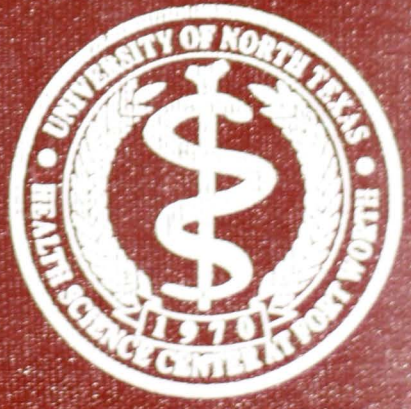
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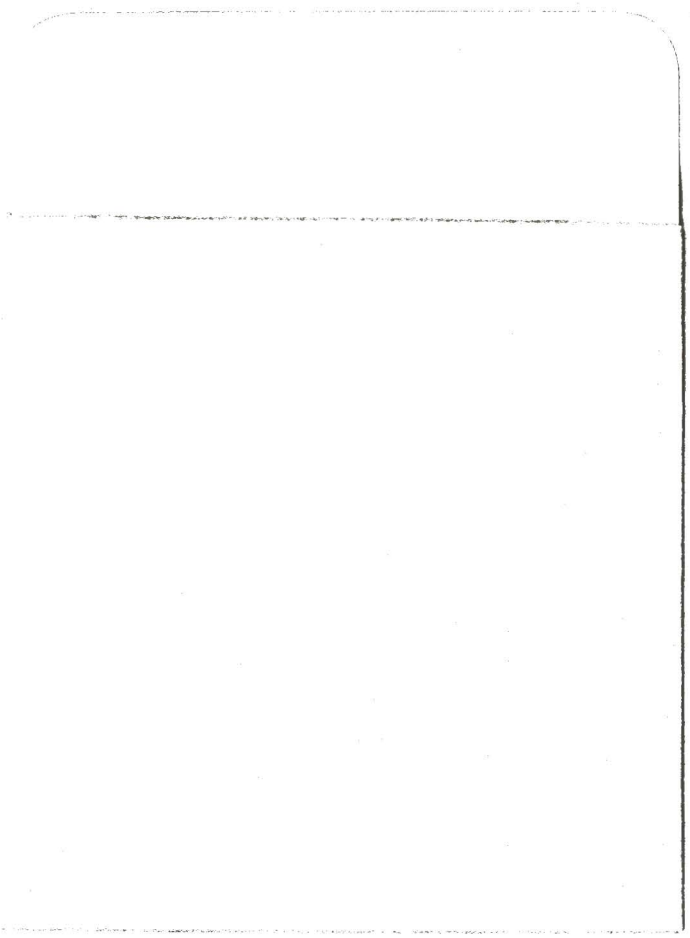

Zhou, Cheng, Molecular Cloning, Expression, and Regulation of the Na^+/Myo-Inositol Cotransporter Gene. Doctor of Philosophy (Biomedical Sciences), August, 1996.

Mammalian cells respond to osmotic stress by accumulation of high concentrations of intracellular osmolytes. Osmotic-induced accumulation of the osmolyte, myo-inositol (MI), is achieved through activation of the Na^+/MI cotransporter. Hypertonic stress results in elevated Na^+/MI cotransporter mRNA abundance and transcription rate, and increased transporter activity. The goals of this dissertation are to establish the osmoregulation of the Na^+/MI cotransporter gene expression in lens cells, and to investigate the transcriptional regulation of the Na^+/MI cotransporter gene.

Expression of the Na^+/MI cotransporter in cultured bovine lens epithelial cells (BLECs) was demonstrated by RT-PCR amplification and Northern blot analysis. Hypertonic stress resulted in induction of the Na^+/MI cotransporter mRNA abundance in cultured BLECs. The induction patterns of the Na^+/MI cotransporter and aldose reductase mRNA abundance by hypertonic stress indicated that osmoregulation of MI and sorbitol accumulations were regulated in concert. Accumulation of MI is an early-onset protective system, which is suppressed by the elevated sorbitol, the late-onset protective system.

5'-RACE analysis indicated that multiple transcription start sites were utilized in controlling of the expression of the Na^+/MI cotransporter. Osmotic stress resulted in preferential utilization of a hypertonic promoter ***a***.

The bovine Na^+/MI cotransporter gene was cloned and analyzed. The regulation of

the Na^+/MI cotransporter expression was investigated by transient transfection assays using promoter-luciferase constructs. Although multiple promoters were functional in cultured BLECs, only the hypertonic promoter ***a*** was osmotically responsive. Characterization of this osmotic-responsive promoter ***a*** localized the osmotic-responsive element(s) between -536 to -300 bp upstream of the hypertonic transcription start site ***a***.

The studies presented in this dissertation refined the osmoregulation of the Na^+/MI cotransporter gene expression. Hypertonicity induces MI accumulation by activation of an osmotic-responsive promoter. The consequences of the activation of this promoter lead to more cotransporter mRNA, more cotransporter protein, and higher transporter activity, resulting in accumulation of a higher concentration of intracellular MI.

MOLECULAR CLONING, EXPRESSION, AND REGULATION

OF THE Na^+/MYO-INOSIOTL COTRANSPORTER GENE

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MOLECULAR CLONING, EXPRESSION, AND REGULATION OF THE Na^+/MYO-INOSITOL COTRANSPORTER GENE

DISSERTATION

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INTRODUCTION

Statement of the Problem

The function of the lens is to provide the high refractive index necessary to focus images on the retina. The lens is an avascular, transparent organ surrounded by a lens capsule and consisting of two cell types: a single layer of epithelial cells on the anterior lenticular aspect of the capsule and the end-stage differentiated fiber cells. The normal lens is transparent because it does not absorb or scatter visual light to any significant degree. However, biochemical alterations in the lens cells, such as aggregation of soluble proteins, or membrane disintegration, may cause a loss of lens transparency resulting in cataract formation.

A cataract is defined as a lens opacity that causes, or is accompanied by, loss of visual acuity (Cataract Management Guideline Panel, 1993). It is the single largest cause of blindness accounting for as much as 42% of existing global blindness with some 16-17 million people affected (Thylefors *et al.*, 1995; Foster and Johnson, 1990). It is estimated that cataracts are also responsible for the visual impairment of 30 to 45 million

people worldwide (World Health Organization Program Advisor Group, 1989).

Cataract formation is a common response of the lens to a physical, mechanical or chemical insult (Harding and Crabbe, 1984). Etiologically, there are multiple risk factors for human cataractogenesis: aging, diabetes (or sugar-induced), radiation exposure (ultraviolet-B), congenital anomalies, trauma, and the side effect of medical treatment (Paterson and Delamere, 1992). Sugar cataracts, which are caused by high concentrations of a variety of sugars such as glucose (uncontrolled diabetes) and galactose (galactosemia in infants), are extensively studied. It has been known for years that diabetic patients are prone to develop cataracts (O'Brien, Molsberry, and Allen, 1934; Klein, Klein, and Moss, 1985; Howie and Drury, 1978). In diabetic patients under 60, the prevalence of cataracts are three to four times higher than that found in the normal population (Ederer, Hiller, and Taylor, 1981). As shown in the Framingham survey (Leibowitz *et al.*, 1980), cataracts were more prevalent in diabetics (19.1%) versus the normal population (11.6%). Flanagan (1993) has shown that 9.5% of insulin-dependent and 12.3% of insulin-independent diabetics either have cataracts or have had cataract surgery.

There are two morphological forms of sugar cataracts: (1) senile cataracts, which appear similar to age-related cataracts. However, in diabetic patients, cataracts not only happen earlier, more frequently, but also progress more rapidly (Harding and Crabbe, 1984); (2) true sugar cataracts, which happen rarely in severe juvenile diabetics (Harding and Crabbe, 1984; Neilson and Vinding, 1984). They were characterized by "snowflake" cataracts in the anterior and posterior cortex (Mclaren and Halasa, 1975;

Bron *et al.*, 1993).

Hypotheses of Sugar Cataractogenesis

Sugar cataract formation has been extensively studied but the precise metabolic mechanism(s) responsible for the etiology of cataractogenesis remain elusive. It has been shown that the concentration of glucose in the circumstances such as in response to diabetes mellitus is elevated in aqueous and vitreous humors in diabetic cataract patients (Davies *et al.*, 1984) and it can lead to the onset of cataractogenesis (Davson, 1990). Currently, there are several popular hypotheses to explain sugar cataractogenesis: polyol pathway-osmotic stress hypothesis, nonenzymatic glycosylation/glycation hypothesis, oxidative stress hypothesis, and myo-inositol depletion hypothesis.

