbit kidney microsome protein; lane 2, 12 ug rabbit kidney microsome protein supplemented with 5 ug BSA; lane 3, 12 ug mouse kidney microsome protein supplemented with 5 ug BSA; lane 4, 12 ug mouse kidney microsome protein; lane 5, molecular weight markers (180 kD, 116 kD, 84 kD, 58 kD, 48.5 kD, 36.5 kD, 26.6 kD); lane 6, 200 Day 4 mouse embryos with BSA.
Figure 6. Western blot of mouse embryos on Days 1 through 4 of development. Lane 1, 220 Day 4 mouse embryos; lane 2, 400 Day 3 mouse embryos; lane 3, 640 Day 2 mouse embryos; lane 4, 942 Day 1 mouse embryos; lane 5, molecular weight markers; lane 6, .75 ug mouse kidney microsome protein supplemented with .38 ug BSA; lane 7, 1.5 ug mouse kidney microsome protein supplemented with .75 ug BSA; lane 8, 3.0 ug mouse kidney microsome protein supplemented with 1.5 ug BSA; lane 9, 6 ug mouse kidney microsome protein supplemented with 3.0 ug BSA. Correlation coefficient for standard curve of ug mouse kidney microsome protein by band intensity, r=.999.
Figure 7. Northern blot of RNA extracted from rabbit embryos on Days 4 through 6 of development. Lane 1, 821 Day 4 mouse embryos; lane 2, 16 Day 4 rabbit embryos; lane 3, 6 Day 6 rabbit embryos; lane 4, 16 Day 6 rabbit embryos; lane 5, 44 Day 6 rabbit embryos; lane 6, 0.4 ug sheep kidney total RNA; lane 7, 2 ug sheep kidney total RNA; lane 8, 9 ug sheep kidney total RNA; lane 9, 9 ug chicken liver total RNA; lane 10, ethidium bromide stained RNA molecular weight markers.
Figure 8. Western blot of rabbit embryos on Days 3 through 6 of development. Lane 1, 3 ug rabbit kidney microsome protein supplemented with 1.5 ug BSA; lane 2, 1.5 ug rabbit kidney microsome protein supplemented with .75 ug BSA; lane 3, .75 ug rabbit kidney microsome protein supplemented with .38 ug BSA; lane 4, 1 Day 6 rabbit embryo; lane 5, 3 Day 5 rabbit embryos; lane 6, 18 Day 4 rabbit embryos; lane 7, 36 Day 3 rabbit embryos; lane 8 molecular weight markers. Correlation coefficient for standard curve of ug rabbit kidney microsome protein by band intensity, r=.941.
Discussion

**Na\(^+\)/K\(^+\) ATPase \(\alpha\) subunit mRNA levels in early mouse embryos**

**Na\(^+\)/K\(^+\) ATPase \(\alpha\) subunit mRNA content of the mouse embryo increased 45-fold from Day 1 to 4.** There was a 33-fold increase in Na\(^+\)/K\(^+\) ATPase \(\alpha\) subunit mRNA levels from Day 1 to 3, which may be compared to a 6-fold increase in total RNA and rRNA per embryo over the same time period (Table 2). Actin mRNA content increases 69-fold between Day 1 and 4 of mouse embryo development (Table 6). During this same period cell number increases about 50-fold and total protein content and diameter of the mouse embryo is relatively constant (Table 3). Because of the complexity of biochemical and morphological changes occurring during early mouse embryo development, a universal basis of comparison is unavailable. Comparing the increase in Na\(^+\)/K\(^+\) ATPase \(\alpha\) subunit mRNA (33-fold) to the increase in total RNA (6-fold) between Day 1 and 3 of development, indicates that Na\(^+\)/K\(^+\) ATPase \(\alpha\) subunit mRNA is being regulated differently than the bulk of embryonic RNA.

