SODIUM-DIVALENT CATION EXCHANGE IN ERYTHROLEUKEMIA CELLS

by

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ABSTRACT

Magnesium and calcium are required for many cell functions. Accordingly, it is important that their intracellular levels be closely regulated for normal metabolism. Our understanding of the cellular control of these two cations is not completely clear. In this study we examined two separate transport systems, Na^+/Mg^{2+} exchange and Na^+/Ca^{2+} exchange, in three erythroleukemia cell lines. Intracellular free magnesium, $[Mg^{2+}]_i$, is in the order of 0.50 mM, allowing for Mg^{2+} to enter cells passively down an electrochemical gradient. Mg^{2+} exit from the cell, however, must be active and is thought to be mediated by a sodium-dependent mechanism. We developed an expression system to test this notion. Since most studies regarding Na^+/Mg^{2+} exchange have been performed in red blood cells, where Mg^{2+} affects volume regulatory processes, we used mRNA isolated from erythroleukemia cells for these expression studies. Poly $(A)^+$ RNA was microinjected into *Xenopus laevis* oocytes and incubated for 36-48 hours prior to assaying transport. Na^+/Mg^{2+} exchange was determined either by quantitation of radioisotopic ^{22}Na or by atomic absorption measurement of magnesium to assess influx and efflux, respectively. The observed transport was dependent on the amount of mRNA injected, with 50 ng resulting in maximal ^{22}Na influx. Magnesium efflux was dependent on the concentration gradient for magnesium across the oocyte membrane. Sodium-dependence of magnesium efflux was demonstrated by inhibition with amiloride and quinidine. These studies indicate that genetically encoded Na^+-dependent Mg^{2+} transport can be expressed in *Xenopus* oocytes. This approach may be employed to expression clone the cDNA coding the Na^+/Mg^{2+} exchanger protein.

The Na^+/Ca^{2+} exchanger plays an important role in maintaining cytosolic Ca^{2+} concentration. Reports are inconclusive as to whether erythrocytes express a Na^+/Ca^{2+} exchanger. The human (K-

562 and HEL) and mouse (GM979) erythroleukemia cell lines have been extensively used as model systems for studying intracellular Ca^{2+} involvement in cellular proliferation and differentiation. The present studies were designed to provide molecular evidence for the presence of Na^{+}/Ca^{2+} exchanger in these cells and to identify the molecular isoforms expressed. The cDNA coding the Na^{+}/Ca^{2+} exchanger contains an alternatively spliced site which determines, in part, tissue specific expression. The cDNA encodes seven different alternatively spliced isoforms containing combinations of exons A-F. Oligonucleotide primers were designed from conserved regions flanking the alternatively spliced region of the Na^{+}/Ca^{2+} exchanger. Homology based RT-PCR was then performed with mRNA from the three erythroleukemia cell lines. Cloning and sequencing of RT-PCR products from all three cell lines demonstrated the presence of a ~ 280 bp cDNA which represented the NACA3 isoform of the Na^{+}/Ca^{2+} exchanger, consisting of exons B and D. The B and D exons in K-562 and HEL cells were identical with those of the human B and D exons whereas the expressed exons identified in GM979 mouse cells shared 98% nucleotide and 95% amino acid sequence identity with the B and D exons of rat kidney cDNA. These results demonstrate the presence of Na^{+}/Ca^{2+} exchanger transcripts in human and mouse erythroleukemia cell lines and show that the alternatively spliced isoform expressed in these cells consists of exons B and D.

The role of these exchangers in diseases such a sickle cell disease and abnormal erythroid differentiation is unknown but would be better understood by identifying the protein(s) involved with transport.

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Key words: *Xenopus laevis* oocytes, expression, transport, Na^+/Mg^{2+} exchange, Na^+/Ca^{2+} exchange, Southern hybridization, poly $(A)^+$ RNA, cDNA sequence, alternative splicing, murine erythroleukemia (GM979) cells, human leukemia (K-562) cells, human erythroleukemia (HEL) cells, calcium, magnesium, atomic absorption

Abbreviations:

HEL	human erythroleukemia
GM979	mouse erythroleukemia
K-562	human leukemia
RT	reverse transcriptase
PCR	polymerase chain reaction
bp	base pair
$[Mg^{2+}]_i$	intracellular ionized magnesium
$[Ca^{2+}]_i$	intracellular ionized calcium
$[Mg^{2+}]_o$	extracellular ionized magnesium
$[Ca^{2+}]_o$	extracellular ionized calcium
RBC	red blood cell
Na^+/Mg^{2+} exchanger	sodium-magnesium exchanger
Na^+/Ca^{2+} exchanger	sodium-calcium exchanger
SCD	sickle cell disease
cTAL	cortical thick ascending limb
CHO	Chinese hamster ovary

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Chapter One Introduction

Magnesium and calcium play essential roles within the cell and influence many biological and physiological functions within the body. Intracellular Mg^{2+} is required for numerous metabolic functions such as activation of enzymes, especially those involved in phosphorylation and dephosphorylation reactions (e.g. ATPases, phosphatases, and kinases), stabilization of negatively charged substances like DNA or RNA, and protein synthesis (reviewed in 9,35). Furthermore, this divalent cation has been shown to regulate the activity of the Na/K/Cl (37) and K/Cl (28,72) cotransport systems which are involved in cell volume regulation, as well as influence flux through Ca^{2+}, K^+, Cl^-, and Na^+ channels (66). Likewise, intracellular Ca^{2+} also plays an important role in cell function by acting as an intracellular messenger, affecting many downstream enzymatic processes such as activation of kinases, and controlling channel activity (89,94). Finally, intracellular Ca^{2+} plays an essential role in erythroid cell proliferation and differentiation (44,59,74,81).

The diverse functions of magnesium and calcium are mediated, in part, via changes in the intracellular ionized concentrations of magnesium ($[Mg^{2+}]_i$) and calcium ($[Ca^{2+}]_i$) which are normally closely regulated within narrow limits. This regulation is dependent upon 1) the presence of molecules which bind magnesium and calcium in the cytoplasm e.g. Mg-ATP, 2) the movement of magnesium and calcium into and out of intracellular stores, and 3) the transport of these cations across the plasma membrane (9). Specialized transport systems must exist that are sensitive enough to maintain Mg^{2+} and Ca^{2+} homeostasis in the cell. These include putative Na^+/Mg^{2+} exchange (54), Na^+/Ca^{2+} exchange (11), and Ca^{2+}-ATPase (17). A Mg^{2+}-ATPase involved with Mg^{2+} transport has not yet been demonstrated (31).

1.1 Renal Handling of Magnesium and Calcium

The kidney is the major regulator of both magnesium and calcium homeostasis as it is responsible for reabsorption and excretion of filtered ions. In the proximal tubule, 20-25% of filtered magnesium is reabsorbed (Fig. 1). This compares with 65% of filtered calcium reabsorbed in the same segment. About 65% of filtered magnesium is reabsorbed (about twice that of calcium) within the thick ascending limb of the loop of Henle. This occurs passively through the paracellular pathway which is dependent on transepithelial voltage. The high degree of reabsorption in this segment reflects, in part, the increased delivery of magnesium to this segment compared with calcium. About 10% of filtered magnesium and calcium is reabsorbed within the distal tubule leaving approximately 1-3% that is excreted into the urine. Magnesium and calcium absorption within the distal tubule is thought to be active and transcellular in nature (29,98).

Calcium entry into polarized cells, such as epithelial cells composing the distal tubule, is passive and might be mediated by selective channels which move Ca^{2+} down a transmembrane electric gradient (Fig. 2) (98). A similar scenario is postulated for magnesium transport (23,92). $[Mg^{2+}]_i$ and $[Ca^{2+}]_i$, are about 0.5 mM and 100 nM, respectively, whereas the external concentrations are in the order of 0.75 and 2.0 mM, respectively (52). In addition to the existing chemical difference, the resting cell membrane voltage of -70 mV constitutes a large driving force for divalent cation entry. If Mg^{2+} or Ca^{2+} were allowed to distribute themselves at equilibrium with the membrane voltage, the intracellular concentration of Mg^{2+} would be ~200 mM with an extracellular concentration of 0.75 mM and that of calcium would be ~500 mM intracellularly and 2.0 mM extracellularly (2,9,114). Values are much lower than would be expected from a passive distribution of Mg^{2+} or Ca^{2+} across the cell membrane. Accordingly, specific cellular mechanisms must exist to

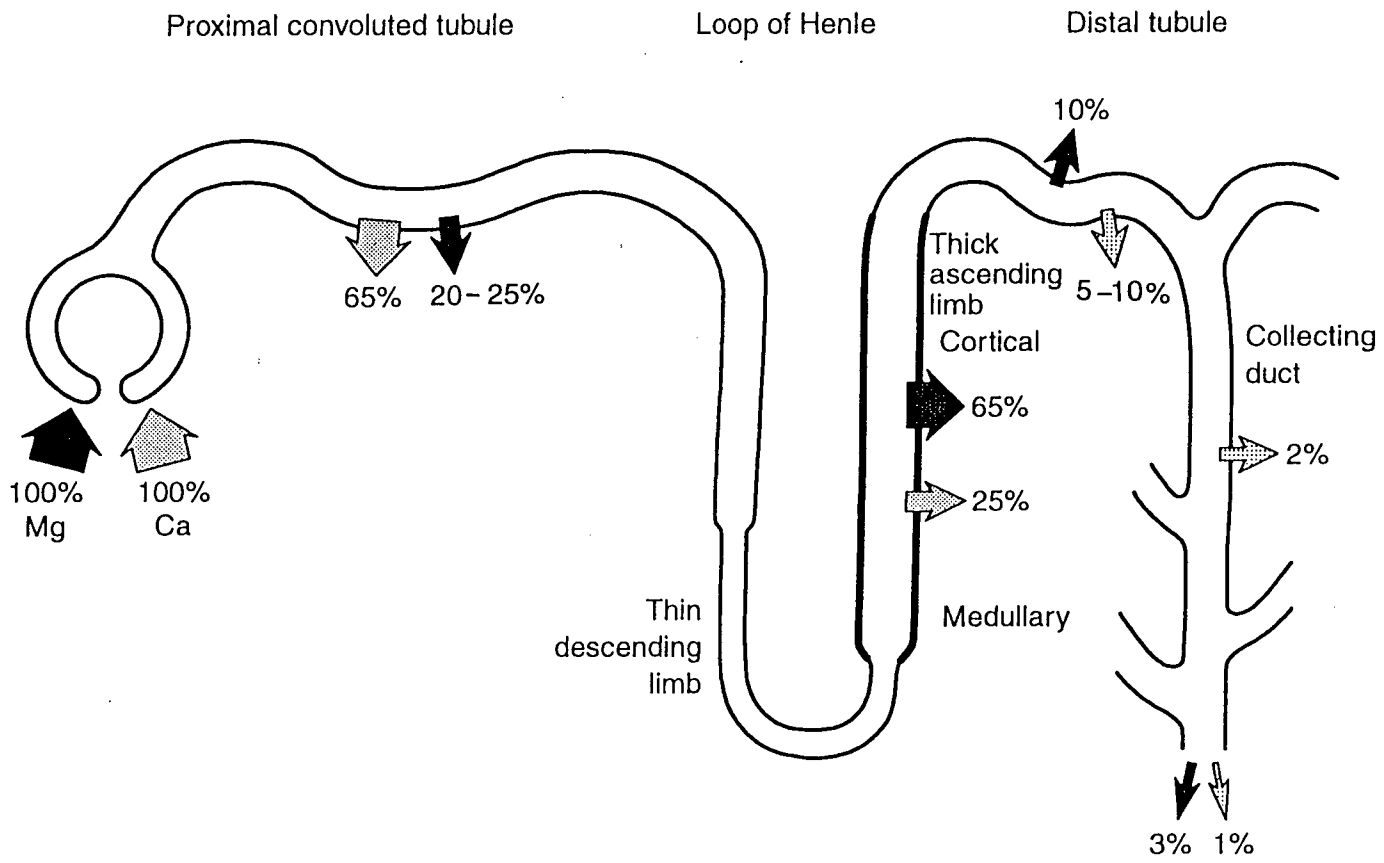

Fig. 1 Schematic representation of a nephron and the proportions of magnesium and calcium reabsorption in each segment. The majority of calcium reabsorption takes place in the proximal convoluted tubule whereas the bulk of magnesium is reabsorbed in the thick ascending limb.

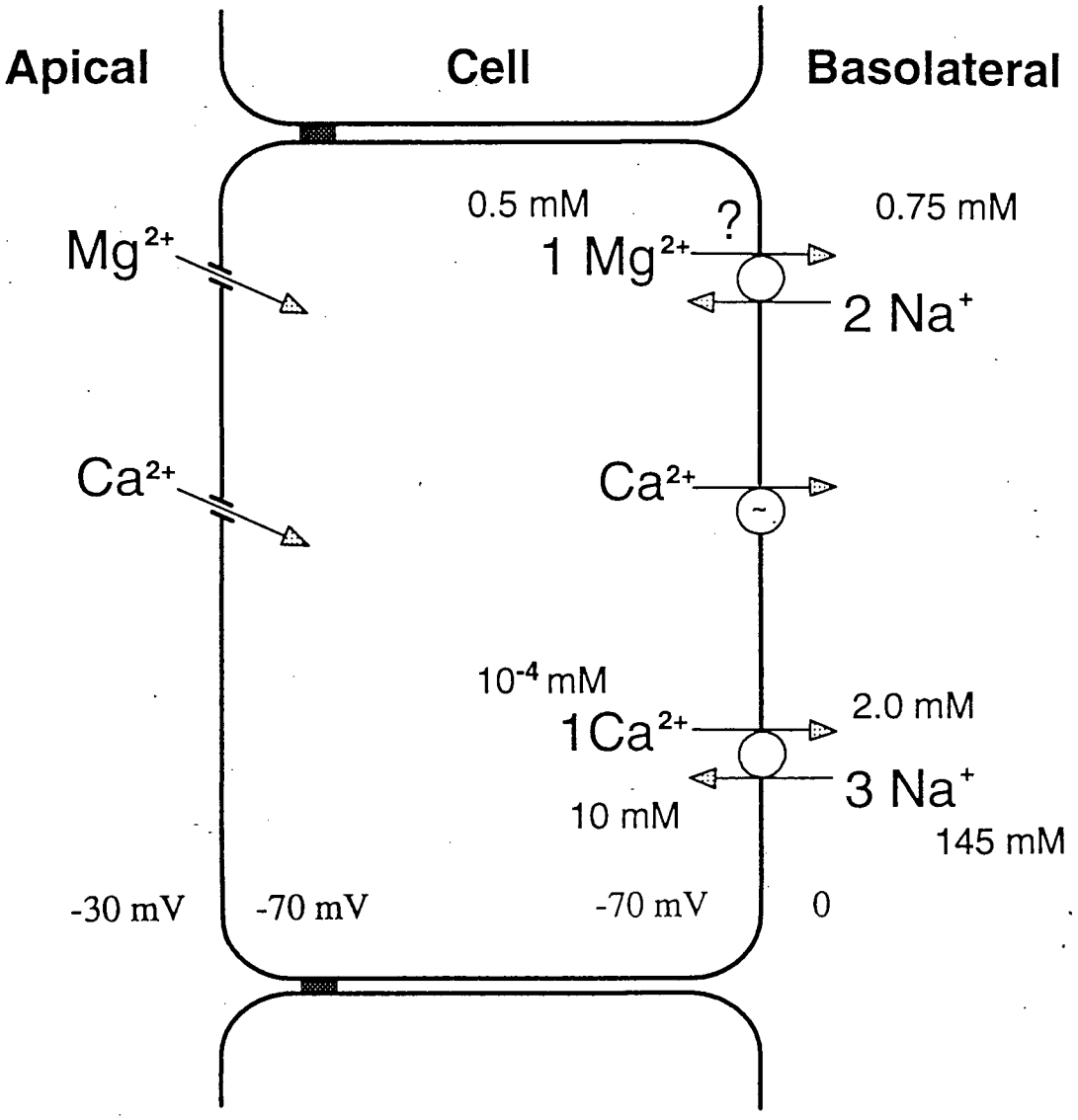

Fig. 2 Model of magnesium and calcium transport in polarized cells of the distal tubule. Both magnesium and calcium enter the cell through selective channels down an electrochemical gradient. They are then transported to the blood by basolaterally located exchangers. For calcium, a -ATPase exists or electrogenic transport can take place via exchange of $3Na^+$ for $1Ca^{2+}$. The putative Na^+/Mg^{2+} exchanger for magnesium transport is electroneutral, transporting two Na^+ for every Mg^{2+} transported out of the cell. Similar scenarios would be expected in non-polarized cells such as cardiomyocytes, smooth muscle cells, neurons, or red blood cells.