Polyol Pathway-Osmotic Stress Hypothesis

The polyol pathway was first demonstrated by van Heyningen (1959) in lens from the lenses of diabetic rats and of young rats maintained on high galactose or xylose diets (Fig. 1). The enzymes involved in the polyol pathway, namely: aldose reductase (Polyol:NADP oxidoreductase, EC 1.1.1.21) and polyol dehydrogenase (L-iditol dehydrogenase, EC 1.1.1.14), were discovered shortly thereafter (van Heyningen, 1962). Pirie and van Heyningen (1964) were the first to demonstrate the presence of sorbitol in the human diabetic cataractous lens, an observation that was later confirmed

by Varma, Schocket and Richards (1979) and Jedziniak et al. (1981). Aldose reductase, which has an NADPH binding site (Harlder and Crabbe, 1985), has been localized in the eye (cornea, retina, lens), but not exclusive in the eye (kidney, myelin sheath, and erythrocyte) (Narayanan, 1991; Crabbe, 1991).

The major glucose-utilizing metabolic pathway in the lens is via hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1), to enter the Embden-Meyerhof glycolytic pathway, for the major energy source of the lens, or the pentose phosphate pathway. Alternatively, glucose can be converted to sorbitol through aldose reductase. Although aldose reductase has a high Michaelis constant, in diabetic lens, the elevated glucose concentration can saturate hexokinase thereby lead to sorbitol production via aldose reductase (Kador and Kinoshita, 1984). Sorbitol will then slowly be reduced to fructose by the enzyme sorbitol dehydrogenase. Galactose, another aldose, is converted to galactitol via aldose reductase. Sorbitol and galactitol will accumulate intracellularly because neither sorbitol nor galactitol is efficiently metabolized or able to rapidly penetrate through membranes. This creates an osmotic gradient resulting in an infusion of water into the cells. Lens hydration leads to cell swelling and eventual lens rupture. Cell swelling also leads to disruption of electrolyte balance, changes in membrane permeability, and depressed metabolism (Kador and Kinoshita, 1984). The consequence of these changes is opacification of the lens.

The importance of aldose reductase in sugar cataract formation is largely supported by indirect evidence. *In vitro* culture studies of lens in medium supplemented with high concentrations og glucose or galactose to mimic severe hyperglycemic or

severe hypergalactocemic conditions respectively have shown a positive correlation between polyol accumulation and lens hydration, eventually leading to opacity formation (Chylack and Kinoshita, 1969; Obazawa *et al.*, 1974).

It has been shown by Varma and Kinoshita (1974) that congenitally glycemic mice do not form sugar cataracts. In these mice lenses, although all other enzyme activities (including polyol dehydrogenase) tested were similar to those in rats, the aldose reductase activity was found to be lower. In this situation, the sorbitol formed from glucose was quickly converted to fructose by polyol dehydrogenase. The combination of low aldose reductase activity and relatively high polyol dehydrogenase activity resulted in an insufficient polyol accumulation and failed to create sufficient osmotic stress to cause cataract onset. These data suggested that osmotic stress generated by aldose reductase might cause sugar cataract onset. This conclusion was also supported by the studies of the South American *degu* (Octodon). Aldose reductase activity in its lens was three times higher than that in rat lens, and it formed sugar cataracts at lower hyperglycemic levels and developed cataracts more rapidly (Varma *et al.*, 1977).

These observations were further supported by the finding that aldose reductase inhibitors were able to delay or prevent sugar cataract formation in experimental animals. Aldose reductase inhibitors interact with aldose reductase at a common stereospecific site containing a nucleophilic residue (Kador *et al.*, 1981; Kador and Sharpless, 1983). Tetramethyleneglutaric acid (TMG) was the first aldose reductase inhibitor to be studied. TMG treatment abolished sorbitol accumulation, decreased lens

hydration, and maintained transparency in lenses cultured in high concentrations of galactose (Kinoshita *et al.*, 1968) or glucose (Chylack and Kinoshita, 1969). Since then, a wide variety of compounds, which consist of a planar aromatic ring system with an electrophilic group, have been identified as aldose reductase inhibitors including: flavonoids (Varma et al., 1977), AY22284 (alrestain) (Dvornik *et al.*, 1973; Chylack *et al.*, 1979) and Sorbinil (Fukushi *et al.*, 1980; Hu *et al.*, 1983).

Currently, the strongest evidence supporting this hypothesis came from experiments using transgenic mice that overexpressed the aldose reductase gene (Lee *et al.*, 1995). All transgenic mice lines exhibited increased aldose reductase activity (8- to 100-fold higher than normal). Those transgenic mice that had high aldose reductase levels developed sugar cataracts when they became galactosemic or diabetic. It was further demonstrated that transgenic mice with a sorbitol dehydrogenase-deficient mutation exhibited greater accumulation of sorbitol and further acceleration of sugar cataract formation.

There is little doubt that aldose reductase plays an important role in sugar cataract formation in animal models albeit the precise mechanisms remains to be established. However, the role of aldose reductase in human sugar cataractogenesis has yet to be established. Human lenses exhibit one tenth the aldose reductase activity of rats, but have relatively higher sorbitol dehydrogenase activity (Halder and Crabbe, 1984). Since sorbitol dehydrogenase in human lenses is more active than aldose reductase, little sorbitol accumulation would be expected in the human diabetic lens. There will be insufficient accumulated sorbitol to generate osmotic damage. This

observation leads support to the suggestion that other biochemical changes influenced by osmotic stress may be involved in sugar cataract formation.

Nonenzymatic Glycosylation/Glycation Hypothesis

One alternative hypothesis for sugar cataract formation is that nonenzymatic glycosylation of lens proteins leads to protein aggregation. Nonenzymatic glycosylation in lens is a process whereby sugar moieties covalently link to certain lens protein amino acid residues, usually the ε-amino group of lysine (Harding, 1985). This reaction results in the formation of carbonyl groups which can cross link. These functional groups may cause protein conformational changes, disulfide bond formation, and protein aggregation. Eventually, these changes would lead to light-scattering and lens opacification (Cerami *et al.*, 1979).

The nonenzymatic glycosylation hypothesis was supported by the finding that glycosylation was increased in experimental hyperglycemic rats. Rat lens crystallins incubated with glucose or glucose-6-phosphate were glycated on the ε-amino groups of lysine residues. These glycosylated crystallins underwent major conformational changes and formed high molecular weight aggregates (Stevens *et al.*, 1978). Further support came from observations that diabetic human lens or experimental diabetic rat lens had increased glycated crystallins relative to normal lens (Galick *et al.*, 1984; Rao and Coltier, 1986; Perry *et al.*, 1989). Fluorescence studies of α-crystallins from young, old,

and diabetic lenses suggested a role for protein glycosylation in the onset of diabetic cataracts (Liang, 1987). Further studies indicated that glycosylated α-crystallins from diabetic human cataractous lenses showed a greater change in protein tertiary structure than nonglycosylated proteins (Liang and Chylack, 1987). Recently, Unakar *et al.* (1995) reported that germanium-132, a derivative of amino acid containing germanium which prevents advanced glycation end product formation (Nakamura *et al.*, 1991), was able to effectively delay the progression of galactose-induced cataracts by 3 to 4 days.

However, this hypothesis still lacks direct evidence. Moreover, analyses of protein glycosylation in human lenses to date have shown no correlation between the degree of glycosylation and cataract formation (Ansari *et al.*, 1980; Pande *et al.*, 1979). Furthermore, study of lenses of rats maintained on a high-galactose diet, both with and without sorbinil, indicated that there was no difference in the degree of glycosylation in lenses of galactosemic rats irrespective of sorbinil treatment (Chiou *et al.*, 1980). While all of the galactose-fed rats developed cataracts, treatment with the aldose reductase inhibitor, sorbinil, prevented cataract formation in those galactose-fed rats with sorbinil. These results strongly suggested little to no correlation between nonenzymatic glycosylation and sugar cataract formation.

Oxidative Stress Hypothesis

Lenses require a highly reduced intracellular environment to maintain clarity. It has been shown that the level of reduced glutathione (GSH) in the lens is significantly

higher than in other tissues (Harding and Crabbe, 1984). The lens is vulnerable to oxidative stress because its membrane permeability increases when membrane thiols are oxidized (Duncan *et al.*, 1991). Furthermore, a reactive thiol is located at Na^+,K^+-ATPase active site (Rossier *et al.*, 1987). Oxidation at this thiol will affect enzymatic activity.

Sugar cataracts induced by hyperglycemia results in decreased intracellular glutathione (Sippel, 1966; Reddy *et al.*, 1976). A number of antioxidants have been reported to significantly delay or prevent sugar cataract formation in galactocemic and diabetic rats. These antioxidants include vitamin E (Creighton and Trevithick, 1979; Trevithick *et al.*, 1981), glutathione or glutathione ester (Creighton and Trevithick, 1979; Martensson *et al.*, 1989), and butylated hydroxytoluene (Linklater *et al.*, 1986; Srivastava and Ansari, 1988; Ansari and Srivastava, 1990).