It is of interest to compare the timing of increase in Na\(^+\)/K\(^+\) ATPase \(\alpha\) subunit mRNA to the timing of morphological changes observed during mouse embryo development (Figure 4). Compaction occurred on Day 2 of development. Blastocoel formation, which is believed to be dependent on Na\(^+\)/K\(^+\) ATPase, begins between Day 2 and 3 of development.
(Table 10). The dramatic increase in \( \text{Na}^+/\text{K}^+ \) ATPase subunit mRNA content occurred during the time when the percentage of blastocysts also increased. Transcription inhibitors have been used to demonstrate that the transcriptional events required for blastocoel formation occur within a few hours of the start of this process (Kidder and McLachlin, 1985). Whether \( \text{Na}^+/\text{K}^+ \) ATPase subunit mRNA is one of the critical messages controlling the timing of blastocoel formation is not known. Slot blots indicated that a limited amount of \( \text{Na}^+/\text{K}^+ \) ATPase subunit mRNA is present in Day 1 embryos, well in advance of blastocoel formation. It is possible that the low level of \( \text{Na}^+/\text{K}^+ \) ATPase subunit mRNA found in the Day 1 mouse embryo is utilized for a housekeeping function of \( \text{Na}^+/\text{K}^+ \) ATPase and not for trans-trophectodermal fluid transport. \( \text{Na}^+/\text{K}^+ \) ATPase is present in most, if not all, mammalian cell membranes where it functions to regulate cell volume and ion concentration. It therefore seems reasonable that the early embryo would also contain a low level of \( \text{Na}^+/\text{K}^+ \) ATPase prior to appearance of the transporting epithelium-like \( \text{Na}^+/\text{K}^+ \) ATPase. This latter possibility is supported by results of Powers and Tuppers (1975, 1977) who demonstrated the presence of \( \text{Na}^+/\text{K}^+ \) ATPase activity in mouse eggs and 2-cell embryos. Whether the \( \text{Na}^+/\text{K}^+ \) ATPase subunit mRNA detected in the Day 1 mouse embryo is of maternal or embryonic origin is unknown.

Northern blots revealed only one band (3.65 kb) in both the mouse embryo and mouse kidney RNA samples under a variety of hybridization and washing stringencies. The \( \alpha+ \) and \( \alpha_{III} \) mRNAs have
76.5 and 76.2% nucleotide homology of protein coding regions with the α form (Shull et al., 1986). Under low stringency conditions the α subunit cDNA may be expected to bind to α+ and αIII mRNAs. If mRNAs encoding α+ and αIII subunits were present and cross-hybridizing to NKA-1 in embryo RNA samples, they would be expected to appear as separate bands of 5.1 kb (α+) and 3.55 kb (αIII) on northern blots (Shull et al., 1986). However, if present the level of α+ and αIII subunit mRNA in embryo samples was below the detection limit of NKA-1 on our northern blots. Probing northern blots of embryo RNA samples with specific cDNAs for α+ and αIII subunits may reveal the presence of other transcripts.

Swiss Webster mouse embryos have a dramatically higher blastocyst formation rate compared to CF1 x Swiss Webster embryos (Table 10). If this difference in timing of blastocoel formation was due to effects on Na+/K+ ATPase gene regulation, we would expect to observe differences in the regulation of the gene during early development. Despite the differences in developmental rate, CF1 x Swiss Webster embryos had a similar rate of increase in Na+/K+ ATPase α subunit mRNA as Swiss Webster embryos (Table 11). It is possible that differences in Na+/K+ ATPase gene regulation due to strain of embryo exist but that they occur at a post mRNA level, or through the regulation of the γ subunit. Alternatively, the differences in development may be unrelated to Na+/K+ ATPase.
Western blotting of early mouse embryos revealed three protein bands migrating with similar mobilities as \( \text{Na}^+/\text{K}^+ \) ATPase \( \alpha \) subunit and one band comigrating with the \( \beta \) subunit. On a per embryo or per mg of protein basis, the intensity of the \( \alpha \) bands remained constant from Day 1 to 4 of mouse embryo development, whereas the \( \beta \) subunit increased dramatically (9-fold). Our results demonstrating a constant level of \( \alpha \) subunit are in contradiction to results obtained by immunofluorescent localization (Watson and Kidder, 1988). Watson and Kidder (1988) demonstrated that \( \text{Na}^+/\text{K}^+ \) ATPase \( \alpha \) subunit first appears at the morula stage. Ultrastructural cytochemistry based on enzyme activity also first detected \( \text{Na}^+/\text{K}^+ \) ATPase at the morula stage (Vorbrodt et al., 1977). However, these reports disagree on the location of the enzyme at the morula stage. Immunofluorescent localization demonstrated cytoplasmic localization of the enzyme, whereas cytochemistry revealed basolateral membrane localization of the enzyme activity. Furthermore, early work evaluating ouabain inhibitable ion fluxes indicated the presence of \( \text{Na}^+/\text{K}^+ \) ATPase in the mouse egg (Powers and Tuppers, 1975) and the mouse 2-cell embryo (Powers and Tuppers, 1977). The actual timing of appearance and localization of \( \text{Na}^+/\text{K}^+ \) ATPase remain uncertain. The sensitivity, specificity and molecular weight determination available with Western blotting make it an ideal technique for studying \( \text{Na}^+/\text{K}^+ \) ATPase in the embryo. The increased sensitivity and improved availability of
antibody–antigen interactions with western blotting compared to immunofluorescent localization may explain the discrepancy in results. It is possible that Na⁺/K⁺ ATPase is below the limit of detection by immunofluorescent localization in pre-morula stage mouse embryos. Because the morphology of the embryo is dramatically changing during early development, it is possible that morphological arrangement may affect detection of the enzyme by immunofluorescent localization in certain developmental stages. Also, a different Na⁺/K⁺ ATPase antibody preparation was used in the immunofluorescent localization technique than in this study, and it apparently was not analyzed by western blotting of embryo samples to determine cross-reactivity. Although the Na⁺/K⁺ ATPase antibody used in the immunofluorescent localization study (Watson and Kidder, 1988) was a polyclonal antiserum specific for the α subunit in other systems, it is possible that it was also detecting the β subunit or other proteins in the mouse embryo.