actively remove Mg^{2+} and Ca^{2+} from the cell. The presence of a sodium-magnesium (Na^+/Mg^{2+}) exchanger has been postulated (54). A sodium-calcium (Na^+/Ca^{2+}) exchanger located on the basolateral membrane of epithelial cells transports Ca^{2+} against its electrochemical gradient (98). In human and ferret erythrocytes, the membrane potential is approximately -10 mV (39,99), suggesting the electrochemical gradient for magnesium is slightly inward. Therefore, similar systems would be expected in nonpolarized cells such as cardiomyocytes, smooth muscle cells, neurons, or erythrocytes.

1.2 **Magnesium Transport**

Magnesium transport processes have not been well defined due to the unavailability of ^{28}Mg, the only radioactive isotope of magnesium. Sodium-dependent magnesium transport was first described in squid axon by Baker and Crawford (7) and, subsequently, by DeWeer (30). Employing radioisotopic ^{28}Mg, when it was available, they detected a transport system in squid axon which catalyzed ^{28}Mg efflux in the presence of cytosolic ATP and external Na^+. The putative Na^+/Mg^{2+} exchanger was later reported in chicken erythrocytes by Günther et al. (54) and in human red blood cells (RBC) by Féray and Garay (32). These groups employed atomic absorption to measure Mg^{2+} fluxes. Although many reports have implied the presence of sodium-dependent magnesium transport processes, none to date have provided molecular evidence for a Na^+/Mg^{2+} exchanger.

1.3 Na^+/Mg^{2+} Exchange in Erythrocytes

The most studied and apparently most active Na^+/Mg^{2+} exchanger is present in RBCs (36,50), where it has been detected in many species: birds (46,49,54), rats (33,55), ferrets (38,39), ground

squirrels (110), hamsters (110,111), and humans (32,40,78). Regulation of intracellular Mg^{2+} in erythrocytes is important, as cytosolic Mg^{2+} affects volume regulatory processes in these cells by controlling the swelling- and shrinkage-activated cotransporters, K/Cl and Na/K/Cl, respectively. Modulation of cell volume by $[Mg^{2+}]_i$ has been reported in many red cells including those of ferrets (37), ducks (105), and dogs (91). Increases in $[Mg^{2+}]_i$ inhibit K/Cl cotransport in sheep (28), human (15), duck (105), dog (91), and rabbit (64) erythrocytes, whereas decreases in $[Mg^{2+}]_i$ activates K/Cl efflux (28,72). Accordingly, high levels of free cytosolic Mg^{2+} will activate red cell Na/K/Cl cotransport. It would therefore be favourable for RBCs to posses an efficient means of tightly regulating $[Mg^{2+}]_i$. Abnormal control of $[Mg^{2+}]_i$ may lead to aberrant modulation of RBC volume and contribute to pathological disorders such as sickle cell disease (SCD) (27).

$[Mg^{2+}]_i$ changes may directly or indirectly affect ionic K/Cl and Na/K/Cl cotransport. Cellular Mg^{2+} concentrations affect K^+-channels, which may in turn be relevant in the modulation of volume activated K/Cl and Na/K/Cl cotransport. Magnesium modulation of the K/Cl and Na/K/Cl cotransporters might also be explained in terms of an equilibrium between phosphokinases and phosphatase reactions (63). This would be in keeping with the catalytic role of magnesium in many kinase reactions and some phosphatase reactions. During high $[Mg^{2+}]_i$, phosphorylation may activate Na/K/Cl cotransport and inhibit K/Cl flux. Similarly, the stimulation of K/Cl cotransport with Mg^{2+} depletion might involve an active unphosphorylated state. Further evidence to support this notion is that phosphatase inhibitors have an inhibitory effect on K/Cl cotransport (63). Consonant with these observations, magnesium inhibited K/Cl flux by 70% in low-K sheep red blood cells in the presence of ATP and not in ATP-depleted cells. This would suggest phosphorylation of a putative component occurs (28,88). On balance, the evidence supports the

notion that intracellular Mg^{2+} plays an important role in volume regulation of erythrocytes. The mode by which magnesium affects volume regulatory paths however, remains undefined at the present time.

1.4 Properties of a Putative Na^+/Mg^{2+} Exchanger

Na^+/Mg^{2+} exchange has been functionally characterized in erythrocytes from different species (32,53,102,106), as well as from ferret cardiac and skeletal muscle (10,12), human platelets (112), neuronal tissue (106), rat thymocytes and HL60 cells (51), rat sublingual acini cells (114), and rat hepatocytes (45). The exchanger demonstrates somewhat different properties and transport capacities in the different species and cell types. On balance, the system seems to be an amiloride, quinidine, and imipramine sensitive Na^+-dependent Mg^{2+} antiporter demonstrating ATP dependence, irreversibility, and electroneutrality. The exchanger is thought to be responsible for maintaining $[Mg^{2+}]_i$ below its electrochemical equilibrium in cells which are at physiological external sodium concentrations (53). We speculate that Na^+/Mg^{2+} exchange, such as that in the RBC, may also be present in epithelial cells such as those comprising the distal tubule of the nephron.

1.4.1 Cation specificity

All studies reported to date state that the putative Na^+/Mg^{2+} exchanger is highly selective for Na^+. Experiments were performed by loading RBC with Mg^{2+} and determining Mg^{2+} efflux into a buffer solution consisting of either Na^+ or other cations in substitution. In rat or chicken erythrocytes, replacement of extracellular NaCl by other monovalent cations such as Li^+, Cs^+, Rb^+, or choline$^+$ did not stimulate magnesium efflux (32,46,54,55). These cations were unable to

substitute for Na^+ in Mg^{2+} efflux. Further evidence demonstrating the dependence of magnesium transport on extracellular sodium comes from studies of depolarized chicken red cells placed in high K^+ media. K^+ did not support Mg^{2+} efflux (54). Additionally, in ferret red cells, extracellular Na^+ replacement by choline$^+$ or N-methyl-D-glucamine (NMDG) reduced magnesium efflux for 30 min, after which transport levels increased as a result of Na^+ leakage from the cell to the surrounding bath (38). Acid media also does not support magnesium efflux, thus ruling out a H^+/Mg^{2+} antiport (46). The evidence indicates that external Na^+ is necessary for Mg^{2+} efflux, supporting the postulate of a Na^+/Mg^{2+} exchanger.

The effect of extracellular divalent cations in addition to sodium has also been tested to determine whether magnesium efflux from hamster, human, and chicken red blood cells is inhibited. Extracellularly applied Mg^{2+} inhibited Na^+ influx and Mg^{2+} efflux, respectively, in hamster and chicken erythrocytes (54,111). Net Mg^{2+} efflux was not influenced by the presence or absence of extracellular Ca^{2+}, up to concentrations of 10 mM, (32,54,111) suggesting that transport is not associated with Ca^{2+} but is specific for Mg^{2+}. Other extracellularly applied di- and trivalent cations such as Sr^{2+}, Ba^{2+}, Be^{2+}, and La^{3+} inhibited Mg^{2+} efflux in human erythrocytes by only 5-15% (32). Mn^{2+}, however, caused significant inhibition of Mg^{2+} efflux and Na^+ influx in human, hamster and chicken Mg^{2+} preloaded red cells (32,54,111). At concentrations between 0.4 and 1 mM, Mn^{2+} inhibited efflux by 50% in human red cells and by approximately 70% at concentrations around 10 mM (32). In human cells, 90% inhibition of Na^+ influx was observed with 1 mM Mn^{2+} included in the incubation medium (111). In more recent studies in the rat, where conditions were designed to lead to reversed transport, Mg^{2+} moving in coupled to Na^+ moving out, Mn^{2+} was competitively taken up by the same transporter as Mg^{2+}, whereas Ca^{2+} was unable to substitute for Mg^{2+} (53). In chicken

erythrocytes, subsequent loading of Mg^{2+}-preloaded cells with Mn^{2+} also inhibited Mg^{2+} efflux (54). Overall, this data suggests that Mn^{2+} can competitively replace or inhibit Mg^{2+} in Na^+/Mg^{2+} exchange both for the internal and external sides and that no other divalent cations are able to do so.

1.4.2 Inhibitors

Although there is no specific inhibitor for Na^+/Mg^{2+} exchange, compounds which generally inhibit Na^+ transport mechanisms are modestly effective. This also provides further evidence for Na^+-dependence of the exchanger. Quinidine and amiloride at concentrations of 2 mM each affected sodium-dependent Mg^{2+} efflux in ferret red cells, inhibiting transport by 60-70% (38). One mM amiloride inhibited exchange in human erythrocytes by 60% and reversibly inhibited Mg^{2+} efflux in chicken RBCs (46,49,50,78). At concentrations between 0.1 and 1 mM, quinidine inhibited Mg^{2+} efflux from 60-80% in human erythrocytes (32). In hamster red cells, 5 mM amiloride caused maximal and essentially complete inhibition of Na^+ influx and Mg^{2+} efflux from Mg^{2+}-loaded cells (111). These compounds also were shown to inhibit Mg^{2+} uptake in rat RBC under reverse conditions i.e. magnesium influx and sodium efflux (53). Incomplete inhibition of Mg^{2+} efflux possibly resulted from additional Mg^{2+} efflux mechanisms, such as a Mg^{2+}-ATPase, Mg^{2+}-channels, or non-specific Mg^{2+} leak, or was due to the low affinity of these inhibitors for the exchanger. Additionally, at these high inhibitor concentrations the lack of selectivity of these drugs permits them to inhibit other Na^+ transport systems, such as Na^+/H^+ exchange and epithelial Na^+ channels (65) perhaps having indirect effects on Na^+/Mg^{2+} antiport.

Characterization of Na^+/Mg^{2+} exchange is hampered by the absence of potent and specific

inhibitory compounds. Féray and Garay tested the effects of tricyclic antidepressent drugs on human RBC Na^+/Mg^{2+} antiport (34). These studies were designed with the notion that Mg^{2+} plays a key role in depressive states and psychiatric disorders (4). The two most potent drugs were imipramine and dothiepine, with an IC_{50} of 25 μM and 40 μM, respectively. Non tricyclic antidepressant drugs such as Nomifensine and Trazodone were less potent in inhibiting exchange, demonstrating an IC_{50} of 0.4 mM or higher. These authors suggested that imipramine is a useful magnesium transport inhibitor as it appeared to be more selective than quinidine (IC_{50}=50 μM) (32), and could be used at concentrations where side effects on other Na^+ transport systems could be avoided (34).

Other types of inhibitors have also been employed to determine whether additional modes of transport may be responsible for sodium-dependent magnesium efflux. Treatment of human red cells with vanadate did not affect Mg^{2+} efflux (40). Since the concentrations of vanadate used effectively block other cation pumps, such as ATPases involved with Ca^{2+} or Na^+ transport, it was concluded that Mg^{2+} efflux was not due to a Mg^{2+} pump. Other drugs active on cation transport, such as ouabain (Na^+/K^+ pump inhibitor), furosemide and bumetinide (Na/K/Cl blockers), acetazolamide and DIDS (Cl channel blocker and anion exchange blockers) had no effect on Mg^{2+} efflux in human RBCs (32,38,46). This Mg^{2+} transport system is therefore different from typical cation pumps, K^+-cotransporters, or anion carriers.

1.4.3 ATP Dependence

Cellular ATP content has been reported to influence the activity of Na^+/Mg^{2+} exchange in red cells from a variety of species (32,40,54,78). Metabolic poisoning with potassium cyanide, iodoacetate, or dinitrophenol (dNP), as well as ATP depletion, by preincubation with 2 deoxyglucose

in place of glucose, have been shown to reduce Mg^{2+} efflux in magnesium-loaded cells by 20-85% (32,40,53,54,55,78). In Frenkel et al.'s studies using metabolically starved cells, ATP incorporation when resealing RBCs restored magnesium transport. Non-hydrolysable analogues of ATP had no restituting effect on magnesium extrusion (40). This suggested that sodium-dependent magnesium efflux is somehow dependent on intracellular ATP and that ATP hydrolysis may be necessary to activate transport .