However, the oxidative stress hypothesis also lacks direct evidence. Moreover, in some studies, it has been shown that treatment with the antioxidant, butylated hydroxytoluene, had no effect on sugar cataractogenesis (Woolard *et al.*, 1990). Vitamin E also failed to prevent sugar cataractogenesis in rats fed with a 33% galactose diet in short-term (Libondi *et al.*, 1985) and a 50 % galactose diet in long-term studies (Creighton *et al.*, 1985).

Myo-inositol Depletion Hypothesis

Since neither of the above hypotheses is able to explain the sugar cataract

formation perfectly, other hypotheses have been proposed regarding the onset of cataractogenesis (Hothersall *et al.*, 1988; Srivastava *et al.*, 1990; Narayanan, 1993). The myo-inositol depletion hypothesis is one of them (Narayana, 1993).

Myo-inositol, a cyclohexylic polyol, is an essential growth factor for cultured cells (Eagle et al., 1957). Myo-inositol and its phospholipid metabolites play a key role in intracellular regulation (Berridge, 1984). Second messengers, such as 1,2-diacylglycerol and inositol triphosphate (released from phosphoinositides), constitute important functions in signal transduction pathways for various hormones, neurotransmitter, and growth factors. Myo-inositol is also important in the modulation of Na^+,K^+-ATPase (Charalampous, 1971; Greene *et al.*, 1987). Recently, it has been suggested that myo-inositol might function as an antioxidant (Ruf *et al.*, 1992; Raj *et al.*, 1995).

The role of myo-inositol depletion in diabetic complications has been extensively studied in diabetic neuropathy. It was first reported by Stewart *et al.* (1967) that diabetes resulted in decreased myo-inositol concentration in ocular lenses and peripheral nerves. Reduction of myo-inositol content was also observed in cultured rat glomerular mesangial cells (Haneda *et al.*, 1990) and neuroblastoma cells (Yorek *et al.*, 1989). The accumulation of intracellular polyol contributed to the depletion of myo-inositol (Greene *et al.*, 1985). Aldose reductase inhibitors can prevent the myo-inositol depletion (Gillon and Hawthorne, 1983; Myer and Tomlinson, 1983). Winegrad and Greene (1976) first showed that myo-inositol depletion resulting from hyperglycemia caused impairment of nerve conduction. Myo-inositol supplementation in diabetic rats restored the depleted

myo-inositol and corrected the reduced nerve conduction without changes in associated glucose and sorbitol concentrations (Stewart *et al.*, 1967; Kim *et al.*, 1991). It has also been suggested that myo-inositol plays an important role in modulating Na^+,K^+-ATPase (Charalampous, 1971). The depletion of myo-inositol may be responsible for reduced Na^+,K^+-ATPase activity in diabetic rats (Clements, 1979; Greene et al., 1985) since phosphatidylinositol is identified as a specific activator of Na^+,K^+-ATPase (Mandersloot et al., 1978). Diet supplemented with myo-inositol prevented the decrease of Na^+,K^+-ATPase activity in diabetic rats (Greene and Lattimer, 1983; Yorek et al., 1993).

Although the role of myo-inositol depletion in diabetic complications is mainly from studies of diabetic neuropathy, it has been suggested that diabetic complications in peripheral nerve (diabetic neuropathy), lens (sugar cataracts), and retina (diabetic retinopathy) are likewise caused via a common myo-inositol-dependent mechanism (Fig. 2) (Winegrad, 1987). Depletion of myo-inositol has been reported in diabetic and galactosemic rat lenses (McCaleb and Sredy, 1992; Kawaba *et al.*, 1986), human diabetic lenses (Pirie and van Heyningen, 1964; Belpoliti and Maraini, 1993), and lens epithelial cells cultured in a high galactose medium (Cammarata *et al.*, 1990; Lin *et al.*, 1991). Depletion of myo-inositol in the lens positively correlated with polyol accumulation associated with diabetes and hyperglycemia (Kinoshita, 1974; Narayanan, 1993). Administration of aldose reductase inhibitors have been shown to be able to correct the reduced myo-inositol accumulation when lens epithelial cells were cultured in a high galactose medium (Cammarata *et al.*, 1990). The strongest evidence so far to support a role of myo-inositol depletion in sugar cataractogenesis stems from

experiments using dietary myo-inositol to delay cataract formation. Treatment of diabetic rats with D-myo-inositol for 7-8 weeks resulted in reduced cataract incidence by 26-44%, and also delayed cataract development in diabetic rats for a few weeks (Ruf *et al.*, 1992). Similar results have been reported by Beyer-Mears et al. (1989). Since myo-inositol is potentially also acting as an antioxidant (Ruf *et al.*, 1992; Raj *et al.*, 1995), this finding expands the role of myo-inositol depletion in sugar cataract formation and links the myo-inositol depletion hypothesis with the oxidative stress hypothesis.

Osmoregulation of myo-inositol and sorbitol accumulation

The associated role of myo-inositol and sorbitol in sugar cataractogenesis leads to extensive studies of the osmoregulation of myo-inositol and sorbitol accumulation, mechanisms which normally function to protect cells from osmotic stress.

Cells in hypotonic conditions initially swell by influx of osmotically obliged water. Cell swelling will then undergo a compensatory shrinkage called regulatory volume decrease (RVD) by activating volume-sensitive ion channels and channels for organic molecules (Hoffmann and Dunham, 1995). These changes result in efflux of electrolytes, certain organic osmolytes and the concomitant loss of water.

When exposed to hyperosmotic stress, cells initially shrink by losing water to the hypertonic environment to increase intracellular electrolyte concentrations to compensate against water stress. Osmotic shrinkage then activates uptake systems for

ions such as, $Na^+/K^+/2Cl^-$ cotransporter, Na^+/H^+ and Cl^-/HCO_3^- extrangers, to restore cell volume to original levels in a process called regulatory volume increase (RVI) (Hoffmann and Dunham, 1995). Since inorganic electrolytes perturb functions of macromolecules by an increase Km values (Kinne, 1993), exposure of cells to long-term osmotic stress (several hours) results in accumulation of non-perturbing organic osmolytes to replace perturbing electrolytes.

Living organisms, including bacteria, plants, and animals, respond to water stress by the accumulation of nonperturbing, compatible, osmotically active, organic solutes (osmolytes) (Yancey et al., 1982). Under long-term osmotic stress, accumulation of these nonperturbing, organic osmolytes will increase intracellular osmolarity and maintain cellular osmotic balance without dramatically changing intracellular electrolyte concentrations. Since these osmolytes do not perturb the structure and function of biological macromolecules, such as proteins, RNA, and DNA, their accumulation does not affect normal cell function (Kinne, 1993).

In mammals, renal cells of inner medulla are normally exposed to hyperosmolar conditions during the urine concentration process. Non-renal cells can be subject to osmotic stress in pathological conditions, such as: diabetes, congestive heart failure, renal dysfunction, etc. The organic osmolytes comprise three classes of compounds: polyols, neutral free amino acids and their derivatives, and methylamines plus urea. Using mass and nuclear magnetic resonance spectrometry, several organic osmolytes have been identified in the renal medulla and a variety of cultured cells (Bagnasco *et al.*, 1986; Nakanishi *et al.*, 1992; Burg, 1994), namely: sorbitol and myo-inositol (polyols),

glycerophosphorylcholine (GPC) (a methylamine), betaine and taurine (amino acid derivatives).

Sorbitol is synthesized from glucose catalyzed by aldose reductase, and converted to fructose by sorbitol dehydrogenase. Accumulation of sorbitol is controlled by the aldose reductase. GPC is synthesized from phosphatidylcholine catalyzed by phospholipases and lysophospholipases (Dawson, 1955). It is degraded to choline by GPC diesterase (Baldwin and Cornatzer, 1968) and degradation of GPC controls GPC level. Myo-inositol (Nakanishi *et al.*, 1989; Yamauchi *et al.*, 1991), betaine (Nakanishi *et al.*, 1990; Yamauchi *et al.*, 1991) and taurine (Uchida *et al.*, 1991) are accumulated from extracellular sources through specific sodium-dependent cotransporters. Osmotic stress induces accumulation of these osmolytes by activation of expression of those genes that regulate osmolyte concentrations.