The presence of three α subunit bands in our embryo samples was unexpected. Whether these three bands represent three different α subunit isoforms (e.g. α, α⁺, α III), three different levels of processing of one isozyme, or cross-reactive proteins has not been conclusively demonstrated. It would be of interest to further characterize the type of α subunit present in the early embryo and measure the relative changes in the level of each isozyme. Our work with Na⁺/K⁺ ATPase α subunit mRNA suggest that at least the α subunit is being actively transcribed in the early mouse embryo.
The dramatic increase in the level of $\alpha$ subunit over the first 4 days of mouse embryo development is in agreement with changes in enzyme activity (Vorbrodt et al., 1977). It is possible that Na$^+/K^+$ ATPase enzyme activity and blastocoel formation in the preimplantation mouse embryo are regulated by $\alpha$ subunit levels. The $\alpha$ subunit is a glycosylated protein and it has been demonstrated that blocking glycosylation in the early mouse embryo blocks blastocyst formation (Iwakura and Nozaki, 1985). Because the function of the $\beta$ subunit of Na$^+/K^+$ ATPase is unknown, the meaning of the difference in $\alpha$ and $\beta$ stoichiometry during early mouse embryo development is unclear. We are unaware of other studies demonstrating a dramatically different level of regulation of the $\alpha$ and $\beta$ subunit, and the active enzyme is believed to be composed of 2 and 2 subunits. Further characterization and study of the regulation of the $\beta$ subunit in embryos may be enlightening.

Comparison of Na$^+/K^+$ ATPase $\alpha$ subunit and $\alpha$ subunit mRNA content in the early mouse embryo

Whereas the $\alpha$ subunit mRNA content increases 45-fold on a per embryo basis, the $\alpha$ subunit protein content remains relatively constant during the first 4 days of mouse embryo development. The meaning of these results is uncertain. Total protein content of the mouse embryo during this time period remains constant (Table 3) whereas total RNA content increases dramatically (Table 2).
Comparing \( \alpha \) subunit mRNA content on a total RNA basis and \( \alpha \) subunit protein on a total protein basis decreases differences in the relative changes. The concentration of \( \alpha \) subunit mRNA in total embryonic RNA only increased about 5-fold between Days 1 and 3, whereas the concentration of \( \alpha \) subunit in total embryonic protein remained relatively constant during this period. Additionally, the Na\(^+/K\(^+\) ATPase \( \alpha \) subunit mRNA, along with other transcripts, may be accumulating until the translational machinery of the embryonic cells is highly functional. The rRNA level is also increasing during this time period (Table 2) and may be a limiting factor in rate of translation of embryonic mRNAs into proteins. It has been demonstrated that the percentage of poly(A)\(^+\) RNA tracts present in an active translating form increases during blastocyst differentiation (Kidder and Conlon, 1985). Further analysis of the exact Na\(^+/K\(^+\) ATPase isozymes present in the mouse embryo may lead to a more accurate comparison and better understanding of the regulation of Na\(^+/K\(^+\) ATPase in the early mouse embryo. It is possible that Na\(^+/K\(^+\) ATPase is being regulated at the enzyme activity level, and this is supported by reports demonstrating an increased enzyme activity at the morula and blastocyst stages (Vorbrodt et al., 1977).

**Na\(^+/K\(^+\) ATPase \( \alpha \) subunit and \( \alpha \) subunit mRNA levels in early rabbit embryos**

The Na\(^+/K\(^+\) ATPase \( \alpha \) subunit mRNA content increased 35-fold
whereas α subunit protein content increased 22-fold between Day 4 and 6 of rabbit embryo development. These increases correspond with the time period of maximal blastocyst expansion; embryo diameter increase from 300 um to 3 mm. The magnitude of expansion of rabbit blastocysts is similar to other domestic species, whereas mouse blastocysts are minimally expanding similar to human embryos. Because Na+/K+ ATPase is important in blastocyst formation and expansion, and these processes differ greatly in magnitude in the mouse and rabbit, it is reasonable to expect differences in the regulation of Na+/K+ ATPase in the two species.