Günther et al. performed preliminary studies which indicated the involvement of membrane-bound proteins in Mg^{2+} efflux (54). Accordingly, phosphorylation of these proteins may play a role in activating exchange (48). Many transport systems, such as the Na^+/H^+ exchanger (13) or the Na^+/Ca^{2+} exchanger (24), have been found to be controlled by phosphorylation/dephosphorylation reactions that serve to regulate their activation on a short term basis. DiPolo and Beaugé provided evidence in squid axons that ATP may perform a phosphorylative role in activating exchange (31). The ATP analog, ATPγS, which can be used to thiophosphorylate proteins by protein kinases but not by ATPases, was able to replace intracellular ATP. Xu and Willis found that incubation of Mg^{2+}-loaded hamster red cells with the serine/threonine selective protein kinase A (PKA) activator dibutyryl cAMP (db-cAMP), with the protein kinase C (PKC) promoter phorbol ester, or with the ser/thr protein phosphatase inhibitor okadaic acid did not cause any significant effect on the rate of amiloride-sensitive Na^+ influx (111). However, Günther and Vormann's recent studies with reversible Na^+/Mg^{2+} exchange in rat RBCs suggests that PKC may play a stimulatory role in Mg^{2+} transport. They found Mg^{2+} uptake to be stimulated by phorbol myrystate acetate (PMA) treatment, as an activator of PKC, and inhibited by staurosporine, a PKC inhibitor. These results are in contrast to those of Xu and Willis. Furthermore, db-cAMP was ineffective and okadaic acid had a only small

stimulatory effect (53). Overall, these observations suggest that stimulation of Na^+/Mg^{2+} exchange by a PKC pathway may be relevant. Phosphorylation/dephosphorylation events may regulate the Na^+/Mg^{2+} transporter or a protein involved with uptake, or increase binding or affinity of Mg^{2+} to a modified site of the antiporter (53). In summary, the function of ATP with respect to Na^+/Mg^{2+} exchange is, as of yet, undefined. It is clear however, that ATP is required for Na^+/Mg^{2+} exchange to take place in erythrocytes (32,39,40,54,78). Observations to date suggest that ATP most likely plays a phosphorylative regulatory role with respect to this transport sysytem. Cloning of the putative Na^+/Mg^{2+} exchanger will increase our understanding of how ATP affects this transporter.

1.4.4 Reversibility

The Na^+/Mg^{2+} exchanger is not a simple system as it does reverse cation transport in the presence of altered transmembrane gradients of sodium and magnesium (40,111). Early studies in human, rat, and chicken red cells demonstrated that the magnesium content did not change significantly when cells were incubated in media containing high magnesium concentrations, suggesting the inability to reverse transport (47,55,78). In these experiments $[Mg^{2+}]_i$ was low or not reported. Mg^{2+} efflux in rat or chicken erythrocytes was increased at higher $[Mg^{2+}]_i$, with saturation of Mg^{2+} efflux occuring at internal concentrations of 5 to 12 mM. No efflux was observed in chicken red blood cells at normal $[Mg^{2+}]_i$ (0.5 mM) (54,55). A gating mechanism might exist at the inner cell surface which is actived only at increased concentrations of internal Mg^{2+} (55). Günther and Vormann's most recent studies using rat erythrocytes suggested that in order to reverse Na^+/Mg^{2+} exchange intracellular Mg^{2+} was required (53). In the presence of intracellular Mg^{2+}, transport was dependent on the direction of the Na^+ gradient. This reversibility was also observed in ferret

erythrocytes, but was not detected in human RBCs (53). In summary, Na^+/Mg^{2+} antiport may be reversible in some species under controlled conditions. Physiologically, Mg^{2+} uptake possibly does not play a role in vivo because high $[Na^+]_i$ in combination with low $[Na^+]_o$ would never be observed in circulating RBCs or any other cells. The Na^+/Mg^{2+} antiporter is most likely present to actively transport Mg^{2+} out of the cell to maintain $[Mg^{2+}]_i$ homeostasis rather than oriented to increase Mg^{2+} entry (53).

1.4.5 Stoichiometry

The stoichiometric relationship of Mg^{2+} efflux with Na^+ influx is also controversial. Günther's work on chicken and rat RBCs suggests that efflux of one Mg^{2+} is coupled with uptake of two Na^+ (53,54,55). They used magnesium-loaded chicken red cells, in which intracellular sodium and magnesium were measured within the first 30 min. Cellular magnesium content was reduced by 1.5 mM, and cellular sodium content was increased by 3 mM. This suggested electroneutral transport with a 2:1 Na^+ to Mg^{2+} ratio. It was assumed that other significant sodium influx paths other than the Mg^{2+}-dependent one were absent under the experimental conditions. Dipolo and Beaugé employed voltage-clamp studies to demonstrate electroneutral transport in squid axons (31). Magnesium efflux was not affected with changes in membrane potential. Similarly, in hamster red cells, studies comparing Vmax values demonstrated a ratio of $2Na^+:1Mg^{2+}$, with the Vmax of Mg^{2+} efflux being about half that of Na^+ influx (111). These predictions are lower than that of Féray and Garay whose studies on human erythrocytes showed that Na^+ influx and Mg^{2+} efflux were coupled in a stoichiometry of about 3:1 (34). Frenkel et al. and Flatmann and Smith suggested transport with a stoichiometry of 1:1 in human and ferret red cells, respectively (38,40).

The stoichiometry of Na^+/Mg^{2+} coupling investigated so far ranges from 1/1 to 3/1 in different preparations and different species. Therefore, Na^+/Mg^{2+} exchange may demonstrate different properties depending on the cell type or tissue origin. Further studies are required to clearly define this parameter.

1.4.6 Objectives and Approach

In these studies, we attempted to express Na^+-dependent Mg^{2+} exchange in *Xenopus laevis* oocytes with the intention of later expression cloning the Na^+/Mg^{2+} exchanger. Poly $(A)^+$ RNA (mRNA) isolated from three erythroleukemia cell lines was injected into *Xenopus* oocytes prior to assaying for Na^+-dependent Mg^{2+} transport. Radioisotopic ^{22}Na measurements were employed in some of the experiments. Additionally, due to the absence of ^{28}Mg, we had to rely on atomic absorption measurements of Mg^{2+} efflux from cold magnesium. This method requires that cells express an abundance of protein in order for detectable amounts of transport to take place. In these studies, oocytes were loaded with magnesium and transport measured in the presence of sodium in the bathing solution. The exchange observed in the mRNA-injected oocytes was characterized to ensure that the transport expressed resulted from Na^+/Mg^{2+} exchange. Amiloride and quinidine were employed to demonstrate the sodium dependence of this exchanger (5).

A second objective in this study was to try to identify and clone the Na^+/Mg^{2+} exchanger through homology-based studies. Degenerate primers were designed from transmembrane regions of the Na^+/Ca^{2+} exchanger and from the Walker A sequence of the ATP-binding cassette (ABC) and used for RT-PCR. Furthermore, a probe was made from the Na^+/Ca^{2+} exchanger clone and employed to screen erythroleukemia cDNA libraries.

1.4.7 Rationale

Secondary active transport in the form of a Na^+/Mg^{2+} exchanger has been postulated to account for $[Mg^{2+}]_i$ being below its electrochemical equilibrium. Most studies to date have been performed on erythrocytes because this cell appears to express an abundance of exchanger protein. The Na^+/Mg^{2+} exchanger plays an important role in this cell type because changes in $[Mg^{2+}]_i$ affect volume regulatory mechanisms, such as K/Cl and Na/K/Cl cotransport. Although studies with RBCs strongly suggest the presence of a Na^+/Mg^{2+} exchanger, the properties of this exchange system remain to be fully elucidated. On balance, Na^+/Mg^{2+} exchange demonstrates Na^+-dependence of Mg^{2+} efflux that is sensitive to amiloride, quinidine, and imipramine. Exchange is specific for Mg^{2+} with the possible exception of Mn^{2+} which may replace Mg^{2+}. Finally, Na^+/Mg^{2+} transport is likely to be electroneutral, and is regulated by intracellular ATP levels.

Some reports have suggested that the Na^+/Mg^{2+} exchanger may structurally resemble the Na^+/Ca^{2+} exchanger and share some common properties (53). If this is true, one would expect that these two exchangers may also demonstrate similarity in amino acid sequence. Any similarity would most likely exist in areas of the Na^+/Ca^{2+} exchanger which are highly conserved among species, such as within the transmembrane regions. We also postulated that transport similar to the ATP binding cassette (ABC) family of transporters may account for magnesium translocation. In ABC transporters, two short sequences (Walker A and B) constitute a nucleotide binding pocket. The energy from binding and hydrolysis of ATP is used to transport substrates (77). There is the possibility that the Na^+/Mg^{2+} exchanger also functions in this manner, and that this mode of transport provides the driving force for the antiport.

1.5 Na^+/Ca^{2+} Exchange

Calcium transport processes are much better understood relative to magnesium transport. The Na^+/Ca^{2+} exchanger has been extensively described at both the functional and molecular levels. As the Na^+/Ca^{2+} exchange system is fairly well understood, only the important features of Na^+/Ca^{2+} transport will be reviewed only as it pertains to Na^+/Mg^{2+} exchange.

1.5.1 Na^+/Ca^{2+} Exchange in Erythroid Cells

Na^+/Ca^{2+} exchange plays a very important role in many cell types including epithelial cells of the nephron (93). Although somewhat controversial, Na^+/Ca^{2+} exchange is also thought to be present in erythroid cells. Gardner and Balasubramanyam reported that the human leukemia (K-562) cell line did not possess Na^+/Ca^{2+} exchange (43). Varecka and Carafoli have also ruled out the presence of a Na^+/Ca^{2+} exchanger system in human red blood cells (108). However, others have clearly shown that Na^+/Ca^{2+} exchange is present in differentiated red blood cells where it plays a significant role in Ca^{2+} transport (3,80,86,90). Control of intracellular Ca^{2+} concentrations in erythroid cells is important as it is thought to be involved in fundamental changes of red blood cell function, such as growth and differentiation (44,59,74,81). The early studies of Smith et al. showed that a Na^+/Ca^{2+} exchanger may function in reverse mode to increase cytosolic Ca^{2+}, a step which they postulated is essential for the commitment of erythroleukemia cells to terminal differentiation (102). Others have reported that differentiation results from increases in cytosolic Ca^{2+} achieved either by intracellular Ca^{2+} release and/or influx through Ca^{2+} channels (20,44). Whatever the means by which cytosolic Ca^{2+} is increased, Ca^{2+} signalling within erythroleukemia cells, and thus cell differentiation, will be influenced by expression of a Na^+/Ca^{2+} exchanger.

1.5.2 Molecular Cloning of the Na^+/Ca^{2+} Exchanger

Sodium-dependent transport of calcium was initially demonstrated in the squid giant axon (6). Following many functional studies, the Na^+/Ca^{2+} exchanger was ultimately cloned from the canine heart (83) and, subsequently, from other tissues and species (25,41,68,70,75,76,97,113). This exchanger comprises an amino terminal cleaved signal sequence, a short glycosylated extracellular region, a domain of five hydrophobic transmembrane segments, a long cytoplasmic loop, and finally a region of six transmembrane segments at the carboxy-terminal end (83) (Fig. 3). The Na^+/Ca^{2+} family is made up of three different members (NCX1, NCX2, NCX3) with 65-74% amino acid identity among them (84). The commonly expressed member is that related to the cardiac exchanger, NCX1. Although a single gene codes for the common form of the Na^+/Ca^{2+} exchanger found in diverse species and tissues, it is expressed as a number of different transcripts. This is due to alternative splicing in the coding sequence located within the cytoplasmic loop (69). Kofuji et al. postulated that the various isoforms could result from the presence of two mutually exclusive exons (A and B) in conjunction with four cassette exons (C-F) that together could allow for the generation of up to 32 possible distinct Na^+/Ca^{2+} exchanger transcripts (69). To date, restriction-enzyme protection analysis and sequencing data have revealed the existence of seven major isoforms: NACA1 (82), NACA2 and 3 (68,97), NACA4 and 5 (41), NACA6 (69), NACA7 (73) (Fig. 4). Lee et al. suggest the expression of Na^+/Ca^{2+} exchanger variants is regulated and influenced by different promoters in a tissue-specific fashion (73). Na^+/Ca^{2+} exchange is functional in both electrically excitable cells such as muscle and neuronal tissue, in addition to being present in nonexcitable cells such as the kidney (6,41,68,82,97). The presence of Na^+/Ca^{2+} exchange in erythroid cells is not yet

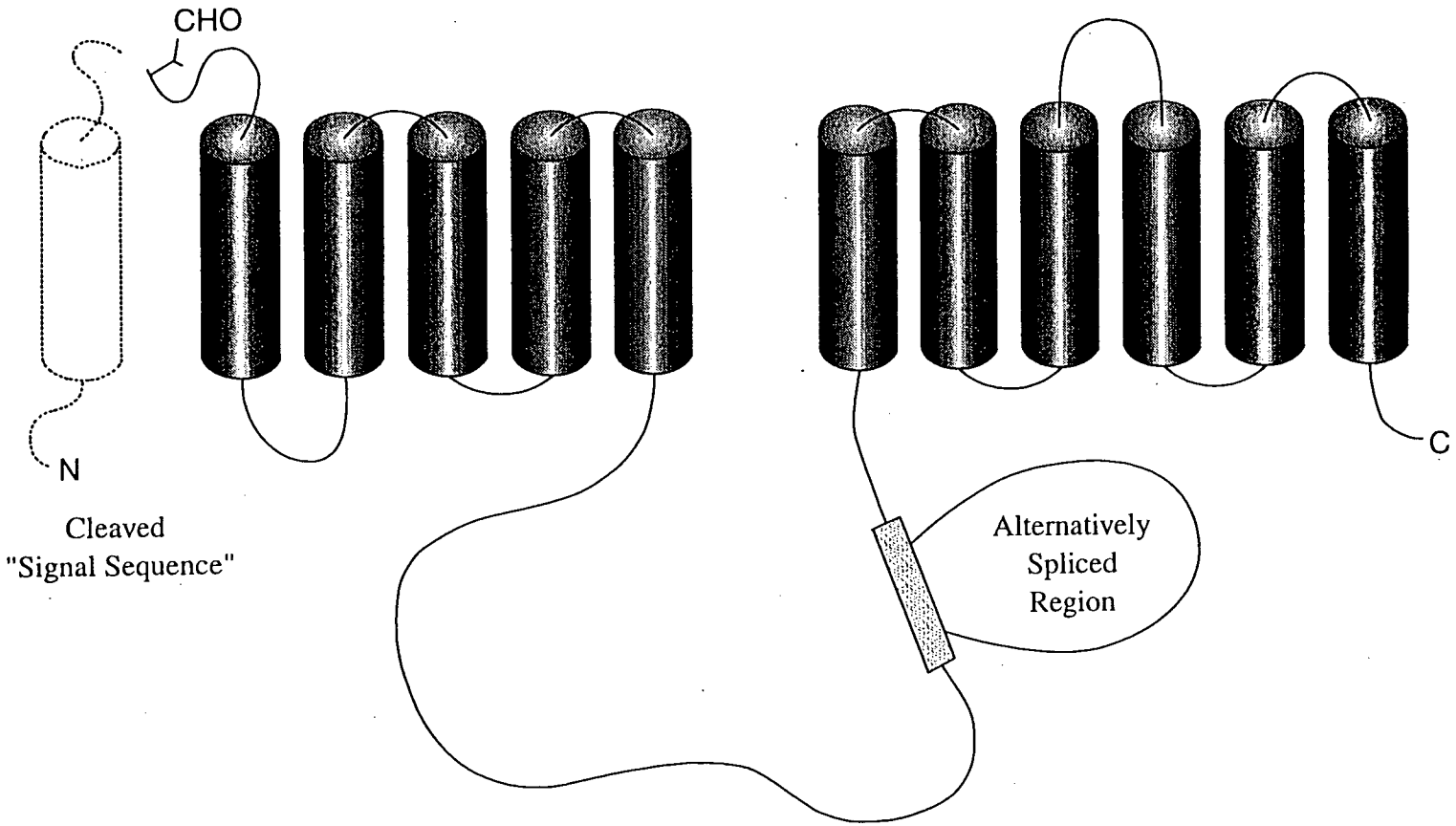

Fig. 3 A model of the Na^+/Ca^{2+} exchanger. The exchanger is composed of a cleaved N-terminal signal sequence followed by five transmembrane domains, a long cytoplasmic loop and another set of six transmembrane domains and a short carboxy tail. The N-terminal end is glycosylated. A portion of the cytoplasmic loop undergoes alternative splicing depending on the cell type, and may also act as a regulatory domain due the presence of potential phosphorylation sites.