Osmoregulation of aldose reductase gene expression

Aldose reductase was first identified in seminal vesicles and placental tissue (Hers, 1960). It was later found in various other tissues, including lens, kidney, peripheral nerve, and brain (Gabby, 1975). Osmotic stress has been shown to increase aldose reductase activity over a 3-4 day period in renal cells (Bagnasco *et al.*, 1988). Similar results have been reported in rat kidney mesangial cells and chinese hamster ovary cells subjected to hypertonic insult (Kaneko *et al.*, 1990). The induced aldose reductase activity correlated with the increased amount of aldose reductase protein

(Bedford *et al.*, 1987; Kaneko *et al.*, 1990). The osmoregulation of aldose reductase protein synthesis in renal medullary cells was investigated using pulse-chase with [^{35}S]methionine and immunoprecipitation with anti-aldose reductase antiserum to measure the rate of aldose reductase protein synthesis and degradation (Moriyama *et al.*, 1989). The results indicated that osmotic stress-induced aldose reductase protein amount was mainly attributed to increased rate of protein synthesis while no significant change in the rate of its degradation was detected. The amino acid sequences of bovine lens aldose reductase was first determined by Doughty et al. (Doughty *et al.*, 1988). The aldose reductase cDNA was first cloned from rat lens (Carper *et al.*, 1987). Shortly after, aldose reductase cDNAs from rabbit kidney (Garcia-Perez et al., 1989), human liver and placenta (Bohren *et al.*, 1989; Chung and LaMendola, 1989), and human retina and muscle (Nishimura *et al.*, 1990) were cloned. Expression of aldose reductase mRNA has also been detected in rat kidney mesangial cells and chinese hamster ovary cells (Kaneko *et al.*, 1990), cultured dog lens epithelial cells, cultured human retinal capillary pericytes, Y79 human retinoblastoma cells, as well as rat tissues of lens, retina, sciatic nerve, kidney medulla (Nishimura *et al.*, 1988), and cataract rat lens epithelium (Bekhor *et al.*, 1989). Northern blot analysis demonstrated that osmotic stress resulted in the induction of aldose reductase mRNA abundance in renal medullary cells, dog lens epithelial cells (Carper *et al.*, 1990), retinal pigment epithelial cells (Henry *et al.*, 1993), chinese hamster ovary cells, and cataractous rat lens. Nuclear run-on experiments indicated that hypertonicity increased aldose reductase mRNA abundance at the same extent as the enhanced transcription rate (Smardo *et al.*, 1992). Therefore, the enhanced

transcription rate of the aldose reductase gene accounts for the increased mRNA content. The osmoregulation of aldose reductase gene expression in renal medullary cells is summarized in Fig. 3 (Smardo *et al.*, 1992). Furthermore, increased aldose reductase gene expression has been associated with diabetic complications in human and experimental animals (Vinores *et al.*, 1988; Ghahary *et al.*, 1989; Hamada *et al.*, 1991).

The human (Graham, A *et al.*, 1991), rat (Graham, C *et al.*, 1991) and bovine (Ferraris *et al.*, 1994) aldose reductase genes have been cloned. Recently, a region of 5'-flanking sequence of the aldose reductase gene has been shown (Ferraris *et al.*, 1994; Iwata and Carper, 1996) to contain an osmotic-responsive element.

Osmoregulation of Na^+/myo-inositol cotransporter gene expression

Myo-inositol is found in bacteria, fungi, higher plants, and mammalian tissues (Holub, 1986). It is actively transported in a sodium and energy-dependent manner in many mammalian tissues and cells (Johnstone and Sung, 1967; Varma *et al.*, 1970; Caspary and Crane, 1970; Spector and Lorenzo, 1975; Chen and Vu, 1979; Prpie *et al.*, 1982; etc).

The ocular lens maintains a high myo-inositol concentration, about 70 fold higher than that in posterior chamber aqueous humor (van Heyningen, 1957; Reddy *et al.*, 1970). Accumulation of myo-inositol in the lens is primarily by active transport from the aqueous humor (Varma *et al.*, 1970; Cotlier, 1970). There is little to no ouabain-resistant passive uptake of myo-inositol in lens cells (Cammarata *et al.*, 1991), and no

appreciable amount of myo-inositol is being synthesized in the lens (Kinoshita *et al.*, 1963).

In cultured bovine lens epithelial cells, myo-inositol transport is a sodium-dependent, ouabain-sensitive, active transport process (Cammarata *et al.*, 1992a). This process appears to be distinct from glucose transport. Two myo-inositol transport sites were identified by kinetic analysis: a glucose-sensitive, sodium-dependent, high-affinity myo-inositol transport site; and a sorbitol-sensitive, sodium-dependent, low-affinity myo-inositol transport site (Cammarata *et al*, 1992b). Hypergalactosemic treatment resulted in reduced myo-inositol uptake activity (Cammarata *et al*, 1990; Cammarata *et al.*, 1991). Aldose reductase inhibitors were able to prevent the reduction in uptake activity which suggested that polyol accumulation by aldose reductase contributed to this attenuation. Further study indicated that polyol accumulation inhibited myo-inositol uptake at both the high- and low-affinity sites, with a reduced Vmax and no significant changes in Km (Cammarata and Chen, 1994).

The effects of osmotic stress on myo-inositol uptake in cultured bovine lens epithelial cells has been investigated (Cammarata and Chen, 1994). Hyperosmolarity generated by supplementation of 150 mmol/l of sorbitol, raffinose, mannitol, or NaCl stimulated myo-inositol accumulation with an increased Vmax and no significant changes in the Km suggesting that extracellular hypertonicity induced an increase in the amount of myo-inositol transporter(s). The enhanced uptake activity continued to increase throughout a 72 hour period with an initial lag time of 12 hours, consistent with the time for new transcription, translation and insertion into the plasma membrane.

Switching hypertonically-adapted cells to physiological medium for 24 hours reversed the osmotic response. Myo-inositol cotransporter induction required new protein synthesis since the protein synthesis inhibitor, cycloheximide and colchicine, effectively inhibited the hypertonic-induced myo-inositol accumulation (Cammarata *et al.*, 1994). This result strongly suggested that osmotic-induced myo-inositol accumulation involved new myo-inositol transporter protein synthesis.

Osmoregulation of the Na^+/myo-inositol cotransporter gene expression was first studied by Kwon et al. (1991). Myo-inositol uptake was measured by injection of poly(A)$^+$ RNA from MDCK cells, cultured in isotonic or hypertonic medium, into Xenopus oocytes. Myo-inositol uptake in oocytes injected with poly(A)$^+$ RNA from hypertonic cells was markedly elevated compared with oocytes injected with RNA from isotonic cells. These results suggested that hypertonicity-induced myo-inositol uptake involved an increase in Na^+/myo-inositol cotransporter mRNA and subsequent synthesis of transporter protein. The study of the molecular mechanism of hypertonicity-induced Na^+/myo-inositol cotransporter gene expression was dramatically expanded when its cDNA was cloned (Kwon *et al.*, 1992). Northern blot analysis indicated that hypertonicity resulted in approximately a 10-fold induction of the Na+/myo-inositol cotransporter mRNA abundance in MDCK cells. Nuclear run-on assays (Yamauchi *et al.*, 1993) demonstrated that the transcription rate of the cotransporter gene was also induced upon osmotic insult and reached a maximal induction of 15-fold after 16 hours of exposure. Switching cells back to isotonic medium reduced transporter mRNA abundance to that normally seen in isotonically-maintained cells within 8 hours. Taken

together, osmotic-induced Na^+/myo-inositol cotransporter mRNA abundance, the amount of cotransporter protein, as well as uptake activity is primarily regulated by increased cotransporter gene transcription rate. Figure 4 represents a summary of the osmoregulation of the Na^+/myo-inositol cotransporter gene expression in MDCK cells. It should be noted that the molecular mechanism of transcriptional regulation of the Na^+/myo-inositol cotransporter gene expression is presently unknown. In order to address this question, genomic cloning of this gene will initially be required. Furthermore, characterization of this gene's promoter regions will provide the information needed to elucidate how its transcription is regulated.

Experimental Rational and Research Goals

Little has been done to investigate the osmoregulation of the Na^+/myo-inositol cotransporter gene expression in non-renal cells. Since hypertonic-induced myo-inositol accumulation is mainly controlled by the increased transcription rate, the depletion of myo-inositol in the diabetic condition, attributable to polyol accumulation, may also be achieved by suppressing cotransporter gene transcription. Therefore, the study of osmoregulation of the Na^+/myo-inositol gene expression, and its relationship with polyol accumulation, should provide important information as to the mechanism of the onset of diabetic complications.

To investigate the transcriptional regulation of the Na^+/myo-inositol cotransporter gene, genomic cloning is a necessary first step. Since transcription

modulates the osmoregulatory response of the cotransporter, it will be significant to elucidate the nature of the genetic element(s) in the Na^+/myo-inositol cotransporter gene promoter that controls the osmotic response. Is there a common osmotic response element to mediate the response to the osmotic insult by activation of a group of genes in the same manner or are these genes osmoregulated individually through different elements?