Ouabain binding studies indicate that Na+/K+ ATPase content increases 50-fold between Day 4 and 6 in the rabbit embryo (Benos, 1981). Because ouabain binds to the K+ binding site on the α subunit, this would be a measure of α subunit content. Radioactive methionine incorporation indicates the synthetic rate of Na+/K+ ATPase increases 90-fold over the same time period (Benos et al., 1985). Although the α subunit was detected by 2-D polyacrylamide gel electrophoresis, the presence of other isozymes was not mentioned (Benos et al., 1985). Results of our western blots are in reasonable agreement with these results. We only detected Na+/K+ ATPase α subunit isozyme in rabbit embryos and it was the same size as the α subunit identified in rabbit kidney microsomes. Our 22-fold increase in Na+/K+ ATPase α subunit levels in Day 4 to 6 rabbit embryos may be compared to a 20-fold increase in total protein synthetic rate (Benos et al., 1985).
Northern blot analyses indicated a 35-fold increase in Na\textsuperscript{+}/K\textsuperscript{+} ATPase $\alpha$ subunit mRNA levels between Day 4 and 6 in rabbit embryos. Total RNA levels increase 23-fold and total cell number increases 78-fold during the same time period (Table 5). Because $\alpha$ subunit protein levels increase similarly to $\alpha$ subunit mRNA levels (22-fold versus 35-fold) between Day 4 and 6 in the rabbit embryo, it appears $\alpha$ subunit protein is partially regulated at the mRNA level.

**Conclusions**

Our results do not support the hypothesis that Na\textsuperscript{+}/K\textsuperscript{+} ATPase $\alpha$ subunit and $\alpha$ subunit mRNA content increase in a parallel manner before blastocoel formation in the mouse embryo. Because Na\textsuperscript{+}/K\textsuperscript{+} ATPase $\alpha$ subunit content in the mouse preimplantation embryo does not change, blastocoel formation must not be triggered by an increase in the $\alpha$ subunit content of the embryo. The increase in Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity at the morula stage reported by Vorbrodt et al., (1977) apparently is not regulated by the amount of $\alpha$ subunit present. Our results demonstrating a 9-fold increase in $\beta$ subunit content suggest that the $\beta$ subunit may be important in the regulation of Na\textsuperscript{+}/K\textsuperscript{+} ATPase activity and blastocoel formation. The increase in $\alpha$ subunit mRNA is partially accounted for by the increase in total RNA in the mouse embryo occurring at this time. The increase in $\alpha$ subunit mRNA we observed is not followed by a parallel increase in $\alpha$ subunit protein. This difference in $\alpha$ subunit mRNA and protein content may
be due to differences in total RNA and protein synthesis and the translational regulation of protein synthesis.

In contrast our data supports the hypothesis that Na⁺/K⁺ ATPase α subunit and α subunit mRNA content increase in a parallel manner during blastocoel expansion in the rabbit embryo. The amount of α subunit and α subunit mRNA increased in a similar pattern during Days 4 to 6 of rabbit embryo development. These data indicate that Na⁺/K⁺ ATPase is being regulated at the mRNA level during expansion of the rabbit blastocyst.
Bibliography


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Appendix: Details of Laboratory Protocols

RNA extraction from kidneys

1. Sacrifice animal and immediately remove kidneys.
2. Homogenize kidneys with 5 volumes of guanidinium solution:
   - 4 M guanidinium isothiocyanate
   - 5 mM Na citrate pH 7.0
   - 0.1 M B-mercaptoethanol
   - 0.5% sarcosyl
3. Sonicate homogenate 1 – 2 min at maximum setting.
4. Centrifuge for 10 min at approximately 2,000 – 4,000 x g.
5. Transfer supernatant to clean tube.
6. Add 11.2 g cesium chloride (CsCl) to each 15 ml of supernatant and adjust the volume to 28 ml with guanidinium solution (final concentration: 2.5 ml homogenate per g CsCl).
7. Layer 10 ml of a 5.7 M CsCl/EDTA (0.1M) sterile RNase free solution (The Pad) into the bottom of each new 38 ml centrifuge tube.
8. Layer the 28 ml of CsCl – guanidinium homogenate on top of the pad.
9. Centrifuge at 50,000 x g for at least 18 h.
10. Remove the brown supernatant from the pad with a Pasteur pipet. Change the pipet and remove the supernatant down to 1/3 inch from the bottom.

11. Cut off the centrifuge tube at about 2/3 inch and remove remaining supernatant with a sterile Pasteur pipet.

12. Resuspend the lenticular pellet in 200 - 800 ul (minimize the volume; e.g., 400 ul) of 1x TES (1x TES =10 mM Tris pH 7.5, 5 mM EDTA, 1% SDS) and transfer to a microcentrifuge tube.

13. Add 1/20 volume of 5 M NaCl (e.g., 20 ul).

14. Add GDAW (glass distilled autoclaved water) saturated phenol at 1/2 the original volume (e.g., 200 ul) and vortex 1 min.

15. Add 1/2 volume of chloroform (e.g., 200 ul), vortex 1 min and microfuge 3 min.

16. Transfer the aqueous top layer with a sterile Pasteur pipet (previously drawn to a fine tip) to a new microcentrifuge tube.