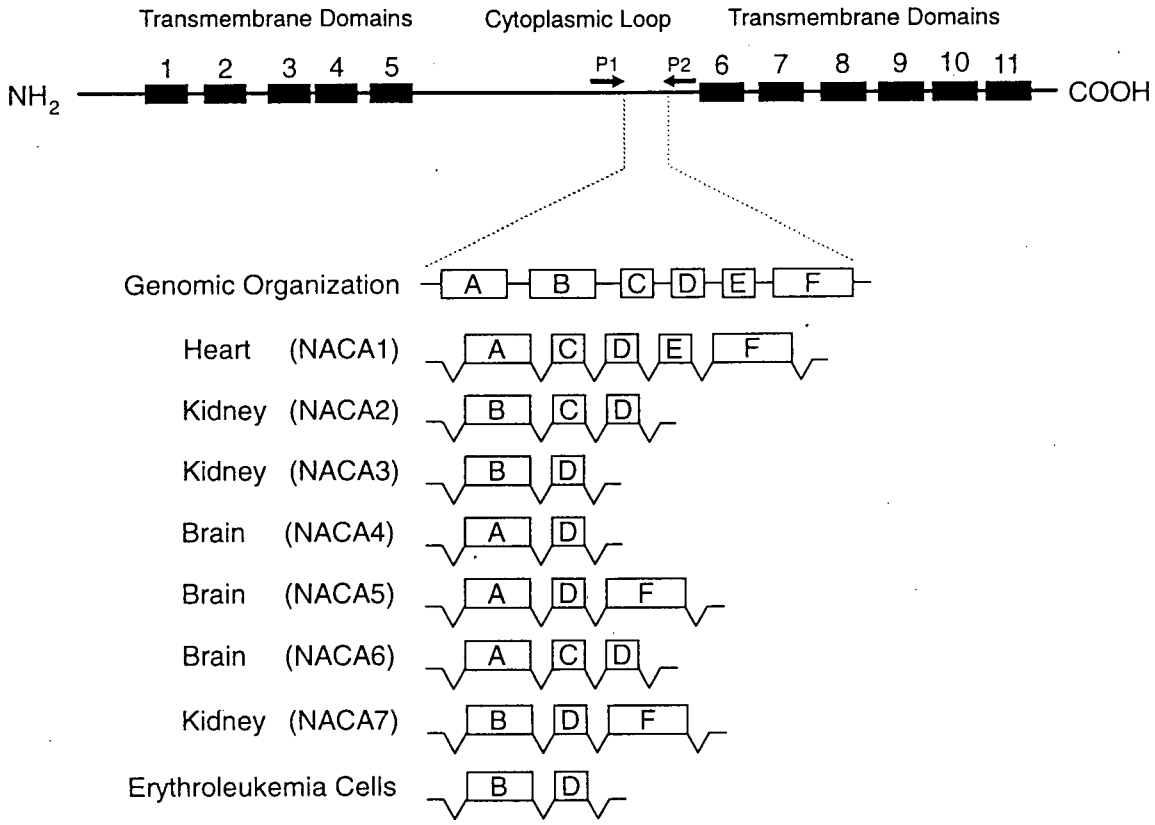

Fig. 4 Schematic representation of the Na^+/Ca^{2+} exchanger gene and the alternatively spliced isoforms. Transmembrane domains 5 and 6 flank a large cytoplasmic loop containing an alternatively spliced site. Primers, P1 and P2, were designed flanking this splice site and used to amplify cDNA from erythroleukemia cells. The variable region may be composed of various patterns of exons A-F to form the given Na^+/Ca^{2+} exchanger (NACA) isoforms. The isoforms of NACA were first isolated in order: NACA1 from dog (82), human (1,70), and rat (73) heart, NACA2 and NACA3 from rabbit kidney (68,96), NACA4 and NACA5 from rat brain (41), NACA6 from rabbit brain (69) and NACA7 from rat kidney (73). This data is, in part, from ref. 69. The NACA isoforms for erythroleukemia cells was isolated in the present study.

determined. The structural complexity of the single gene allows the Na^+/Ca^{2+} exchanger to respond independently to the unique demands of different environments. Accordingly, it is important to determine the expression of these isoforms to better understand their regulation and function.

1.5.3 Cation Specificity and Inhibitors

Both Na^+-dependent Ca^{2+} efflux and influx demonstrates specificity for Ca^{2+}. In renal basolateral membrane vesicles, about 50% inhibition of Ca^{2+} uptake, as measured with ^{45}Ca, was observed when Mn^{2+}, Ba^{2+}, or Sr^{2+} were applied to the intravesicular medium (62). Ba^{2+} and Sr^{2+} may function by serving as substrates for transport (65). Studies with arterial smooth muscle cells indicate that Mg^{2+} competed with Ca^{2+} for binding to the carrier, but was not itself transported (103). La^{3+} was the most potent inhibitor of Na^+-dependent Ca^{2+} transport in a number of systems, including the squid giant axon, where micromolar concentrations inhibited exchange (6,62,71). The inhibitors La^{3+} and Mg^{2+}, when applied extracellularly to cTAL cells, inhibited calcium movement in a sustained fashion upon readdition of extracellular Na^+ (25), suggesting irreversible inhibition perhaps though a conformational change of the exchange protein. In squid axon, Mn^{2+}, Co^{2+}, and Ni^{2+} are effective inhibitors of Na^+/Ca^{2+} exchange for both calcium uptake and efflux (6). The Na^+ selectivity of the Na^+/Ca^{2+} exchanger has also been demonstrated. Different monovalent cations, including Na^+, Li^+, K^+, Rb^+, and choline$^+$ inhibited Na^+/Ca^{2+} exchange (65,71). These studies indicate that Na^+/Ca^{2+} exchange is specific to both Na^+ and Ca^{2+} and is inhibited by cationic substitution of these substrates.

A variety of substances have been shown to inhibit Na^+/Ca^{2+} exchange but their usefulness is limited because they are only weakly effective or nonspecific (71). Amiloride or its analog, bepridil (sodium channel blocker), verapamil (calcium entry blocker), and quinacrine (sodium

transport inhibitor) inhibit Na^+/Ca^{2+} exchange at high drug concentrations (i.e. at mM concentrations) at which other sodium transport systems may also be inhibited (65). In spite of the lack of potent inhibitors to Na^+/Ca^{2+} exchange, this system has been well characterized, as its protein structure has been elucidated by cloning techniques.

1.5.4 ATP Dependence

Na^+/Ca^{2+} exchange, like Na^+/Mg^{2+} exchange, demonstrates ATP dependence. Upon ATP depletion of Chinese hamster ovary (CHO) cells or COS cells transfected with the bovine cardiac Na^+/Ca^{2+} exchange, the rate of rise in $[Ca^{2+}]_i$ with low external sodium was remarkably less than in ATP repleted cells. Ca^{2+} efflux was also decreased in these ATP-depleted cells under conditions of high external sodium (17). Therefore ATP depletion inhibits both Ca^{2+} influx and efflux modes of the Na^+/Ca^{2+} exchanger. Likewise, in heart myocytes (58) and smooth muscle cells (104), ATP depletion with metabolic inhibition reduced Na^+/Ca^{2+} exchange by more than 80%. This data clearly shows some ATP dependence of exchange function.

Regulation of Na^+/Ca^{2+} exchange in cardiovascular cells has received more attention than in epithelial cells (95). There is evidence that protein kinase-dependent phosphorylation is responsible for stimulation of the exchange activity induced by Mg-ATP in heart sarcolemma (18). Phorbol esters, 8-bromoguanosine 3',5'-cyclic monophosphate, norepinephrine, and platelet-derived growth factor (PDGF) stimulate Na^+/Ca^{2+} exchange activity in smooth muscle cells (42,61,95,109). These findings suggest that phosphorylation of the exchanger or associated ancillary proteins may be involved in activation of Na^+/Ca^{2+} exchange in cardiovascular cells. Recently, Iwamoto et al. (61) have shown that PDGF activates exchange through phosphorylation of multiple sites of the exchange

protein, which supports the notion of a direct action on the exchanger. However, other studies performed in CHO cells or COS cells transfected with the bovine cardiac Na^+/Ca^{2+} exchange failed to detect changes in phosphorylation or exchange activity with inhibitors of protein kinases or phophatases (22). In cortical thick ascending limb (cTAL) cells, calmidazolium, a Ca^{2+}-calmodulin inhibitor, decreased exchange activity whereas okadaic acid, a phophatase inhibitor, increased the activity (24). Further studies are necessary to implicate phosphorylative mechanisms in the modulation of Na^+/Ca^{2+} exchange activity.

1.5.5 Reversibility and Stoichiometry

Under normal circumstances, extracellular Na^+ enters in exchange for cytosolic Ca^{2+}, however, depending on the prevailing electrochemical gradient for Na^+, Ca^{2+} may move into the cell increasing cytosolic Ca^{2+} concentration (100). In renal distal tubule membranes, the activity of Na^+/Ca^{2+} exchange was a function of the sodium gradient such that Ca^{2+} uptake by vesicles loaded with 150 mM NaCl decreased progressively as the concentration of Na^+ in the incubation medium was increased (62,93,107). Reeves and Hale suggested that electrogenic transport occured with a stoichiometric ratio of 3 Na^+ to 1 Ca^{2+} in the exchange process (96). Accordingly, the membrane potential also acts as a driving force for net Ca^{2+} movement via the Na^+/Ca^{2+} exchanger. Renal basolateral Na^+/Ca^{2+} exchanger demonstrated Ca^{2+} efflux under conditions of negative membrane potential and Ca^{2+} influx with positive membrane potentials (62,107). In cTAL cells of the nephron transmembrane depolarization also induced a reversible Na^+-dependent Ca^{2+} influx which was dependent on the transmembrane Na^+ concentration and voltage gradients (25). Therefore, it is the coupled movement of sodium ions down their electrochemical gradient that provides the energy

required to move Ca^{2+} out of the cell against its electrochemical gradient.

In summary, Na^+/Ca^{2+} exchange is specific for both Na^+ and Ca^{2+} and is inhibited by sodium transport inhibitors such as amiloride and quinidine, and Ca^{2+} entry blockers such as verapamil. This transport system is modulated through phosphorylation by intracellular ATP, and demonstrates 3 Na^+ : 1 Ca^{2+} transport stoichiometry.

1.5.6 Objectives and Approach

The present studies were designed to determine the expression of Na^+/Ca^{2+} exchanger in three commonly used erythroleukemia cell lines and to characterize the isoform coding the exchange protein. To fulfill these two objectives a homology based RT-PCR cloning strategy and DNA sequencing was employed. PCR primers employed were designed to flank the alternatively spliced region of a rat Na^+/Ca^{2+} exchanger cDNA.

1.5.7 Rationale

Erythroleukemia cells have been the subject of extensive studies as one of the best models for terminal differentiation (8,57,60,79,84). Upon exposure to inducing agents which result in increases in cytosolic Ca^{2+}, these cells exhibit all the characteristics of erythroid cells, including the loss of growth proficiency (60,84). Na^+/Ca^{2+} exchange plays a significant role in maintaining cytosolic Ca^{2+} concentrations in many cell types. In cells of erythroid origin, Na^+/Ca^{2+} exchange may either play a role in elevating intracellular Ca^{2+} concentrations or returning intracellular Ca^{2+} levels to normal following an increase in Ca^{2+} prior to differentiation. Expression of Na^+/Ca^{2+} exchanger in these undifferentiated erythroleukemia cells may also reflect characteristics of mature

RBCs. It would therefore be of relevance to determine whether the Na^+/Ca^{2+} exchanger is present in these cells.

Chapter Two Materials and Methods

2.1 RNA isolation

Human leukemia (K-562) cells were cultured in RPMI 1640 medium (Stem Cell Technologies Inc.; Vancouver, B.C.) supplemented with 10% fetal calf serum (FCS) (GIBCO/ BRL; Grand Island, NY) and an antibiotic mixture (PSN; 50 μg penicillin, 50 μg streptomycin, 100 μg neomycin/100 ml media) (Stem Cell Technologies Inc.; Vancouver, BC) in a humidified atmosphere of 95% O_2/5% CO_2 at 37 °C. Human (HEL) and mouse (GM979) erythroleukemia cells were grown in DMEM NF-12 medium (Stem Cell Technologies Inc.; Vancouver, B.C.) supplemented with 10% FCS and the above mentioned antibiotics. The cells were washed with phosphate-buffered saline (PBS) and poly $(A)^+$ RNA extracted using the Poly A Tract System 1000 (Promega; Madison, WI). mRNA was stored at -80 °C at a working concentration of 1.5 μg/μl.

2.2 Expression of Na^+/Mg^{2+} Exchange

2.2.1 Isolation of oocytes

Adult *Xenopus laevis* were anesthetized with 1.5 gm/l (5.7 mM) 3-aminobenzoic acidethyl ester (Tricaine) (Sigma; St. Louis, MO) and several lobes of the ovary were removed. Fully grown oocytes (1.2-1.3 mm diameter, stages V and VI) were selected after removal of the follicular cell layer by treatment with collagenase (3 mg/ml, type II, Sigma; St. Louis, MO) in ORII buffer [containing (in mM): NaCl, 82.5; KCl, 2.0; $MgCl_4$, 1.0; HEPES/Tris, 10; pH 7.4] for 3 hr at 19 °C with gentle continuous agitation. The oocytes were subsequently washed extensively with ORII buffer and the remaining follicular cells manually stripped off. Oocytes were maintained for 2-5 days in ND96 solution [containing (in mM): NaCl, 96; KCl, 2.0; $CaCl_2$, 1.8; $MgCl_2$, 1.0;

HEPES/Tris, 5.0; pH 7.4] containing 2.5 mM Na pyruvate and gentamicin (5 μg/ml) (Sigma; St. Louis, MO) at 18 °C with continuous gentle agitation and daily changes of solution. All studies were performed following this equilibration period.