The specific goals to explore the molecular mechanism of Na^+/myo-inositol cotransporter gene expression are as follows:

1) Cloning the cDNA for the Na^+/myo-inositol cotransporter in cultured bovine lens epithelial cells (BLECs);

2) Northern blot analysis using the cDNA probes specific to the cotransporter cDNA and aldose reductase cDNA (a gift from Dr. D. Carper) to investigate the effect of hypertonicity and polyol accumulation on the cotransporter and aldose reductase mRNA levels in cultured bovine lens epithelial cells, and the relationship between the expression of these two genes;

3) Identifying the 5'-terminal of the cotransporter mRNA from BLECs treated with different insults and examining the role of hypertonicity on cotransporter gene transcription initiation;

4) Genomic cloning, organization and sequencing of the bovine Na^+/myo-inositol cotransporter gene;

5) Characterization of the cotransporter promoter(s) and identification of the osmotic response element.

The results of this dissertation should help to establish the osmoregulation of the Na^+/myo-inositol cotransporter gene expression in secondary cultures of bovine lens epithelial cells and to investigate the molecular mechanisms governing the transcriptional regulation of the Na^+/myo-inositol cotransporter.

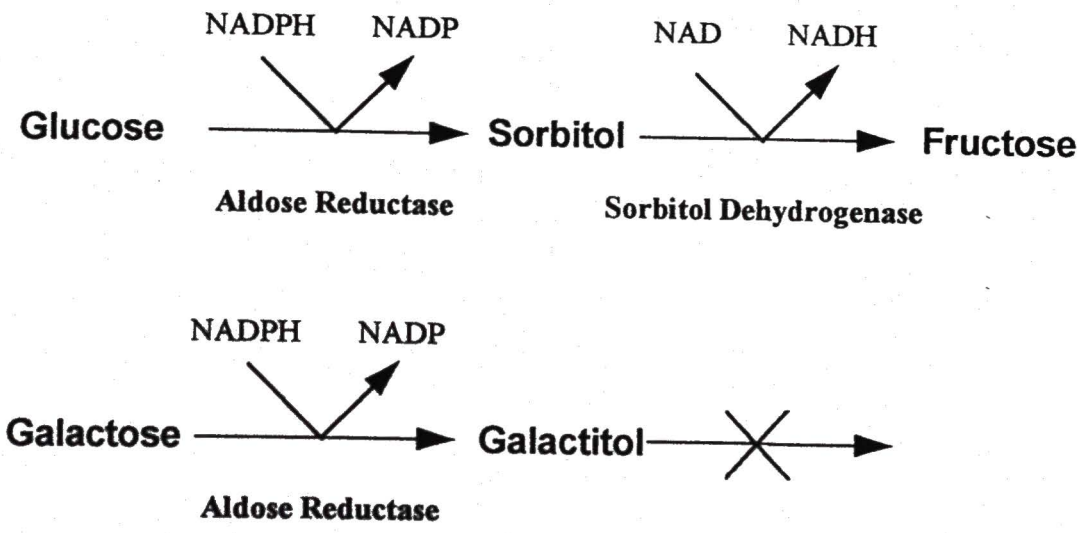

Figure 1. Sorbitol pathway.

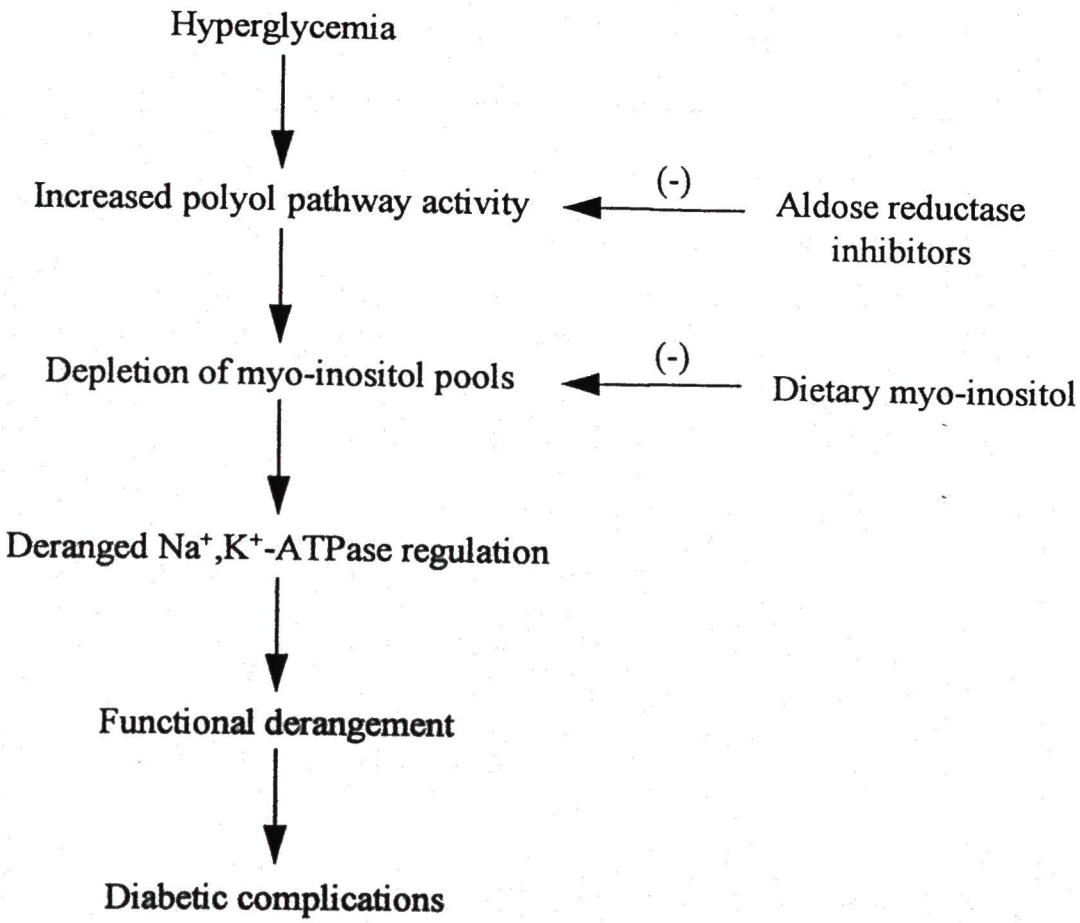

Figure 2. Schematic outline of the common pathway of diabetic complication development (modified from Winegrad. 1987. *Diabetes*. 36: 396-406).

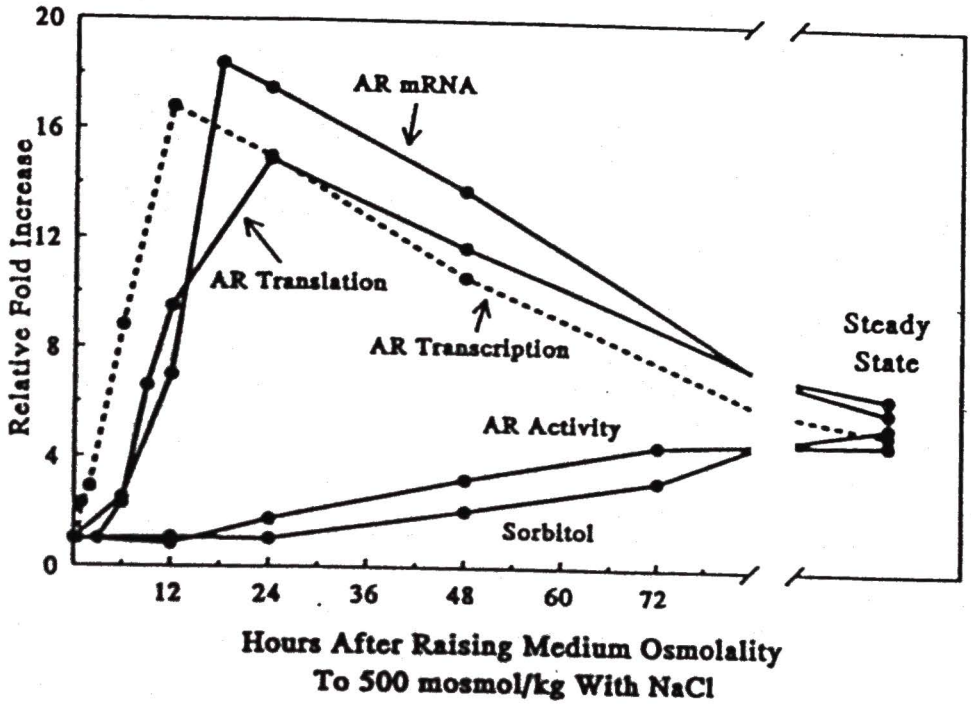

Figure 3. Summary of osmoregulation of aldose reductase gene expression in renal medullary cells (from Smardo *et al.* 1992. *Am J Physiol.* 262: C776-C782). Cells were switched from isotonic to hypertonic medium. mRNA abundance was measured by Northern blot analysis. Aldose reductase translation was determined by metabolic labeling and immunoprecipitation assays. Aldose reductase transcription was estimated by nuclear run-on assays. Aldose reductase activity was assayed by monitoring the decrease in absorbance of NADPH at 340 nm.