17. Add one volume (e.g., 400 ul) chloroform, vortex 1 min, microfuge 1 min and transfer the aqueous top layer to a new microcentrifuge tube.

18. Add 2.5 volumes (e.g., 1000 ul) of 100% ethanol and store at -70 C for at least 1 h (or overnight at -20 C).

19. Microfuge the ethanol RNA mixture for 15 min at 4 C. Remove the supernatant with a sterile fine tipped Pasteur pipet.

20. Add 70% ethanol at 3.5 times the original volume (e.g., 1400 ul).

21. Microfuge the ethanol/ RNA mixture for 5 min. Remove the supernatant with a sterile fine tipped Pasteur pipet.
22. Add 400 ul GDAW to the pellet and heat in 65 C water bath for 5 min with the lid off. Vortex. Heat 5 min at 65 C with lid on. Vortex. Heat 5 min at 65 C with lid on.

23. Microfuge the sample for 3-5 seconds. Store at -20 C (or -70 C) or use immediately.

**Preparation of poly (A)$^+$ RNA**

1. Equilibrate oligo-dT cellulose with loading buffer (20 mM Tris Cl, pH 7.5; 0.5 M NaCl; 1 mM EDTA; 0.1% SDS).

2. Pour a 1 ml column and rinse with 3 column volumes of A (GDAW), B (0.1 M NaOH, 5 mM EDTA) and C (GDAW) until the pH is below 8.

3. Rinse with 5 volumes of loading buffer.

4. Heat the sample to 65 C for 5 min and add equal volume of 2x loading buffer (2x loading buffer = 40 mM TrisCl, pH 7.5; 1M NaCl; 1mM EDTA; 0.2% SDS).

5. Apply the sample to the column at a flow rate of 1 drop/s. Apply the sample a second time.

6. Wash the column with 5 to 10 volumes of loading buffer.

7. Wash the column with four volumes of low salt buffer (20 mM Tris, pH 7.5; 1 mM EDTA; 0.1 M NaCl; 0.1% SDS).

8. Elute the poly (A)$^+$ RNA with 3 volumes of elution buffer (10 mM Tris, pH 7.5; 1 mM EDTA; 0.05% SDS).

9. Ethanol precipitate the RNA.

10. Rinse the column as in step 2 and 3.
11. Resuspend the RNA as in step 4.

12. Repeat steps 5 through 9.

Northern blotting

A. Agarose gel electrophoresis

1. Melt 2 g agarose with 132 ml of GDAW.

2. Cool agarose to 56 C in water bath.

3. Heat 18 ml of 10x MOPS buffer (10x=0.4 M morpholinopropanesulfonic acid, pH 7.0; 100 mM sodium acetate; 10 mM EDTA, pH 8.0) and 33 ml of formaldehyde to 56 C in water bath.

4. Add formaldehyde and MOPS buffer to agarose solution. Incubate at least 6 min at 56 C.

5. Pour the gel in a leveled casting tray and let solidify 1 h.

6. Add 31 ul of master mix (180 ul 10x MOPS buffer, 315 ul formaldehyde, 900 ul deionized formamide) to each 9 ul sample and incubate 15 minutes at 56 C.

7. Add 2 ul of loading buffer (50% glycerol, 1 mM EDTA, 0.4% bromophenol blue, 0.4% xylene cyanol) to each sample.

8. Move the solidified gel to the gel running box and add enough 1x MOPS buffer (1:10 dilution of 10x MOPS buffer) to just cover the surface of the gel.

9. Load the samples.
10. Run the gel at 75 V for about 8 h, or until the bromophenol blue dye is at the end of the gel. Recirculate the buffer.

B. Ethidium bromide staining of the RNA gel.

1. Cut off, mark, measure and remove the lane of gel containing the molecular weight markers.
2. Rinse the marker lane with GDAW at least 3 x 5 min and 1 x 30 min.
3. Add 75 ul of 10 mg/ml ethidium bromide stock solution to the gel in 250 ml GDAW and shake for 10 min.
4. Destain gel for 1 h to overnight in GDAW.
5. Photograph the stained gel with ultraviolet light, polaroid type 107c black and white film and a Cokin A.003 filter with a shutter speed of 3 s and aperture of 4.5.

C. Acridine orange staining of the RNA gel.

1. Mark and remove the entire gel from the gel box.
2. Rinse the gel two times with GDAW.
3. Stain with 33 ug/ml acridine orange in 10 mM NaPO₄, pH 6.5 for 10 min.
4. Destain 3 x 20 min in 10 mM NaPO₄, pH 6.5.
5. Photograph the gel with ultraviolet light.
6. After transfer, photograph the nitrocellulose with ultraviolet light.