2.2.2 Oocyte injection

Microinjection of mRNA into oocytes was performed through the use of micropipettes controlled by a minipump (Hampel, Frankfurt, Germany). In a typical experiment, oocytes were injected with 50 nl of water containing 30-50 ng of mRNA. Negative controls included oocytes injected with water alone. Injected oocytes were incubated at 18 °C for 36-48 hr in 24-well microtiter trays in a total volume of 1 ml. The incubation media was changed daily prior to atomic absorption determinations or radioisotopic ^{22}Na measurements.

2.2.3 Determination of Na^+/Mg^{2+} Exchange

Following a 36-48 hr incubation period, *Xenopus* oocytes, immersed in a LiCl bath, were loaded with 10 nl of a 1 M Mg^{2+} solution to a final cytoplasmic concentration of 15 mM Mg^{2+} (assuming a volume of 700 nl/ oocyte). Two methods were employed to demonstrate Na^+/Mg^{2+} exchange: $^{22}Na^+$ influx and Mg^{2+} efflux. For $^{22}Na^+$ influx, magnesium-loaded oocytes were reimmersed in a solution of $^{22}NaCl$ (96 mM) and incubated for 12 min. Serial washes in ND96 were then performed to dilute $^{22}NaCl$ which remained in the bath solution. Oocytes were transferred to scintillation vials and the counts per minute measured using a Beckman LS 6500 multi-purpose scintillation counter (Fullerton, CA). Alternatively, Mg^{2+} efflux was measured from the bath solution 10-30 min after immersion of 3-10 magnesium-loaded oocytes into 60 ul of a 96 mM NaCl

solution. Atomic absorption measurements (Perkin-Elmer 2380 Atomic Absorption Spectrophotometer; Norwalk, CT) of the bathing solution were taken at a wavelength of 285.2 nm to determine magnesium extrusion into the bathing solution.

2.2.4 Inhibition of transport

Amiloride and quinidine (Sigma; St. Louis, MO) were diluted in dimethyl sulfoxide (DMSO) (BDH Laboratory Supplies; Poole, England) to final concentrations of 5.0 mM and 4.0 mM, respectively, and used to inhibit transport. They were added directly to the NaCl bath in which oocytes were placed following magnesium loading. $^{22}Na^{+}$ uptake measurements were then taken to determine Na^{+}/Mg^{2+} exchange.

2.3 Molecular Studies of the Putative Na^{+}/Mg^{2+} Exchanger

2.3.1 Design of PCR Primers

This approach was designed with the notion that a certain degree of homology may exist between the Na^{+}/Mg^{2+} and Na^{+}/Ca^{2+} exchangers and that the Na^{+}/Mg^{2+} exchanger may contain an ATP-binding cassette (ABC) which accounts for its ATP dependence. An array of degenerate primer pairs were constructed from several areas of conserved sequence within given transmembrane domains of the NCE. F1 cDNA (kindly provided by Dr. J. Lytton; University of Calgary, Alberta, Canada) (73). Primers were also designed from the Walker A sequence of the ABC (77). These primers were synthesized by the Nucleic Acid Service Laboratory (N.A.P.S.), Biotechnology Laboratory, UBC.

2.3.2 Homology based RT-PCR

PCR amplifications were performed using either mRNA from the three erythroleukemia cells lines or cDNA from the K-562 library subsets isolated through Qiagen (Qiagen Inc.; Chatsworth, CA). 1.5 μg mRNA was reverse transcribed for 50 min at 42 °C in a reaction mixture consisting of 25 mM oligo dT or 1 μl downstream primer, 1X first strand buffer, 10 mM DTT, 0.5 mM dNTP mix, and 20 units/μl Superscript II (GIBCO/BRL; Grand Island, NY). The reaction was terminated by heating to 99 °C for 5 min. 2 μl of this first strand cDNA or 1 μl of cDNA from the library was added to the PCR reactions. PCR samples contained 1X PCR buffer, 0.1 mM dNTP mix, 2.25 mM $MgCl_2$, 625 mM of downstream and upstream primers, and 1.25 U *Taq* DNA polymerase (GIBCO/BRL; Grand Island, NY) in a total volume of 40 μl. Amplification with the primers was performed using a programmable thermal cycler (Perkin Elmer Gene Amp PCR system 2400) as follows: hot start at 94 °C prior to addition of cDNA; 35 cycles of 94 °C 30 s, 42 °C 45 s, and 72 °C 1min 10 s; and then 72 °C 7 min preceeding cool down to 4 °C.

2.3.3 Southern analysis

PCR products were separated on a 0.8% agarose gel and visualized by ethidium bromide staining. They were transferred by downward capillary transfer to Genescreen (NEN Research Products; Boston, MA) (21), and the blots were crosslinked in a UV Stratalinker (Stratagene; San Diego, CA). A probe was made using the NCEF.1 cDNA cloned from rat kidney (73). The probe, consisting of 100 ng ECOR1 cut cDNA, 1X Klenow buffer, 1X CGT nucleotide mix, 100 pmol random hexamer, 1 U Klenow, and 50 μCi [^{32}P]-dATP (ICN; Montreal, QUE) in a final reaction volume of 20 μl, was incubated at 37 °C for 30 min and purified with a Sephadex G-50 column.

Membranes were prehybridized at 40 °C for 30 min in a solution of 1% bovine serum albumin (BSA), 30% formamide, 7% sodium-dodecyl sulfate (SDS), and 350 mM sodium phosphate (NaP) solution. Labelled probe was added directly to the prehybridization solution and the membranes hybridized at low stringency (40 °C) overnight. The blots were then washed for 10 min in 150 mM NaP/0.1% SDS at 21 °C and exposed on Kodak X-OMAT film for 20 hr at -80 °C.

2.3.4 Cloning and Sequencing of PCR products

1 µl of fresh PCR product was cloned using the TA cloning kit (InVitrogen; San Diego, CA). Colonies were transferred to Hybond-N^+ (Amersham, UK) nylon membranes. Colony lysis and binding of DNA to the membranes was accomplished by placing the membranes, in order, on Whatmann 3MM paper (Whatmann International Ltd.; Maidstone, England) soaked with 10% SDS for 3 min, denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 5 min, neutralizing solution (1.5 M NaCl, 0.5 M Tris.Cl) for 5 min, and 2 X sodium citrate (SSC) for 5 min. The membranes were then crosslinked in a UV Stratalinker (Stratagene; San Diego, CA). They were subsequently probed, washed, and exposed as previously described in section 2.3.3. Possible positive clones were picked and streaked. Recombinant colonies were selected by blue white selection on LB plates (GIBCO/BRL; Grand Island, NY) containing 50 ng/ml ampicillin (Sigma; St. Louis, MO). Plasmids were isolated from seemingly positive recombinants by overnight minipreps with a midi Qiagen kit (Qiagen Inc.; Chatsworth, CA). Alkaline lysis plasmid preparations, followed by digestion with the restriction enzyme ECOR1 (GIBCO/BRL; Grand Island, NY) and electrophoresis on a 0.8% agarose gel, was employed to confirm cDNA insert size. The clones were sequenced with M13 forward and reverse primers at the N.A.P.S. Laboratory.

2.3.5 Screening of cDNA Libraries

K-562 and GM979 erythroleukemia cDNA libraries were kindly supplied by Dr. Keith Humphries and Dr. Robert Kay, respectively (Terry Fox Lab, B.C. Cancer Research Center; Vancouver, B.C.). These libraries were titrated and plated at appropriate dilutions onto LB plates (Gibco/BRL; Grand Island, NY) containing 50 μg/ml ampicillin (Sigma; St. Louis, MO), 7.5 μg/ml tetracyclin (Sigma; St. Louis, MO) for the GM979 library and 25 μg/ml amp, 10 μg/ml tet for the K-562 library. Colonies were transferred to Hybond-N^+ (Amersham, UK) nylon membranes. The membranes were thereafter handled as in section 2.3.4.

2.4 **Na^+/Ca^{2+} Exchange**

2.4.1 Design of PCR primers

Non-degenerate oligonucleotide primers were designed based upon highly conserved domains of the rat kidney NCEF.1 cDNA (73). The primers spanned the alternatively spliced region of the exchanger transcript, with the sequence of the external primers being 5' GAGGGGAGGATTTTGAGGACACT 3' and 5' AGGGCCAGGTTTGTCTTCTTAAT 3' and the internal primers for the nested PCR reaction comprised 5' CTCGAATTCCAGAATGATGAAAT 3' and 5' CTCTTGAATTCGTAAAATTCTTC 3'.

2.4.2 RT-PCR amplification

RT-PCR was carried out as previously described in section 2.3.2. For these experiments however, external primers were employed in the primary PCR reaction at an annealing temperature of 58 °C. PCR products were reamplified with the internal primers using 1 μl of the initial product

in a second 40 μl reaction at an annealing temperature of 47 °C.

2.4.3 Southern analysis

PCR products were treated as in section 2.3.3 with the exception being that membranes were prehybridized and hybridized at 55 °C. Furthermore, the blots were washed for 10 min at 21 °C and 15 min at 55 °C prior to exposure on film.

2.4.4 Cloning and sequencing of PCR products

1 µl of fresh PCR product was cloned using the TA cloning kit (InVitrogen; San Diego, CA). Recombinant colonies were selected by blue white selection on LB plates (GIBCO/BRL; Grand Island, NY) containing 50 ng/ml ampicillin (Sigma; St. Louis, MO). Plasmids were isolated from overnight minipreps with a midi Qiagen kit (Qiagen Inc.; Chatsworth, CA). Alkaline lysis plasmid preparations, followed by digestion with the restriction enzyme ECOR1 (GIBCO/BRL; Grand Island, NY) and electrophoresis on a 0.8% agarose gel, was employed to confirm cDNA insert size. The clones were sequenced with M13 forward and reverse primers at the N.A.P.S. Laboratory.

Chapter Three Results

3.1 Na^+/Mg^{2+} Exchange

3.1.1 RBC mRNA expression

Xenopus laevis oocytes have long been known to efficiently express proteins when injected with exogenous RNA or DNA (101). During oogenesis, oocytes accumulate large quantities of enzymes, storage proteins, and organelles, which form a reserve for use during early embryonic development. In our study, 0-80 ng of mRNA isolated from the human leukemia (K-562) cell line, mouse erythroleukemia (GM979) cell line, or human erythroleukemia (HEL) cell lines were injected into stage V-VI *Xenopus laevis* oocytes. Oocytes were incubated for 36-48 hours, allowing adequate time for translation of foreign mRNA and expression of protein to take place on the oocyte plasma membrane, prior to assaying for transport. These studies were directed at demonstrating the expression Na^+-dependent Mg^{2+} transport in oocytes. Oocytes were either magnesium-loaded (15 mM final concentration) or not-loaded (~ 0.5 mM Mg^{2+}) and ^{22}Na influx and magnesium efflux determined. Magnesium efflux, as determined with atomic absorption, was associated with the presence of mRNA with about 3 times more transport taking place in mRNA-injected compared to water-injected oocytes (Fig.5). Using the other approach, maximum ^{22}Na transport was detected with 50 ng of mRNA injected for which transport was 5.3-times greater compared to control values. Smaller amounts of mRNA were associated with less uptake showing that transport was dependent on the amount of mRNA injected (Fig.6). Injecting 80 ng of mRNA had no additional effects when compared to 50 ng (Fig.6). Na^+ transport from mRNA injected Mg^{2+}-loaded oocytes was significantly greater than from those not loaded with magnesium (Fig.6), demonstrating the dependence of transport on Mg^{2+}. The high transport in Mg^{2+}-loaded versus non-loaded oocytes

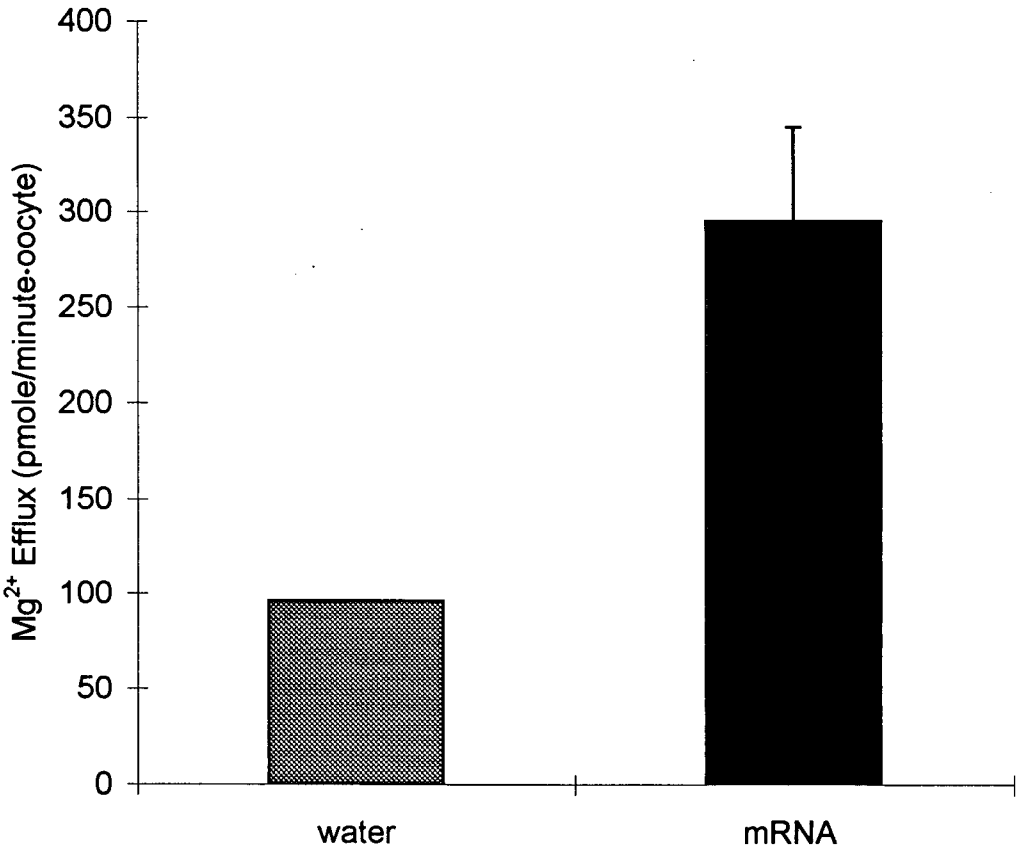

Fig. 5 mRNA dependence of Mg^{2+} efflux. Oocytes injected with water or 50 ng of RBC mRNA were incubated for 36-48 hours. Following magnesium loading to a final intraoocyte concentration of 15 mM, Mg^{2+} efflux was measured. Atomic absorption measurements were recorded at a wavelength of 285.2 nm following a 30 min incubation in sodium-containing medium. N=2 experimental groups of 10 oocytes each for water-injected oocytes. Data is represented as mean +/- SEM, N=5 experimental groups for mRNA-injected oocytes.