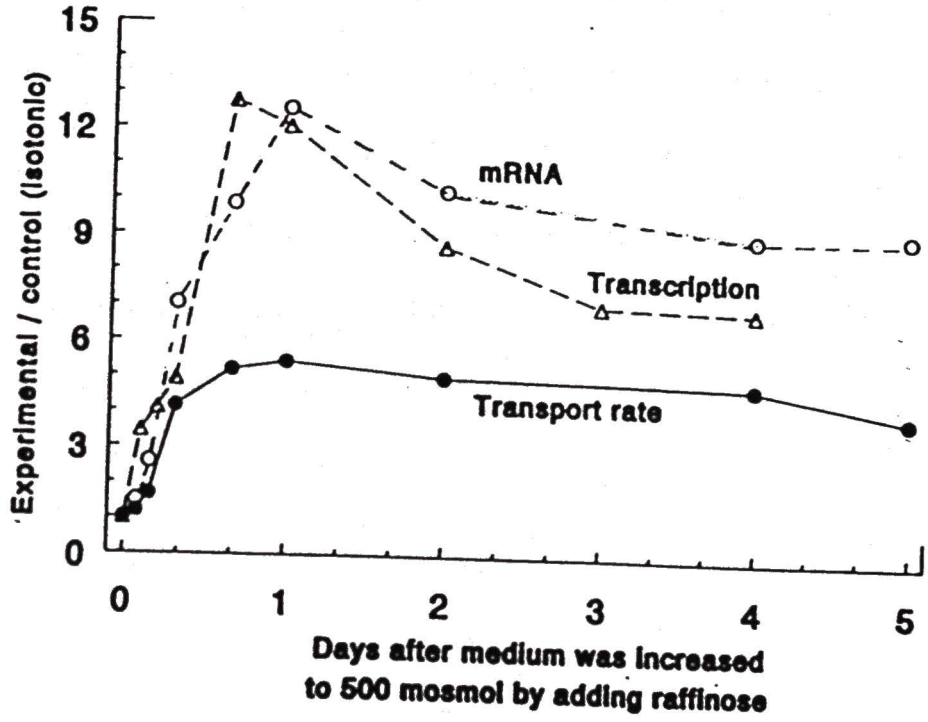

Figure 4. Summary of osmoregulation of the Na^+/myo-inositol cotransporter gene expression in MDCK cells (from Handler and Kwon. 1993. *Am J Physiol.* 265: C1449-C1455). Cells were switched from isotonic to hypertonic medium. mRNA abundance was measured by dot blot analyses and ribonuclease protection assays. Transcription rate was determined by nuclear run-on assays. Tansport rate was determined by measuring myo-inositol uptake into cells.

Previous research by Cammarata et al.(1992a, 1992b) has demonstrated the existence of a sodium coupled myo-inositol transporter in bovine lens epithelial cells by kinetic analysis. In the following study, we cloned a 626 bp Na^+/myo-inositol cotransporter cDNA from lens epithelial cells by RT-PCR amplification. Northern blot analysis using this cDNA as the probe demonstrated the expression of Na^+/myo-inositol cotransporter mRNA in lens cells. Osmotic stress resulted in induction of the cotransporter mRNA abundance.

Chapter II

The following manuscript was published in *Investigative Ophthalmology & Visual Science*, 1994; **35:** 1236-1242.

Osmoregulatory Alterations in Myo-inositol Uptake by Bovine Lens Epithelial Cells. II. Cloning of a 626 bp cDNA Portion of a Na^+/ Myo-inositol Cotransporter, An Osmotic Shock Protein.

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Short Title: Osmotic Shock Increases Na^+/ Myo-inositol Cotransporter mRNA

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Supported by National Public Health Service Award EY05570 (PRC).

Proprietary interest category: N

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Key Words: hypertonicity, upregulation, Na^+/ myo-inositol cotransporter mRNA, lens epithelial cells, cell culture.

Purpose. **Bovine lens epithelial cells (BLECs) accumulate osmotically active organic solutes (i.e. osmolytes) including myo-inositol when exposed to hypertonic stress (osmotic shock). In hypertonic medium, the increase in myo-inositol accumulation is attributed to an elevation in activity of Na^+/myo-inositol cotransporter(s). We report the nature of the hypertonicity-induced enhancement of myo-inositol uptake in cultured BLECs by amplifying a 626 bp cDNA from lens cell RNA.**

Methods. **A portion of cDNA encoding a Na^+/myo-inositol cotransporter was isolated from cultured BLECs using PCR primers designed from an established myo-inositol transporter from Madin-Darby canine kidney (MDCK) cells. Using the reverse transcription-polymerase chain reaction, a 626 bp PCR product was amplified. Its nucleic acid sequence was determined by the dideoxynucleotide method utilizing Sequenase kit. Na^+/Myo-inositol cotransporter mRNA expression in the cultured cells was demonstrated under physiological and hypertonic conditions by northern analysis of poly $(A)^+$ RNA using the lens cell 626 bp cDNA as probe.**

Results. **The BLEC cDNA sequence was 92% identical with the Na^+/myo-inositol cotransporter of MDCK cells. Myo-inositol transporter mRNA was demonstrated in cultured BLECs and was significantly induced by hypertonic stress.**

Conclusions. **These data suggest that cultured bovine lens epithelial cell adaption to hypertonicity involves intracellular accumulation of small organic osmolytes (i.e. myo-inositol) through elevation of myo-inositol uptake activity resulting from the upregulation of transporter mRNA.**

Little is known about the osmoregulatory role of organic osmolytes (including myo-inositol) under normal, hyperglycemic and hypertonic conditions in lens cells. Many cells, including bovine lens epithelial cells, accumulate small organic osmolytes in adaption to water stress in hypertonic environments.[1] In hypertonic medium, bovine lens epithelial cells accumulate myo-inositol over 10 x greater than control cells maintained in physiological medium.[2] This enhancement of myo-inositol uptake capacity is the result of an increase in the Vmax (without significant change in Km) of specific Na^+/myo-inositol cotransporter(s), indicative of an increase in the number of myo-inositol transporters. Recent reports by Kwon et al.[3,4] suggested that the hypertonicity-induced upregulation of the Na^+/myo-inositol cotransporter in MDCK cells involved both an increase in mRNA and synthesis of cotransporter protein(s).

We report the first isolation and sequence of a 626 bp cDNA encoding a portion of a Na^+/myo-inositol cotransporter from cultured lens cells, demonstrate its expression in BLECs by northern analysis and show that hypertonic stress results in an increase in transporter mRNA.

MATERIALS AND METHODS

Cell Culture

Bovine (*Bos taurus)* eyes obtained from a local slaughterhouse were brought on ice to the laboratory where the lenses were removed aseptically. Bovine lens epithelial cells (BLECs) were isolated and cultured in physiological medium (5.5 mM glucose) or 40 mM galactose as described by Cammarata et al.[5] For the induction of myo-inositol (MI) transporter mRNA, BLECs were exposed for 20 hr to serum-supplemented, physiological medium made hypertonic by the additional increase of 6.8 grams/liter NaCl (approximately 116 mM) or 40 mM galactose medium supplemented with 150 mM sorbitol in the presence and absence of the aldose reductase inhibitor, zopolrestat (0.1 mM). Madin-Darby canine kidney (MDCK) cells were maintained in physiological medium and employed as a positive control in the reverse transcription-polymerase chain reaction.

Extraction of lens epithelial cell total RNA

The cells from ten subconfluent 150 cm^2 flasks were dispersed in Ca^2-Mg^{2+}-free physiological medium containing 0.125% trypsin/0.05% EDTA, washed 1x with serum-free, physiological medium and pelleted by centrifugation at 1500 x g for 7 min. Total lens epithelial cell RNA was extracted using RNAzol B kit (Biotex Laboratories, Houston, TX).