D. Capillary transfer of RNA to nitrocellulose.

1. Cut 2 sheets of 3MM paper long enough to hang deeply into the buffer reservoir and wider than the gel, to serve as a wick. Flood the surface with 20x SSC (20x = 175.3 g NaCl, 88.2 g Na citrate, pH 7.0 in 1 liter GDAW).

2. Carefully invert and lay the gel on top of the wick. Remove air bubbles.

3. Place strips of waste film under the edges of the gel.

4. Cut the nitrocellulose to the same size and markings of the gel. Wet the nitrocellulose in GDAW and then soak in 20x SSC.

5. Flood the gel with 20x SSC and carefully lay the nitrocellulose on top of the gel. Remove air bubbles.

6. Flood with 20x SSC and lay 2 sheets of 3MM paper, cut to gel dimensions and presoaked in 20x SSC, on top of the gel. Then add a few sheets of dry 3MM paper.

7. Place a 3 inch stack of paper towels, cut to approximate gel dimensions, on top of the 3MM paper.

8. Place the plexiglass and weight on top of the paper towels and wait about 24 h.

9. After transfer, remove the nitrocellulose and dry under a heat lamp for 30 min.
10. Bake the nitrocellulose in a vacuum oven at 80°C for 2 h.
11. Seal it in a seal-a-meal bag and store it at 4°C until needed for prehybridization.

E. Prehybridization and hybridization of the probe to the nitrocellulose.

1. For prehybridization add 0.2 ml/cm$^2$ of 65°C Stark's buffer (prepared as in F) to the nitrocellulose in the bag. Remove air bubbles and reseal the bag. Prehybridize from 3 h to overnight at 42°C, palpating the bag occasionally.
2. After prehybridization, clip the bag and remove the excess Stark's buffer.
3. For hybridization, denature the radioactive cDNA probe (prepared as listed in the following section) by placing it in a boiling water bath for 5 to 10 min, followed by an ice bath for 5 min. Add the denatured probe to a solution of 4 parts Stark's buffer and 1 part 50% dextran sulfate. The volume of the hybridization buffer should be about 50 ul/cm$^2$ of nitrocellulose. Add the hybridization buffer to the blot, remove the air bubbles, double seal and incubate in 42°C water bath for about 24 h.

F. Preparation of Stark's buffer.
1. Proteinase K digest, sonicate, phenol-chloroform extract and ethanol precipitate 200 mg salmon sperm DNA.

2. Deionize 125 ml of formamide by stirring for 1 h with ion exchange resin. Filter the formamide through 3MM paper.

3. Mix 62.5 ml of 20x SSC, 8.3 ml of 25 mM NaPO₄ (pH 6.5), 5 ml of 50x Denhardt's solution (1 g Ficoll, 1 g polyvinylpyrrolidone, 1 g BSA in 100 ml GDAW) and 44.6 ml GDAW. Stir fast.

4. Add 4.6 ml of boiled salmon sperm DNA (13.5 mg/ml).

5. Add 125 ml of deionized formamide to fastly stirring solution.

G. Washing the northern blot and preparing the autoradiogram.

1. Remove the blot from the bag and rinse 4 x 5 min at room temperature in first blot wash (10% 20x SSC, 0.1% SDS).

2. Wash the blot 2 x 15 min at 50 C in second blot wash (0.5% 20x SSC, 0.1% SDS).

3. Blot the nitrocellulose with 3MM paper to remove excess moisture. Air dry.

4. Wrap the nitrocellulose in Saran Wrap and expose it to x-ray film at -70 C.

5. After exposure, develop the x-ray film.

6. Scan the resulting autoradiogram with a densitometer. Verify the linearity of the film response by analyzing a standard curve composed of decreasing amounts of total RNA from mouse or sheep kidneys.
Preparation of slot blots

1. Resuspend RNA samples in 15 ul TE. Add 10 ul of formaldehyde and 25 ul of deionized formamide.
2. Heat samples at 65 C for 10 min. Add 150 ul of 20x SSC and immediately load the sample onto the hybri-slot apparatus prepared as follows.
3. Saturate 4 sheets of 3MM paper and place on the filter support plate. Wet the nitrocellulose in GDAW and subsequently soak it in 20x SSC. Place the nitrocellulose on top of the filter paper.
4. Block the wells not in use with parafilm. Clamp the sample wells tightly into place and apply a low vacuum. Wash the wells with 500 ul of 20x SSC.
5. Apply the samples. Wash the wells with 500 ul 20x SSC.
6. Remove the nitrocellulose and handle as in Northern blotting, beginning with heating under a heat lamp for 30 min.

Preparation of radioactive Na+/K+ ATPase cDNA probe

A. Preparation of plasmid DNA.

1. Prepare T-broth (10 g NaCl, 10 g tryptone, 5 g yeast extract, pH to 7.5 with 1 M Tris and bring to 1 liter).
2. Streak an agar plate (T-broth with 15 g/liter agar) with the bacteria containing the recombinant plasmid and incubate at 37 C for 24 h.