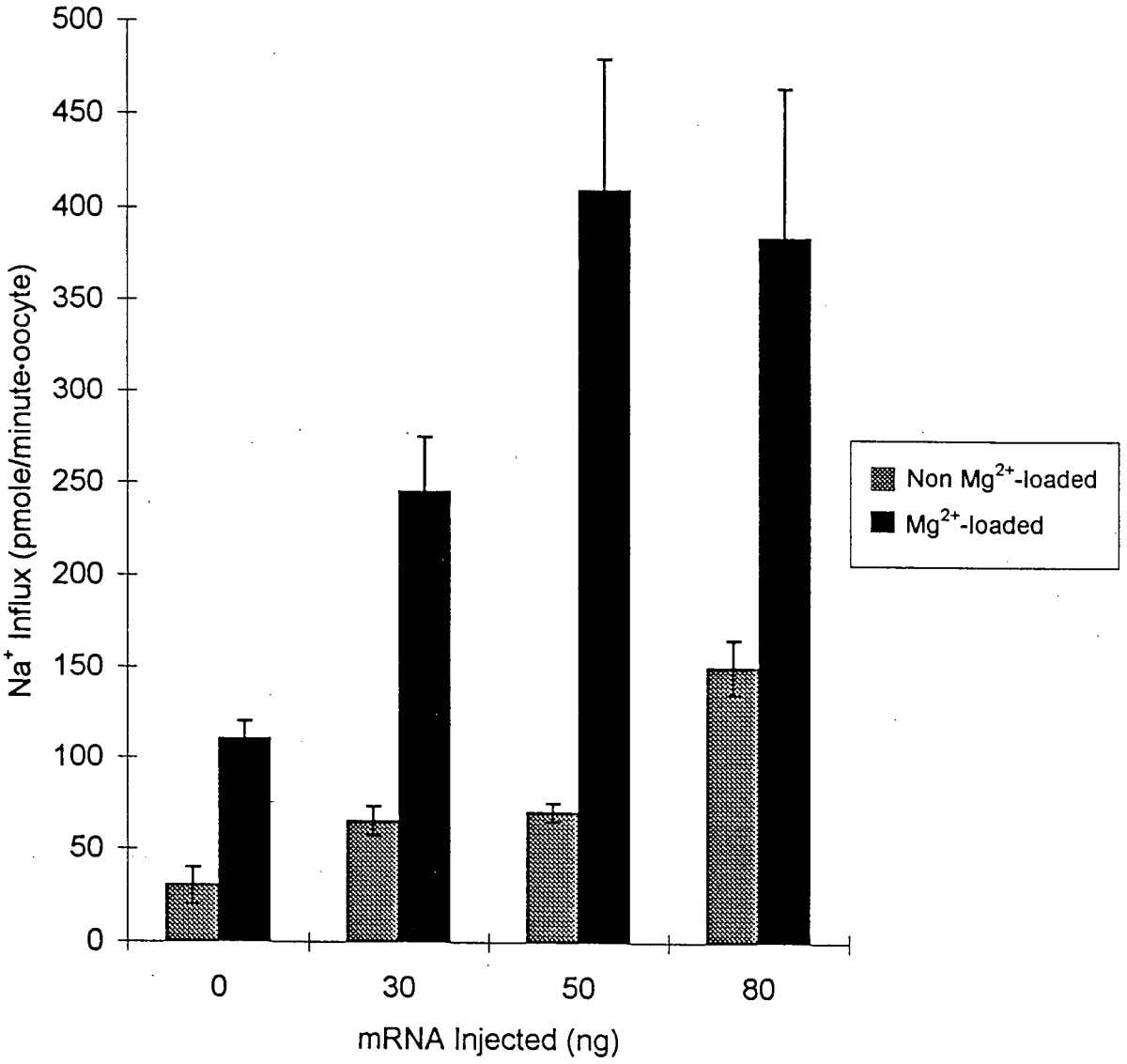

Fig. 6 mRNA concentration dependence of Mg^{2+}-dependent Na^+ influx. 0-80 ng of mRNA was microinjected into *Xenopus* oocytes to determine whether transport was concentration dependent. mRNA- or water-injected oocytes were incubated for 36-48 hours prior to assaying for Mg^{2+}-dependent ^{22}Na transport. Oocytes were placed in a NaCl bathing solution containing ^{22}Na and the radioisotope measured through scintillation counting. Hatched bars represent Na^+ transport in non magnesium-loaded oocytes. Solid bars represent Na^+ transport in magnesium-loaded (9.5 mM, final) oocytes. Transport is reported as pmole Na^+/minute•oocyte. Each bar represents mean +/- SEM, N=10 oocytes.

demonstrates that the transport observed is due to Na^+/Mg^{2+} exchange and not a result of other Na^+ influx paths. Interestingly, the high background level in non-magnesium loaded oocytes and non-mRNA injected oocytes might suggest the presence of endogenous transport (Fig.5 and Fig.6). Oocytes may possess endogenous magnesium transport systems such as Mg^{2+}-channels, a Mg^{2+}-pump, or a Na^+/Mg^{2+} exchanger. Transport may be overexpressed with injection of mRNA isolated from the erythroleukemia cells. Alternatively, magnesium measured in the efflux bath may be accounted for by non-specific magnesium leak from the oocyte.

3.1.2 Effect of Intraoocyte Magnesium

The effect of the intraoocyte magnesium concentration on magnesium-stimulated Na^+/Mg^{2+} exchange was investigated. Oocytes injected with 50 ng mRNA were loaded to differing concentrations of magnesium, from 0.5 mM (not loaded, endogenous $[Mg^{2+}]_i$) to 25 mM, to determine whether the transmembrane magnesium gradient affected transport. In this study, 5-6 oocytes were assayed in a volume of 60 ul. The maximal increase in magnesium-dependent Na^+ transport (stimulated minus background) was observed with about 15 mM intraoocyte magnesium (Fig. 7a). At this concentration of magnesium, about a 6-fold increase in ^{22}Na transport was observed in comparison to endogenous values (300 pmol/min•oocyte with 15 mM magnesium versus 50 pmol/min•oocyte in 0.5 mM magnesium). Furthermore, a single experiment demonstrated that magnesium efflux increased 4-fold with 10 mM intraoocyte magnesium, and 2.5 fold with 15 mM compared to endogenous transport values (Fig. 7b).

3.1.3 Sodium Dependence of the Exchanger

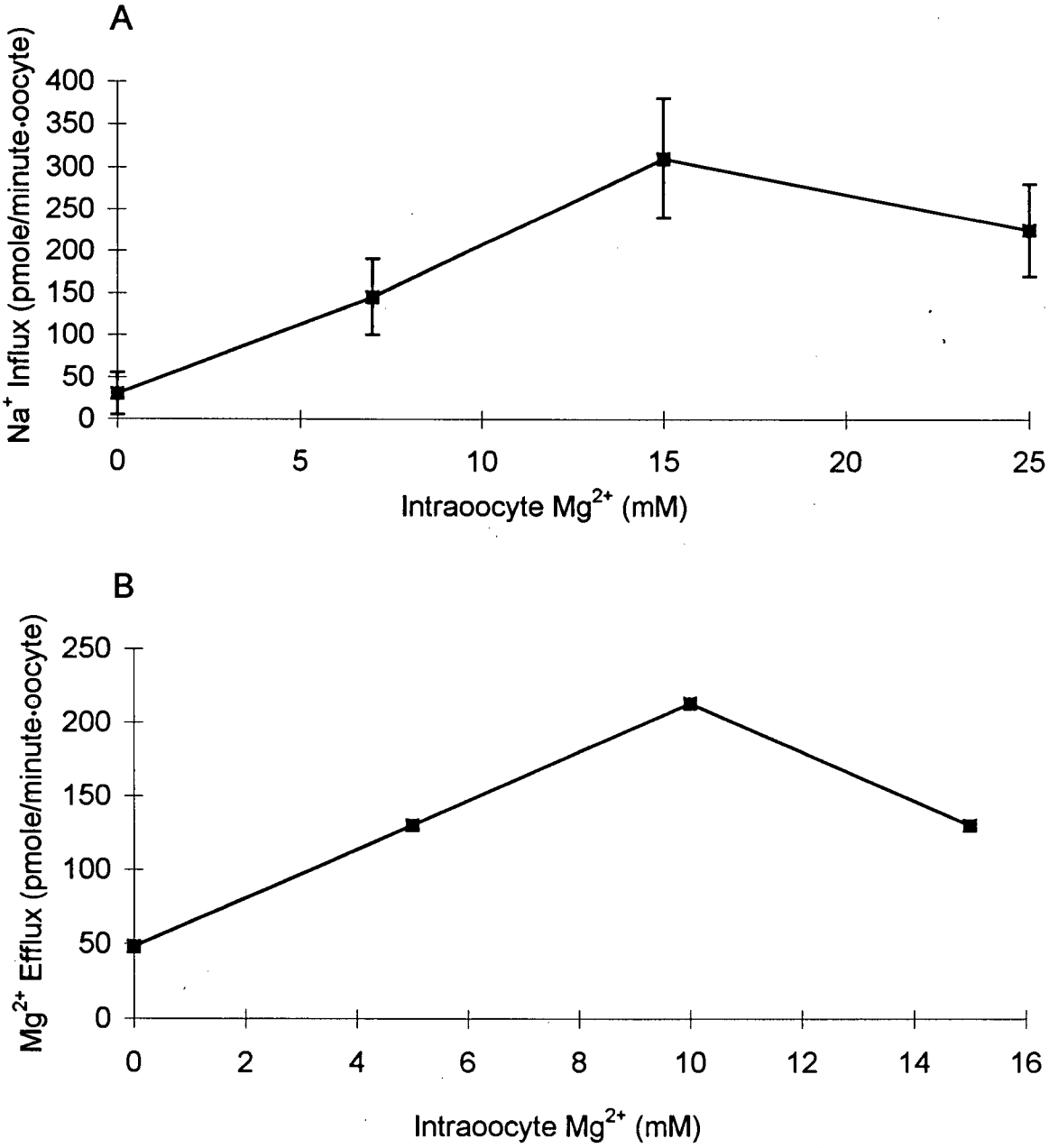

Fig.7 Effect of the membrane concentration gradient for Mg^{2+} on ion transport. mRNA-injected oocytes were loaded with different concentrations of magnesium (0 25 mM final intraoocyte concentration). Magnesium and ^{22}Na transport was measured as described in Fig. 5 and Fig. 6. A) Values for Na^+ influx represent the mean +/- SEM (stimulated minus background), N=10 oocytes for each data point. B) Mg^{2+} efflux measurements represent one experimental group.

We were interested to determine whether transport was inhibited with sodium transport agonists. Amiloride and quinidine at concentrations of 5.0 mM and 4.0 mM, respectively, were added to the oocyte bath solution (ND 96), and incubated for 10 min prior to assaying for ^{22}Na uptake. Both compounds inhibited transport by more than 50%, essentially reducing transport to control uptake values (475 pmole/min•oocyte vs 225 and 250 pmole/min•oocyte for quinidine and amiloride, respectively) (Fig. 8). Endogenous transport did not appear to be affected by treatment with these compounds.

3.1.4 Stoichiometry

A comparison of sodium influx measurements to those for magnesium efflux from separate experiments, revealed an approximate transport ratio of 1.65 Na^+ to 1 Mg^{2+} (Fig. 9). This suggested electroneutral transport; i.e. two Na^+ for one Mg^{2+}. Further electrophysiological studies are required to confirm these studies.

3.2 Na^+/Ca^{2+} Exchanger and ABC Homology-Based Studies of the Na^+/Mg^{2+} Exchanger

Screening the K-562 and GM979 cDNA libraries with a NCE.F1 (69) ^{32}P-labelled probe was unsuccessful because all the colonies on the membrane hybridized probe, whether they contained an insert or not. This pattern may be the result of vector sequences being recognized by the probe at the low stringency conditions employed.

Homology-based PCR employing Na^+/Ca^{2+} exchanger and ABC primers with cDNA derived from the libraries was also unsuccessful. Low stringency conditions were employed; accordingly, many PCR products were amplified and subsequently cloned and sequenced. Most of the clones

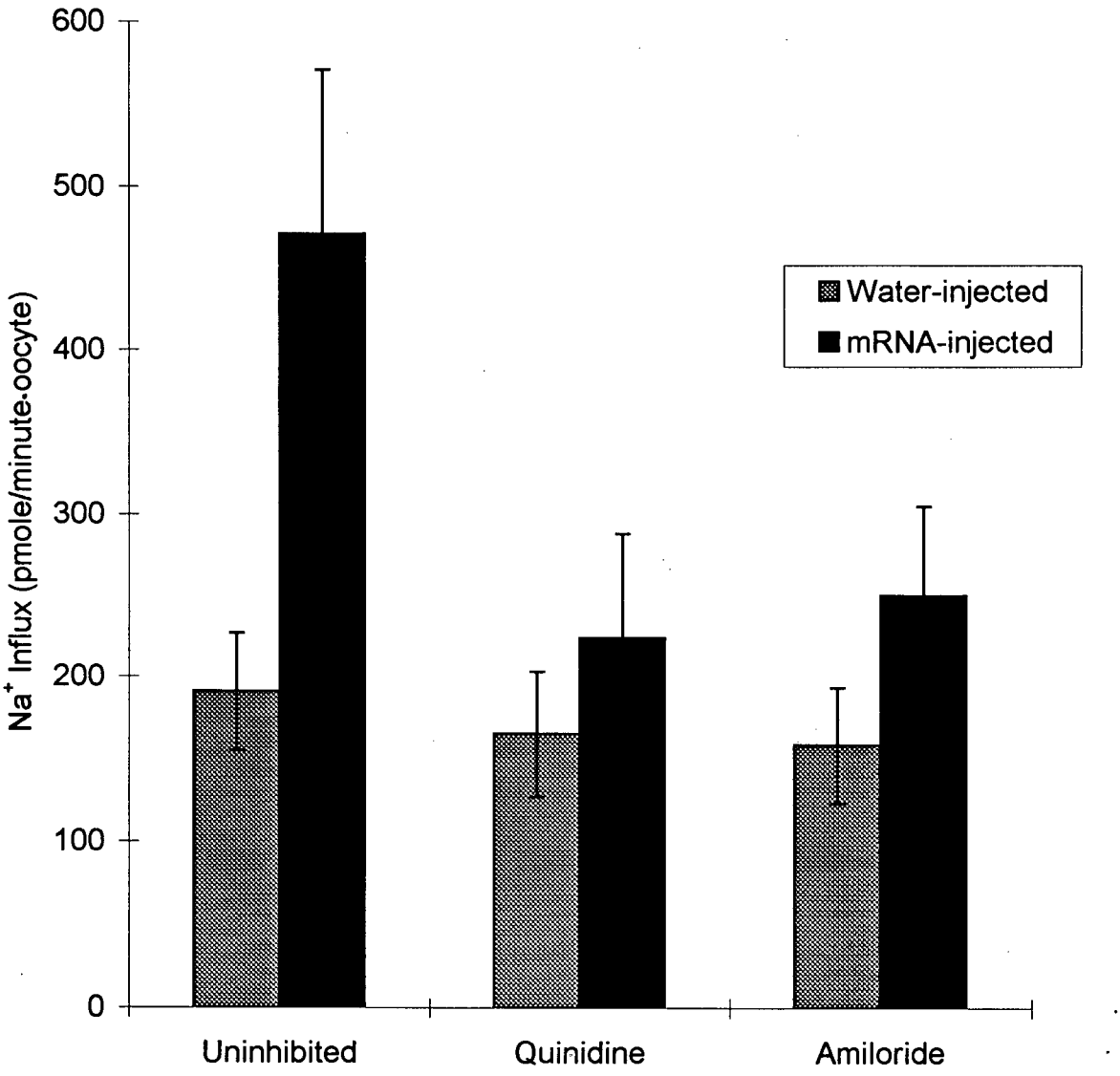

Fig. 8 Sodium-dependence of the Na^+/Mg^{2+} exchanger. Amiloride and quinidine were added, at concentrations of 5.0 mM and 4.0 mM, respectively, to the oocyte bath solution. ^{22}Na uptake was measured from oocytes loaded with 15 mM Mg^{2+}. Each bar represents mean +/- SEM, N=10 oocytes.