Reverse transcription-polymerase chain reaction

First strand cDNA was prepared with M-MLV reverse transcriptase (GIBCO BRL, Grand Island, NY) with the use of oligo$(dT)_{12\text{-}18}$ (Pharmacia LKB, Piscataway, NJ) as the primer. The reaction was performed in a total volume of 100 µl containing: 25 µg of total

lens epithelial cell RNA, 2 μg of oligo$(dT)_{12-18}$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3 mM $MgCl_2$, 700 μM of each dNTP, 1 unit/μl RNasin (Promega, Madison, WI) and 1500 units of M-MLV. The reaction mixture was incubated in 37° C for 1 hr. PCR primers were designed from an established MI cotransporter from Madin-Darby canine kidney cell cDNA.[4] Primer 1 (sense primer) was designed to correspond with the region of 1501-1520 of the MDCK Na^+/ MI cotransporter cDNA.The sequence was 5'AACAGGCCAGGCTTCATCAA 3' and sequence of primer 2 (antisense primer) was designed to be complimentary to region 2110-2129 of the MDCK Na^+/ MI cotransporter cDNA. The sequence was 5'ATTCCAAGTGAACACACAGC3'. Primer 1 and 2 were commercially prepared (Genosys, Houston, TX). The PCR reaction was performed in a total reaction volume of 100 μl containing 0.8 μg total cDNA, 0.4 μM of each of the oligonucleotide primers, 200 μM of each dNTP, 50 mM KCl, 10 mM Tris-HCl (pH8.3), 1.5 mM $MgCl_2$, 0.001% gelatin and 2.5 units Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). Samples were overlaid with 100 μl of mineral oil. Twenty five cycles of denaturation at 94°C (1 min) with annealing at 50°C (2 min) and extension at 72°C (2 min) was the reaction condition employed.

Subcloning the cDNA fragment

The PCR amplified 626 bp cDNA fragment was recovered using a DEAE membrane (Schleicher and Schuell, Keene, NH) after running the PCR reaction mixture in a 1% agarose gel as described by Sambrook et al.[6] The recovered cDNA fragment was subcloned using the TA cloning system kit (Invitrogen, San Diego, CA) The construct (hereafter defined as pCR-1) was prepared by ligating the amplified 626 bp cDNA

fragment to the pCR vector using T4 DNA ligase as per the instructions of the Invitrogen kit.

A minipreparation of pCR-1 plasmid DNA was obtained by the alkaline lysis method Sambrook et al.[6] The pCR-1 plasmid was subsequently digested by EcoRI (Boehringer Mannheim, Indianapolis, IN). The DNA insert was isolated in 1% agarose gel.

Nucleotide sequence analysis

Utilizing a Sequenase version 2.0 kit (United States Biochemical, Cleveland, OH), the nucleotide sequence of the 626 bp, PCR amplified lens cell cDNA was determined by using the purified, RNA-free pCR-1 DNA as template as per the instructions in the Sequenase kit. The DNA sequence was determined by use of DNA Scan, an image-processing and analysis computer program for the Unix SparcStation as developed by Protein Data Imaging (Huntington Station, New York). Subsequent nucleotide and amino acid comparative analysis was processed with GeneWorks Software (version 2.2.1, Mountain View, CA).

Northern blot analysis

Poly $(A)^+$RNA from cultured bovine lens epithelial cells exposed for 20 hr under experimental conditions as described above was isolated by using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Purified lens epithelial cell Poly $(A)^+$ RNA was separated by electrophoresis in 1.0 % agarose-formaldehyde denaturing gels as described by Lehrach et al.[7]and blotted to a nylon membrane (Schleicher and Schuell, Inc., Keene, NH) for a period of 16 hr in 20 x SSC.

Following the blotting, the nylon membrane was baked at 80°C for a period of 1 1/2 hr under vacuum. The blot was then subjected to hybridization with ^{32}P-oligolabelled 626 bp lens cell cDNA using Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) or β-actin genomic DNA[8] probes using the procedure described by Agarwal et al.[9] After hybridization, the blot was subjected to a stringent wash procedure. The blot was first washed twice with 2x SSC with 0.1% SDS at room temperature for a period of 15 min and subsequently washed in 0.1x SSC with 0.1% SDS at 42°C for a period of 30 min with shaking. Afterwards, the blots were subjected to fluorography utilizing an intensifying screen and Hyperfilm (Amersham, Arlington Heights, IL) at -70°C. The density of both the lens cell mRNA transcript and of the actin mRNA was determined by use of Quantity One, an image-processing and analysis computer program for the Unix SparcStation as developed by Protein Data Imaging (Huntington Station, New York).

RESULTS

The viability of the cell cultures appeared unaffected by exposure to high ambient galactose or hypertonic medium for 20 hr as cells were characteristically flattened and attachment to the culture dish was high, with few floating cells observed.

Figure 1 shows that the lens epithelial RT-PCR product (lane 4) comigrated with the MDCK RT-PCR product (lane 3) in 1% agarose gel and was larger than 615 bp but smaller than 738 bp as determined with a 123 bp DNA ladder (lane 1, GibcoBRL, Grand Island, N.Y.). No detectable DNA was observed when the PCR reaction was performed in the absence of DNA template (i.e. RT product, lane 2).

The nucleotide and predicted amino acid sequences of the RT-PCR product are shown in Fig. 2. A comparison of the nucleotide sequence from the BLEC 626 bp RT-PCR product to that of the comparative published sequence of the MDCK Na^+/myo-inositol cotransporter[4] cDNA showed that they were 92% identical (Fig. 2A). Sequence comparison between the published MDCK Na^+/myo-inositol cotransporter cDNA and the RT-PCR product from MDCK was virtually identical (data not shown). The 626 nucleotide cDNA predicts a polypeptide of 208 amino acids. The polypeptide shows a highly significant (Fig. 2B) sequence and a remarkably conserved hydropathy profile (Fig. 3) to the Na^+/myo-inositol cotransporter in Madin-Darby canine kidney cells.[4] There was 96% amino acid identity as compared to the MDCK Na^+/myo-inositol cotransporter based on translation in the same reading frame. Despite several amino acid substitutions, hydropathy analysis revealed that the overall hydrophilic nature of the polypeptide was not compromised.

The lens cell 626 bp cDNA probe was used to identify and calculate relative content of the Na^+/myo-inositol cotransporter mRNA in control and osmotically shocked lens cells (Fig. 4). Northern blot analysis of poly $(A)^+$ RNA from lens epithelial cells using the lens 626 bp cDNA as probe showed a number of minor bands and one consistently prominent band; the size of the predominant BLEC Na^+/myo-inositol cotransporter mRNA was approximately 10.5 Kb (Fig. 4A) as determined by a 0.24 - 9.5 Kb RNA ladder (GibcoBRL, Grand Island, N.Y.) , which agreed with that previously reported for the MDCK Na^+/myo-inositol cotransporter mRNA transcript.[4] Northern blot analysis of the BLEC Na^+/myo-inositol cotransporter mRNA revealed that the greatest increase in

messenger RNA was for cells maintained for 20 hr in physiological medium supplemented with an additional 116 mM NaCl (Fig. 4B, lane 5). An increase in messenger RNA, albeit not nearly as great as with the NaCl insult, was apparent for cells exposed to 40 mM galactose made hypertonic by the addition of 150 mM sorbitol (a nonionizing osmolyte) plus the aldose reductase inhibitor (ARI), zopolrestat (lane 4). The addition of 150 mM sorbitol to 40 mM galactose (in the absence of added zopolrestat) also increased the concentration of messenger RNA for the Na^+/myo-inositol cotransporter but at a level which was blunted relative to the same treatment in the presence of the ARI (lane 3). Incubation of cells in 40 mM galactose (lane 2) alone failed to induce the mRNA content above that compared to control cells in physiological medium (lane 1). In order to confirm that amounts of poly $(A)^+$RNA from control and osmotically shocked cells were comparable, the blots for Na^+/myo-inositol cotransporter cDNA were stripped and subsequently reacted with a genomic probe for β-actin.

Cells exposed to high ambient NaCl increased 8-fold in Na^+/myo-inositol cotransporter mRNA over the control; that of the galactose/sorbitol/zoplorestat treatment increased 2.4-fold as quantified by laser densitometry from the northern blot in Fig. 4 and summarized in Table 1. The suppresive effect of 40 mM galactose on the induction of the BLEC Na^+/myo-inositol cotransporter mRNA in 150 mM sorbitol *in the absence of zopolrestat* vs. the increase in mRNA observed with 40 mM galactose and 150 mM sorbitol *in the presence of zopolrestat* is also calculated in Table 1.