3. Select an isolated colony and add it to 5 ml of T-broth containing 13 ug/ml tetracycline. Shake overnight at 37 C.

4. Add the 5 ml culture to 450 ml T-broth in a Fernbach flask and shake at 37 C. Check the optical density of the culture at 600 nm after 3h.

5. When the optical density is 0.5 to 0.7 add chloramphenicol to a final concentration of 170 ug/ml and shake overnight at 37 C.

6. Centrifuge the bacterial culture at 4,000 x g for 15 min at 4 C.

7. Resuspend the bacterial pellet in 100 ml of cold STE (10 mM Tris, pH 8.0; 0.1 M NaCl; 1 mM EDTA) and repeat the centrifugation.

8. Resuspend the bacterial pellet in 7.5 ml of cold solution I (75 mM glucose; 37.5 mM Tris, pH 8.0; 15 mM EDTA). Add 2.5 ml lysozyme (made fresh in cold 0.25 M Tris, pH 8, 20 mg/ml). Divide into 2-50 ml centrifuge tubes and let stand at room temperature for 5 min.

9. Add 10 ml of Solution II (0.2 N NaOH, 1% SDS) per tube. Mix by gently inverting the tube and let stand on ice 10 min.

10. Add 7.5 ml per tube of ice cold potassium acetate solution (60 ml of 5 M potassium acetate, 11.5 ml acetic acid, 28.5 ml GDAW). Mix contents and let stand on ice 10 min.
11. Centrifuge at 20,000 to 30,000 x g for 1 h at 4 C. Transfer equal quantities (9 ml) of the supernatant to 4 centrifuge tubes.

12. Add 0.6 volumes (5.5 ml) of isopropanol to each tube. Mix well and let stand at room temperature for 15 min or longer.

13. Recover the DNA by centrifuging the tubes at 11,000 x g for 10 to 30 min at room temperature.

14. Wash the pellet with 70% ethanol, spin 5 min.

15. Resuspend the pellet in the smallest convenient volume (1.5 ml/tube) of TE (10 mM Tris, pH 8.0; 1 mM EDTA) and pool the tubes.

16. Add 7.0 g CsCl to 7 ml of solution, mix.

17. Add 0.8 ml of 10 mg/ml ethidium bromide stock solution, mix. Transfer to an ultracentrifuge tube.

18. Spin at 106,000 x g for 36 - 48 h at 20 C.

19. Use long wave ultraviolet light to visualize the bands. Pipet off the band containing the supercoiled plasmid DNA. Transfer the supercoils to a fresh ultracentrifuge tube and add 0.5 ml of 10 mg/ml ethidium bromide stock solution. Top the tube off with CsCl solution (100 ml TE with 100 g CsCl).

20. Spin at 106,000 x g for 36 - 48 h at 20 C.

21. Pipet off the supercoiled plasmid DNA and transfer it to a centrifuge tube.

22. Extract the supercoils 3 - 5x with GDAW saturated butanol.
23. Transfer the aqueous phase to a fresh tube. Add 3 volumes of GDAW. Add 2.5 times the new volume of 95% ethanol. Precipitate at 20 C for 2 h to overnight.

24. Spin the ethanol precipitate at 10,000 x g for 15 min at 4 C. Wash the pellet with 70% ethanol.

25. Resuspend the pellet in 1 ml TAE (10 mM Tris, pH 8.0; 0.3 M Na acetate; 5 mM EDTA). Heat in 65 C water bath 5 min.

26. Add 2.5 volumes of 95% ethanol and precipitate at -20 C 2 h to overnight. Centrifuge for 15 min; wash pellet with 70% ethanol.

27. Resuspend the pellet in 400 ul TAE, heat in 65 C water bath. Transfer to a microcentrifuge tube.

28. Phenol - chloroform extract, chloroform extract twice , ethanol precipitate and 70% ethanol wash.

29. Resuspend the DNA pellet in 300 ul TE. Determine the DNA concentration by measuring the absorbance at A260 and A280.

B. Restriction digest of plasmid DNA and separation of the cDNA insert.

1. To 10 ug of plasmid DNA in 20 ul of TE add 5 ul of 10x buffer A (10x =100mM Tris, pH 7.5; 100 mM MgCl₂; 0.5 M NaCl), 5 ul of 10 mM dithiothreitol, 2 ul of 10 unit/ul PVU II, 2 ul of 10 unit/ul Bgl II and 16 ul of GDAW.

2. Incubate at 37 C for 2 h.
3. Add 1/5 volume stop dye (75 mM EDTA, 0.1% xylene cyanol, 0.1% bromophenol blue, 50% glycerol) and incubate at 65°C for 15 min.

4. Run the DNA in two wells on a 1% agarose gel.

5. Stain the gel with ethidium bromide. Plot the molecular weight markers and determine the molecular weights of the plasmid fragments.