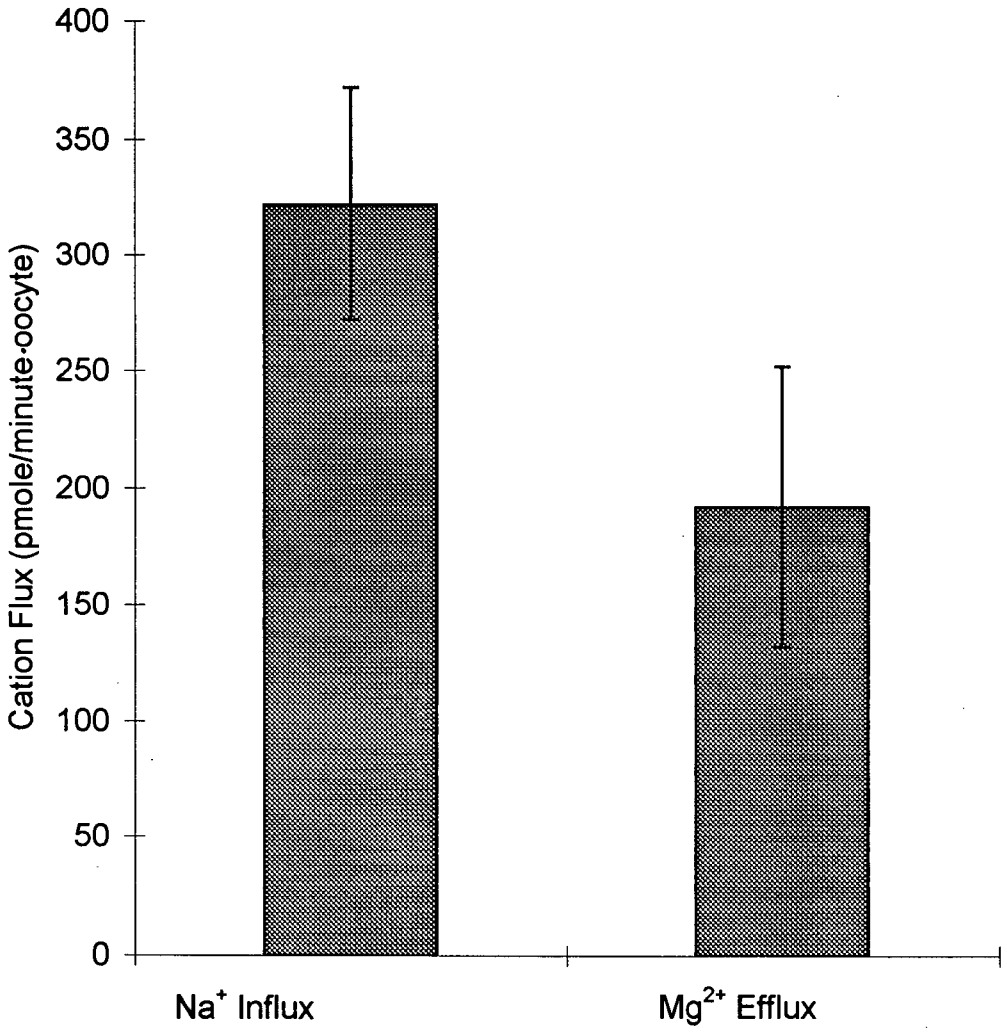

Fig. 9 Stoichiometry of the Na^+/Mg^{2+} exchanger. Sodium influx measurements were compared to those for magnesium efflux from different experiments to generate an approximate ratio of cation exchange. Results are reported as mean +/- SEM. A ratio of 1.65 Na^+ : 1 Mg^{2+} was shown suggesting an electroneutral exchange.

contained vector sequences. Those clones that did not either contained several stop sequences and/or did not provide any useful information as they did not contain transmembrane- or nucleotide binding-like domains. Furthermore, low stringency Southern analysis of PCR products derived from mRNA or library cDNA did not reveal any homologous cDNA to the Na^+/Ca^{2+} exchanger. In summary, homology-based studies were unsuccessful in identifying a Na^+/Mg^{2+} exchanger. These observations support the notion that a putative Na^+/Mg^{2+} exchanger may be quite different from the Na^+/Ca^{2+} exchanger and from the ABC transporter family.

Of interest, bands which were successfully detected by Southern analysis were of the same size as the positive NCE.F1 control amplified with the appropriate primer pairs. These results indicate of the presence of Na^+/Ca^{2+} exchanger transcript in the erythroleukemia cells. As is discussed in greater detail below, these PCR products were cloned and, through sequencing, were found to be almost identical to portions of the Na^+/Ca^{2+} exchanger .

3.3 **Na^+/Ca^{2+} Exchange**

3.3.1 Analysis of Na^+/Ca^{2+} Exchanger Isoforms in Erythroleukemia Cells

The Na^+/Ca^{2+} exchanger is composed of five amino-terminal transmembrane domains, a large intracellular loop, followed by another six carboxyl transmembrane domains (82). As illustrated in Fig.3, the intracellular loop undergoes alternative splicing to produce multiple isoforms of the Na^+/Ca^{2+} exchanger depending upon the tissue in which it is expressed. The portion of the Na^+/Ca^{2+} exchanger transcript which is alternatively spliced is comprised of two mutually exclusive exons, A and B, and four cassette type exons, C-F (Fig. 4).

RT-PCR was performed with mRNA isolated from three erythroleukemia cell lines; human

(K-562 and HEL) and mouse (GM979). Nested primers were designed from conserved sequences flanking the alternatively spliced region (Fig.4) of NCE.F1 cDNA (73). As predicted, a product of approximately 350 bp was obtained from positive control NCE.F1 cDNA (Fig.10A and 10B, lanes 2 and 1, respectively). The other bands that were present probably represent nonspecific PCR products. Amplified human K-562 mRNA resulted in three bands (Fig.10A, lane 1) which were smaller than the positive control, with the middle band (~ 280 bp) being most prominent. GM979 mouse cells, in which reverse transcription was initiated with a downstream primer rather than oligo dT, showed a single PCR band ~ 280 bp in size (Fig.10B, lane 3). HEL mRNA RT-primed with oligo dT resulted in multiple bands (Fig.10B, lane 4) of which the ~ 280 bp was most abundant. Although these bands were visible prior to the nested amplification reaction, the product was present in very small quantities (results not shown). This may suggest that Na^+/Ca^{2+} exchanger transcript is not abundant in undifferentiated erythroleukemia cells. No product was observed in the PCR tubes containing reaction mixtures without the cDNA template (Fig. 10A and 10B, lanes 3 and 2, respectively). Furthermore, since our primers spanned the entire splice region, i.e. exons A-F, the products obtained from our test samples did not arise from genomic DNA contamination.

Although several products were present following RT-PCR of K-562, GM979, and HEL mRNA, Southern blot analysis showed only one major PCR product (~ 280 bp) that bound to the NCE.F1 probe (Figs. 11A and 11B, lanes 1 and 3,4 respectively). Subcloning and sequencing of RT-PCR products revealed only one isoform, NACA3, in both the human and mouse erythroleukemia cell lines (Fig.4). Therefore, the other PCR products most likely represented nonspecific products. Multiple independent cDNA clones were sequenced to account for PCR extension mistakes. As illustrated in Fig.12, the two human clones, K-562 and HEL, shared 100%

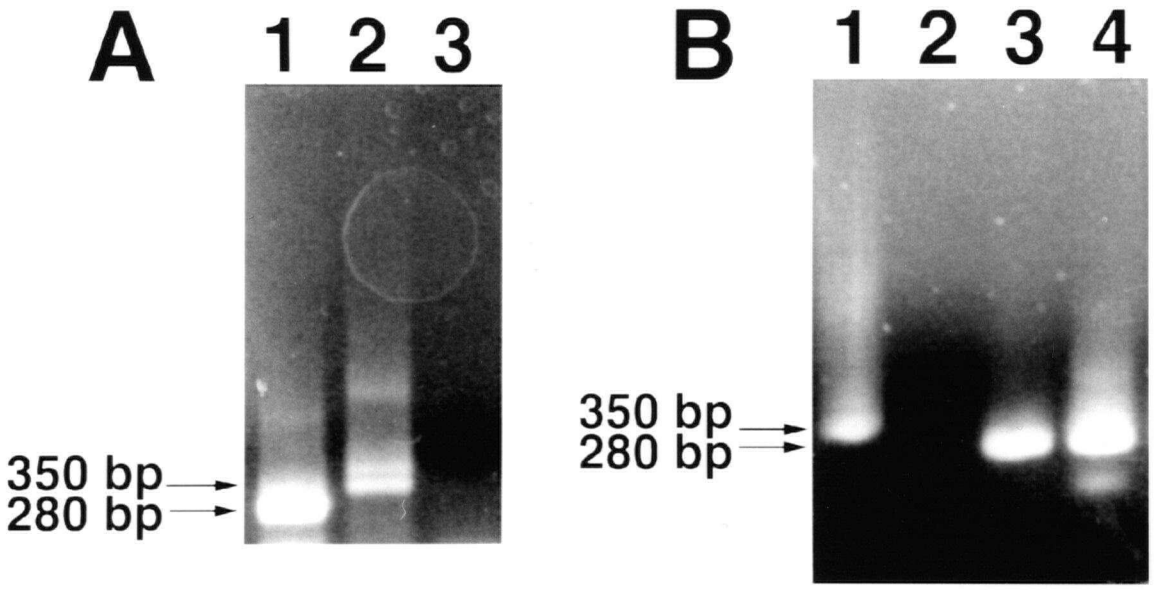

Fig. 10 RT-PCR amplification of the alternatively spliced region of K-562, HEL, and GM979 mRNA. NCE.F1 cDNA and reaction buffer were employed as positive (+) and negative (-) controls, respectively. The products were separated on a 0.8% agarose gel and visualized by ethidium bromide staining. (a) lane 1, K-562; lane 2, (+) control; lane 3, (-) control; (B) lane 1, (+) control; lane 2, (-) control; lane 3, GM979; lane 4, HEL. A band of ~ 350 bp is detected in the positive control lanes. Other bands present represent nonspecific products. Amplification using GM979 cDNA as template yields a single band of ~ 280 bp. The predominant band present in the HEL and K-562 lanes is ~ 280 bp in size. As expected, no product is amplified from the negative controls which are lacking the cDNA template.

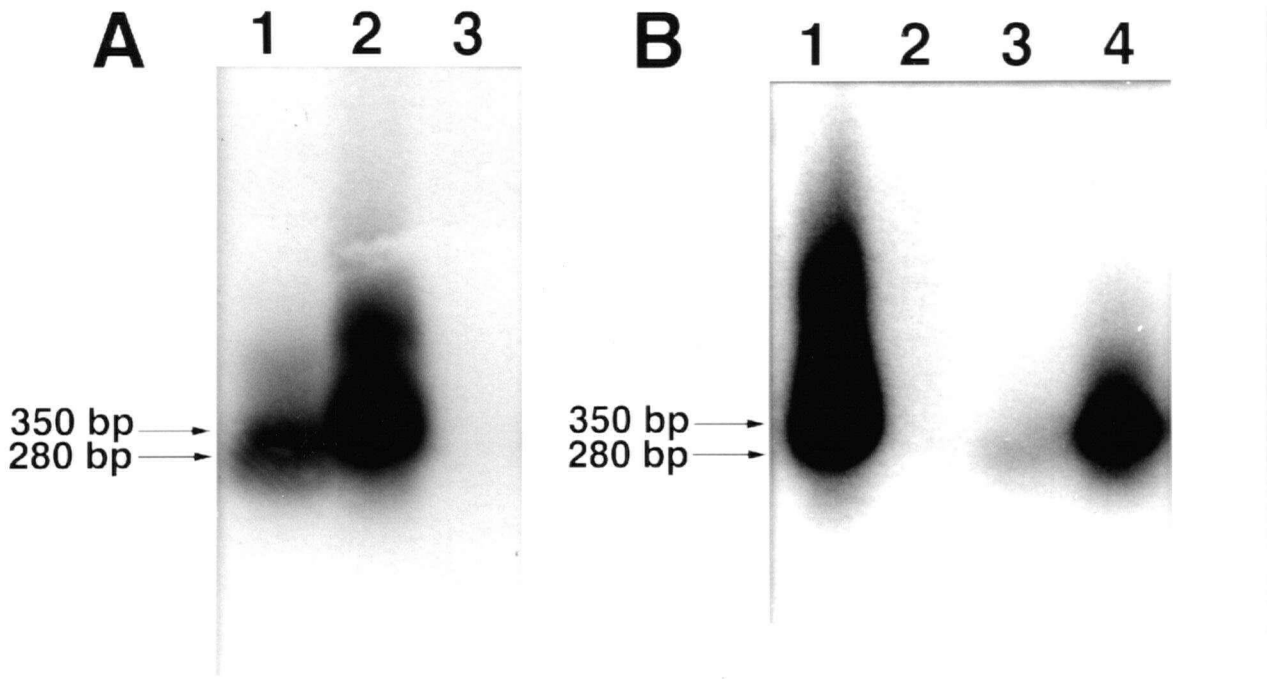

Fig. 11 Southern blot analysis of RT-PCR products from K-562, HEL, and GM979 mRNA. RT-PCR products were separated on a 0.8% agarose gel and transferred to Genescreen. Membranes were probed with [^{32}P] dATP-labelled NCE.F1 cDNA. (A) lane 1, K-562; lane 2, (+) control; lane 3, (-) control; (B) lane 1, (+) control; lane 2, (-) control; lane 3, GM979; lane 4, HEL. As expected, in the positive control lane a ~ 350 bp product binds to NCE.F1 probe. A single band of ~ 280 bp is detected in the three erythroleukemia cell lines.