6. Cut the band containing the desired fragment out of the gel with a razor blade.

7. Set up a gel casting tray with the comb in the center of the gel. Place the two bands of gel 1–2 mm before the comb and pour the gel.

8. After the gel has set, remove the comb and fill the wells near the gel fragment with Biogel HTP powder in 1x electrophoresis buffer (1 liter of 10x buffer contains 48.5 g Tris base, 16.4 g Na acetate, 8.63 g EDTA, pH to 8 with acetic acid; dilute this 1:10 for 1x electrophoresis buffer).

9. Run the gel at 100 V for about 4 h. View the gel with a long wave ultraviolet light to determine if all of the DNA has moved into the HTP powder.

10. Remove the HTP powder from the well and run it through an ion exchange column prepared as follows. To a siliconized Pasteur pipet add a small piece of glass wool and 1 cm of cation exchange gel (BioRad AG-50W-x8). Run one pipet volume of electrophoresis buffer through the pipet. Add Biogel P-60 gel to the neck of the pipet.
11. Layer the HTP powder on the column and rinse with electrophoresis buffer. When the column is dry elute the DNA with 250 ul of 1 M NaPO₄, pH 7.

12. Rinse the column with 250 ul of electrophoresis buffer.

13. Rinse the column with 400 ul of electrophoresis buffer and collect the eluant at this point into a 1.5 ml microcentrifuge tube.

14. Add 25 ug of yeast tRNA and 1/10 volume of 3 M Na acetate to the DNA eluant.

15. Phenol – chloroform extract, chloroform extract and ethanol precipitate the DNA.

16. Resuspend in 20 ul TE, and determine the DNA concentration by fluorometry or mini gel electrophoresis.

C. Radioactive labeling of cDNA with a random primer labeling kit.

1. Mix the DNA with GDAW such that 33 ul contains 20 – 100 ng of DNA.

2. Add 10 ul of 5x PT-C reaction buffer and vortex briefly.

3. Boil DNA in buffer for 3 min.

4. Place the DNA on ice and subsequently microfuge for 10 s.

5. Add 1 ul BSA mix, 1 ul Klenow (5 units) and 5 ul of 32 P – dCTP (3,000 Ci/mM, 10 mCi/ml).

6. Incubate at room temperature for 3.5 h.

7. Add 50 ul of Stop buffer, vortex briefly.
8. Heat at 65 C for 15 min.

9. Load sample onto D-50 spun columns and centrifuge for 4 min at 5,000 x g.

Western Blotting

1. Run protein samples on 10% polyacrylamide gels containing 0.1% SDS.

2. After electrophoresis, place the polyacrylamide gel and a piece of nitrocellulose of the same size in the blotting sandwich of a Western Genie Blotting apparatus.

3. Electrophoretically transfer the proteins from the gel to the nitrocellulose with a 12 volt battery charger for 35 min.

4. Remove the nitrocellulose from the apparatus and add 5% nonfat dry milk in PBS(0.39 g KH$_2$PO$_4$, 1.64 g K$_2$HPO$_4$-3H$_2$O, 8.5 g NaCl in 1 liter distilled water). Shake and incubate at least 30 min.

5. Pour off 5% nonfat dry milk and add the first antibody (rabbit anti-guinea pig Na+/K+ ATPase, 1:100 dilution in 0.1% nonfat dry milk). Shake and incubate at least 30 min.

6. Pour off the first antibody and wash 3 times for 5 min with 0.1% nonfat dry milk.

7. Add the second antibody (goat anti-mouse IgG conjugated to alkaline phosphatase, 1:2,000 dilution in 0.1% nonfat dry milk). Shake and incubate at least 30 min.
8. Wash the blot 3 times for 5 min in PBS.
9. Add the substrate (Vector II kit) and watch for the color reaction.
10. When the reaction is complete, wash the blot several times with distilled water.
11. Scan the Western blot with a densitometer.

**Preparation of mouse and rabbit kidney microsomes**

1. Sacrifice the animal and immediately remove the kidneys.
2. Mince the kidneys on ice.
3. Add 10 ml ice cold homogenization buffer (5% sorbitol; 5 mM histidine pH to 7.5 with 5 mM imidazole; 0.5 mM EDTA; 0.5 mM phenylmethylsulfonyl fluoride; 1 ug/ml leupeptin) per g of tissue.
4. Homogenize on ice.
5. Centrifuge the homogenate at 6,000 x g for 15 min, save the supernatant and the pellet.
6. Resuspend the pellet by homogenizing in the original volume of buffer.
7. Centrifuge at 6,000 x g for 15 min; save the supernatant.
8. Combine the supernantants and centrifuge at 48,000 x g for 30 min.
9. Resuspend the microsomal pellet in homogenization buffer to a final concentration of 5 - 20 mg protein/ml. Store at -20 or -70°C.