EXON B

rat kidney	AAGATCATTACCATTAGAATATTTGACCGTGAGGAATATGAGAAAG
human	AAGATCATTACCATTAGAATATTTGACCGTGAGGAATATGAGAAAG
HEL	AAGATCATTACCATTAGAATATTTGACCGTGAGGAATATGAGAAAG
K-562	AAGATCATTACCATTAGAATATTTGACCGTGAGGAATATGAGAAAG
GM979	AAGATCATTACCATTAGAATATTTGACCGTGAGGAATATGAGAAAG

rat kidney	AGTGCAGTTTCTCCCTTGTGCTTGAGGAACCAAAATGGATAAGAAG
human	AGTGCAGTTTCTCCCTTGTGCTTGAGGAACCAAAATGGATAAGAAG
HEL	AGTGCAGTTTCTCCCTTGTGCTTGAGGAACCAAAATGGATAAGAAG
K-562	AGTGCAGTTTCTCCCTTGTGCTTGAGGAACCAAAATGGATAAGAAG
GM979	AGTGCAGTTTCTCCCTTGTGCTTGAGGAACCAAAATGGCTAAGAAG

EXON D

rat kidney	AGGAATGAAA|GGTGGCTTCACATTAACA
human	AGGAATGAAA|GGTGGCTTCACAATAACA
HEL	AGGAATGAAA|GGTGGCTTCACAATAACA
K-562	AGGAATGAAA|GGTGGCTTCACAATAACA
GM979	AGGATTGAAA|GGTGGCTTCACATTAACA

Fig. 12 Nucleotide sequence comparison of the NACA3 isoform cloned from HEL, K-562, and GM979 cells. The two exons comprising the variable region of NACA3 are B and D. The human K-562 and HEL cells are identical to the reported human B and D exon sequences (Genbank Accession numbers X91614 and X91214, respectively). Underlined bases denote substitutions between mouse GM979 cells and rat kidney NCE.F1 cDNA (23). The GM979 mouse clone shares 98% nucleotide and 95% amino acid sequence identity with the rat exchanger. Conserved amino acid substitutions are present in GM979 cells, with the two changes being isoleucine to leucine and methionine to leucine, respectively.

identity at the nucleotide level to the reported human exon B and D sequences (Genbank Accession numbers X91614 and X91214, respectively). The cDNA sequence of the GM979 mouse cell line shared 98% nucleotide and 95% amino acid homologies to exons B and D of the published rat NCE.F1 cDNA (69). The full-length mouse Na^+/Ca^{2+} exchanger cDNA has not been reported.

Chapter Four Discussion

4.1 Na^+/Mg^{2+} Exchange

Erythrocyte magnesium content is an essential modulator of RBC volume and volume regulatory mechanisms. This cation acts by modulating the activity of the Na/K/Cl and K/Cl cotransporters (27,37,72). Magnesium's role in the modulation of volume regulatory processes may be relevant to some pathological states. In human sickle cell disease (SCD), RBCs homozygous for abnormal hemoglobin undergo cellular dehydration (16). Normally, cell maturation and aging render the K/Cl cotransporter silent (56) but in RBCs of patients with sickle cell disease, there are high levels of K/Cl cotransport (14,16), causing low density erythrocytes to quickly dehydrate and become dense and nonfunctional (27). The characteristics of these cells were investigated by Brugnara et al. and were found to be similar to low-K^+ sheep RBCs with respect to volume dependency and Mg^{2+} sensitivity (15). K/Cl cotransport is sensitive to cell Mg^{2+} concentration; i.e. an increase in cell Mg^{2+} induces marked inhibition of K/Cl cotransport (72). Accordingly, methods which aim at increasing erythrocyte magnesium content could inhibit sickle cell dehydration by inhibiting K/Cl cotransport. In a transgenic mouse model for sickle cell disease (SAD 1) high-magnesium diets increased erythrocyte magnesium and potassium contents and reduced K/Cl cotransport activity. This was associated with lower levels of RBC dehydration, less K^+ loss, and decreased cell density and reticulocyte counts, suggesting amelioration of the disease. SAD 1 mice treated with a magnesium deficient diet demonstrated a reduction in erythrocyte Mg^{2+} and K^+ contents and increases in K/Cl cotransport, RBC density, reticulocyte count, and RBC dehydration, suggesting a deteriorating disease state (27). These effects were most likely mediated by increased K/Cl cotransport. Therefore, in these mice changes in dietary magnesium modulated K/Cl

cotransport and cell volume. There have been other reports implicating changes in $[Mg^{2+}]_i$ in patients with SCD compared with control individuals (85,87). Thus, dietary magnesium supplementation may be a potential therapeutic strategy to prevent cell dehydration and sickling in diseased patients.

Although magnesium is an essential intracellular cation, little is known about magnesium transport and regulation of cytosolic magnesium. Molecular characterization of the involved protein(s) would provide the basis for a much better understanding of both the mechanisms of Mg^{2+} transport and the regulation of intracellular Mg^{2+} metabolism. To directly demonstrate Na^+/Mg^{2+} exchange we expressed poly $(A)^+$ RNA of erythroid origin in *Xenopus laevis* oocytes. *Xenopus* oocytes are widely used to express plasma membrane proteins encoded in exogenously injected RNA or DNA and used to characterize properties of the expressed receptors, transporters, and channels (19,26,100). However, these heterologous expressed transport proteins have to be differentiated from the endogenous ones present in the native oocyte. Through functional studies we were able to detect expression of sodium-dependent magnesium transport in mRNA injected oocytes. The exchange observed was dependent on the amount of mRNA injected in the oocyte. Studies suggest that with mRNA coding for non-secretory proteins, the amounts of protein synthesized increases with the amount of mRNA injected in the oocyte and that at least up to 100 ng may be injected per oocyte. With high concentrations of injected mRNA, however, there can be significant mRNA degradation resulting in non-linear increases in transport (19). This is possibly what was observed in our studies because the maximal Na^+ influx observed was with 50 ng of injected mRNA and no further stimulation was observed at 80 ng (Fig. 5).

The properties of Na^+/Mg^{2+} exchange expressed in *Xenopus* oocytes from erythroleukemia

cells were similar to those reported for intact erythrocytes (36). First, the level of transport was dependent on the intraoocyte concentration of Mg^{2+}, with maximal magnesium efflux observed when oocytes were loaded to a final concentration of about 15 mM Mg^{2+} (Fig. 7). This result is in keeping with other studies of red cells where transport was undetectable if intact cells were not loaded with magnesium prior to experimental determinations (54). Interestingly, the maximal $[Mg^{2+}]_i$ required to demonstrate exchange was about 15 mM for both RBCs (54,55) and oocytes (data given here). Another common property was the dependence of magnesium transport on extracellular sodium. Magnesium efflux in oocytes was sensitive to high concentrations of the sodium transport inhibitors amiloride and quinidine (5.0 and 4.0 mM, respectively). The degree of inhibition observed at these concentrations (>50%) was comparable to that observed in RBCs (38,111). In summary, the cardinal features of the Na^+/Mg^{2+} exchanger proteins which have been reported for red blood cells also hold true for the expressed proteins underlying magnesium exchange in the mRNA-injected *Xenopus* oocytes. The results suggest that the injected poly $(A)^+$ RNA corresponds to protein(s) which provide a basis for magneisum transport at least in RBCs.

Our results demonstrate high levels of endogenous transport in water-injected oocytes (Fig. 5). This endogenous transport may be accounted for by non-specific Mg^{2+} leak from the oocyte. Other transport mechanisms, aside from the exchanger, which are specific to magnesium, such as a Mg^{2+}-specific channel or pump, may also exist. Therefore, it is possible that the poly $(A)^+$ RNA responsible for expression may act by directing the synthesis of regulatory proteins not directly involved with transport. These proteins may possess a stimulatory effect on endogenous Na^+/Mg^{2+} transport present in the *Xenopus* oocyte. While this possibility seems unlikely, it cannot be ruled out at present. Also unknown is whether the expressed protein(s) involved in Na^+/Mg^{2+} exchange is

modulated by endogenous components, such as intracellular ATP, or whether it requires components encoded with the injected RNA.

Some reports have suggested there may be similarities between the Na^+/Mg^{2+} and Na^+/Ca^{2+} exchangers (53), and that they may share common features. However, the studies involving homology-based RT-PCR with transmembrane regions of the Na^+/Ca^{2+} exchanger were without success. This domain would be expected to posses the most similarity between the two exchangers These results suggest that these exchangers may be quite different in nature.

This is the first demonstration of magnesium transport elicited via expression studies. The role of this exchanger in magnesium transport and cell metabolism remains to be fully determined. Intracellular magnesium is a highly mobile cation moving in and out of the cell relatively rapidly therefore the exchanger may play a role in cellular magnesium regulation. In erythroid cells, Na^+/Mg^{2+} exchange is likely to be involved with cell volume regulation. In the kidney, basolaterally located Na^+/Mg^{2+} exchange may play a role in transporting magnesium across the basolateral membrane. Our understanding of how Mg^{2+} moves across membranes will not be clear until the transport proteins are identified. The present studies show that it may be possible to expression-clone the Na^+/Mg^{2+} exchanger.

4.2 Na^+/Ca^{2+} Exchange

Multipotent hemopoietic cell lines, that can be induced to mature into stable phenotypes, have been extensively used as model systems for studying the regulation of gene expression during proliferation and differentiation (8,60,84). The undifferentiated K-562, HEL, and GM979 cell lines are pluripotent cells which express specific markers of erythroid, myeloid, and megakaryocyte

lineage. Changes in cytosolic Ca^{2+} concentration have been suggested to play a pivitol role in committing these progenitor cells to differentiation (44,59). Erythropoietin, a growth factor that controls erythropoiesis, is among the array of promoters which initiate changes in cytosolic Ca^{2+} in these cells (20,81). Cytosolic Ca^{2+} is controlled by a balance of influx through Ca^{2+} channels and efflux either by an ATP-dependent Ca^{2+} pump or a Na^{+}/Ca^{2+} exchanger (20,86). Although there is some controversy as to the presence of Na^{+}/Ca^{2+} exchange in red blood cells (43,108), it is clear from the present studies that the exchanger is expressed in the above cell lines. The role of this exchanger in erythroid cells, whether to allow Ca^{2+} influx or to maintain cytosolic Ca^{2+} through efflux is not clear (102).

The genes reported to code the Na^{+}/Ca^{2+} exchanger of various tissues and species demonstrate a remarkable similarity in cDNA sequence. There appears to be >90% homology among the many species studies so far, i.e., dog (82), rat (76,113), cow (1), rabbit (69), and humans (67,70). Kofuji et al. showed that six different alternatively spliced isoforms accounted for the Na^{+}/Ca^{2+} exchangers found in rabbit tissues including heart, brain, kidney, and skeletal muscle (69). Cardiac tissue contains the exons A,C,D,E,F; the brain contains A,D,F or A,C,D or A,D; skeletal muscle contains B,D,F or A,C,D,E,F, and the kidney and intestine contain B,D,F or B,D exons (69,73). The kidney may also contain B,C,D (97) (Fig. 4). On balance, the evidence indicates that Na^{+}/Ca^{2+} exchange is present in red blood cells, but to date no reports have been published on the characterization of the molecular isoform expressed in these cells (43,108).

Southern analysis of PCR products from GM979, HEL, and K-562 cells was used to determine the presence of Na^{+}/Ca^{2+} exchanger transcripts. One cDNA fragment (~ 280 bp) was amplified from GM979 cells and detected by Southern analysis. Although several fragments were

amplified from the two human cell lines, only one clear product (~ 280 bp) was found to bind the Na^+/Ca^{2+} exchanger probe. The primers used in PCR were designed from the regions flanking the alternative splice site. Therefore, products of a different size than the control could be expected if the region amplified represented exons other than those in the positive control (B,D,F). This was the case with the ~ 280 bp product that, when sequenced, was shown to represent exons B and D which is consistent with the NACA3 isoform.

In this study, we show that the two human K-562, HEL and one mouse GM979 erythroleukemia cell lines express the NACA3 isoform consisting of exons B and D. Interestingly, the isoform identified is the same alternatively spliced variant that was found in epithelial cells within the renal nephron (25,68) (Fig. 4). The Na^+/Ca^{2+} exchanger expressed in kidney epithelia is involved with maintaining cytosolic Ca^{2+} and calcium homeostasis (113). It is likely that the same isoform of the exchanger plays a role in controlling cytosolic Ca^{2+} levels and Ca^{2+} signalling in the associated differentiation of RBCs.

Chapter Five Summary and Conclusions

By employing an oocyte expression system, we were able to advance from conventional studies involving functional assays, methods which are currently employed by others to characterize Na^+/Mg^{2+} exchange. The objective of this research was first to show that the exchanger may be expressed in *Xenopus* oocytes. This may allow future studies through which the cDNA coding the Na^+/Mg^{2+} exchanger could be identified. Secondly, we were interested to determine if the Na^+/Mg^{2+} exchanger was similar to the Na^+/Ca^{2+} exchanger. Our studies with homology based PCR suggest that these two exchangers are coded by different DNA and are probably different proteins.

Employing oocyte expression of exogenous RNA and RT-PCR, we were able to express Na^+/Mg^{2+} exchange from these erythroleukemia cells and demonstrate the presence of Na^+/Ca^{2+} exchanger transcripts in these cells, respectively. RBCs have been shown to possess a functional Na^+/Mg^{2+} exchanger but the presence of Na^+/Ca^{2+} exchanger is controversial. Our results demonstrate that both exchangers are present in erythroleukemia cells which may reflect cation transport in mature red blood cells. These two exchangers undoubtedly play essential roles in cells of erythroid origin. Na^+/Mg^{2+} exchange most likely regulates intracellular Mg^{2+} concentrations which, in turn, modulates other activities such as enzyme activation and cell volume regulation. Na^+/Ca^{2+} exchange is likely to be involved with cytosolic Ca^{2+} control and signalling during differentiation of erythroid cells. The role of these exchangers in diseases such as sickle cell disease and abnormal erythroid cell differentiation is unknown but would be better understood by identifying the protein(s) involved with transport.

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