DISCUSSION

A tentative structural model for the MDCK Na^+/myo-inositol cotransporter placed the amino and carboxy termini in the cytoplasm and proposed four minor extracellular loops and one major extracellular loop.[4] Our chosen PCR primers were designed to permit the isolation of a PCR product that included the cDNA sequence encoding the polypeptide of this major loop because of the possibility that this prominent extracellular region might contain the active site for myo-inositol binding. If, indeed, this loop is part of the myo-inositol binding region, then several demonstrated amino acid substitutions (relative to the MDCK myo-inositol transporter) did not compromise the integrity of the lens myo-inositol transporter. It is unknown at this time whether the amino acid differences are species or tissue related. Amino acid sequence comparisons with the only two other known myo-inositol transporters (ITR1 and ITR2) from Saccharomyces cerevisiae[10] revealed less than 11% and 7% identity for ITR1 and ITR2, respectively. Moreover, based on the partial data presented, which permits the deduction of a 208 amino acid polypeptide, less than 19% amino acid identity was exhibited as compared with the Na^+/D-glucose cotransporters of intestinal mucosa[11] and kidney proximal tubules.[12] Thus, it is likely that myo-inositol transporters will be classified into a myo-inositol transporter superfamily and be distinct from sodium-dependent glucose transporters.

BLECs exposed to hypertonic stress upregulate Na^+/myo-inositol cotransporter activity and accumulate myo-inositol. In secondary BLEC cultures, the inhibitor of protein synthesis, cycloheximide, suppressed the hypertonicity-induced enhancement of myo-inositol accumulation (Invest Ophthalmol Vis Sci. 1994; 35: 1223-1235), indicating a necessity for *de novo* synthesis of myo-inositol carrier protein(s) for adaption to osmotic

shock. In the present study we have shown that the increased accumulation of myo-inositol in response to hypertonic medium is also the result of the induction of specific Na^+/myo-inositol cotransporter mRNA.

The precise mechanism of regulation for myo-inositol accumulation and its relationship to polyol levels is obscure but probably involves transporter-leak components. In previous studies, we examined the relationship between sorbitol accumulation and myo-inositol depletion when cultured bovine lens epithelial cells were exposed to high ambient glucose[13,14] and reported that glucose inhibits the high-affinity myo-inositol transport site and sorbitol inhibits the low-affinity transport site. In the accompanying article, we demonstrated that while neither the high- nor the low-affinity transport sites are affected by galactose, galactitol inhibits both the high-affinity transport site and the low-affinity transport site. Exposure of cultured BLECs to 40 mM galactose for as much as five days does not enhance myo-inositol efflux from cells to media.[15] Thus, in isotonic medium, accumulation of sorbitol or galactitol leads to the depletion of cellular myo-inositol largely due to the (partial) suppression of the myo-inositol uptake mechanism(s). In hypertonic medium this inverse relationship between polyol and myo-inositol appears analogous, if not identical. Hypertonic stress leads to the upregulation of Na^+/myo-inositol cotransporter activity in BLECs both by an increase in specific transporter mRNA and synthesis of cotransporter carrier protein(s). Under hypertonic conditions, the rate of efflux of myo-inositol is virtually identical for cultured bovine lens cells as compared to control cells maintained in isotonic medium (unpublished observation). That is, irrespective of medium tonicity, the uptake mechanism is predominant and the "leak" arm

of the transporter-leak mechanism appears to play a relatively minor role because of little, to no, demonstrable difference in cell permeability under these defined experimental conditions.

Hypertonic adjustment of 40 mM galactose with 150 mM sorbitol *significantly increased, but failed to normalize* myo-[^{3}H]inositol uptake. The inability to normalize myo-inositol uptake was attributed to the fact that intracellular galactitol attenuated myo-inositol uptake by inhibiting high- and low-affinity transport sites , *albeit presumably under conditions which prompted increased synthesis of transport carrier protein(s)* (Invest Ophthalmol Vis Sci. 1994; 35: 1223-1235, Figs. 6 and 9). Only when the osmotic adjustment was accompanied by the presence of an aldose reductase inhibitor did myo-[^{3}H]inositol uptake exceed the accumulation otherwise noted with physiological medium. In conjunction with these data, the observations in this present study demonstrate that osmotically adjusted galactose medium decreased the expression of Na^+/myo-inositol cotransporter mRNA compared to osmotically adjusted galactose medium *in the presence of an aldose reductase inhibitor* (Fig. 4). This finding is consistent with the notion that high ambient galactose caused increased galactitol formation and accumulation which, in turn, downregulated Na^+/myo-inositol cotransporter mRNA expression. Similar findings have been reported with the suppression of aldose reductase messenger RNA due to galactitol formation in rat medullary cells[16] and with human retinal pigment epithelium.[17]

In summary, these data suggest that the osmoregulation of intracellular myo-inositol in cultured BLECs involves multiple mechanisms: (1) modulation of myo-inositol uptake by polyol-mediated inhibition of high- and low-affinity transport sites, (2) modulation via

polyol-mediated downregulation of Na^+/myo-inositol cotransporter mRNA and (3) by an as yet, poorly understood mechanism, modulation (by polyol ?) of myo-inositol efflux. It remains to be rigorously proven whether intracellular galactitol accumulation is a *specific* suppressor of Na^+/myo-inositol cotransporter mRNA transcripts, or a *general* toxic suppressor of cellular transcription.

Acknowledgments

This work represents partial fulfillment of the requirements for the doctoral degree for C.Z. The authors express their gratitude to Dr. Umit Yuksel for the nucleotide and amino acid comparative sequence analysis and Ms. Katherine O'Rourke for helpful discussions.

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Figure 1. RT-PCR analysis for the putative lens Na^+/myo-inositol cotransporter. Using known MDCK Na^+/myo-inositol cotransporter cDNA sequences, the primers were designed as detailed in Materials and Methods. The RT-PCR product was separated on 1% agarose gel and stained with ethidium bromide to visualize the DNA. 123 bp DNA ladder (lane 1), control without DNA template (lane 2), MDCK RT-PCR product (lane 3) and BLEC RT-PCR product (lane 4).

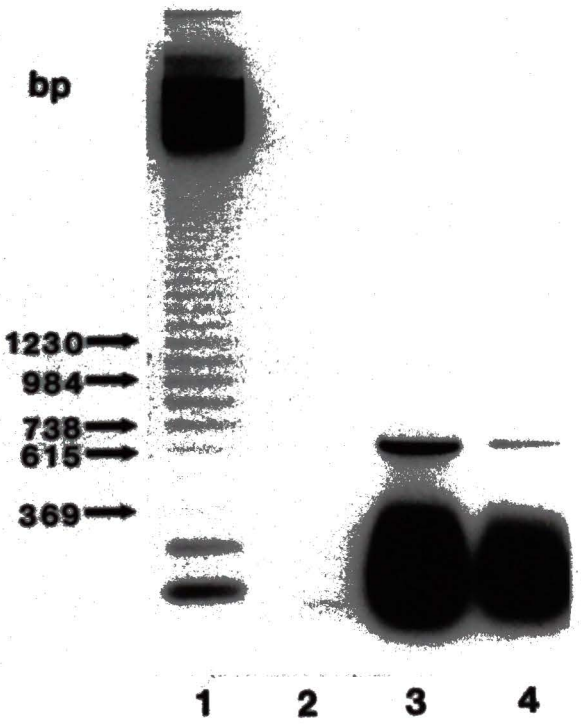

bp
1230
984
738
615
369
1
2
3
4

Figure 2. (A) Nucleotide and (B) amino acid sequences of 626 bp cDNA from BLECs. A, alignment of nucleotide sequence of 626 bp cDNA (BLEC) and established Na^+/myo-inositol cotransporter (MDCK). Identical nucleotide sequences are designated by (.). B, alignment of deduced amino acids of 626 bp cDNA (BLEC) and established Na^+/myo-inositol cotransporter (MDCK). Identical amino acid sequences are designated by (.). (-) designates either a nucleotide or amino acid deletion. The amino acids are given by their single-letter abbreviations.

A

```
Cons.   AACAGGCCAG GCTTCATCAA AGACATCCAT TACATGTATG TGGCCACAGC RTTGTTTTGG
BLEC    .......... .......... .......... .......... .......... G.........   60
MDCK    .......... .......... .......... .......... .......... A......... 1560

Cons.   RTCACAGGAC TCATTACWGT AATTGTTAGC CTTCTSACAC CACCTCCYAC RAAGGAACAG
BLEC    A......... .......T.. .......... .....C.... .......T.. G.........  120
MDCK    G......... .......A.. .......... .....G.... .......C.. A......... 1620

Cons.   ATTCGYACCA CCACYTTTTG GTCTAAGAAG AGCCTGGTGG TSAAGGARAG CTGCTCCCCG
BLEC    .....C.... ....T..... .......... .......... .C.....G.. ..........  180
MDCK    .....T.... ....C..... .......... .......... .G.....A.. .......... 1680

Cons.   AAAGATGAAC CMTACAAAAT GCAAGAGAAG AGCATTCTGA GRTGCAGTGA GAAYAGTGAG
BLEC    .......... .A........ .......... .......... .A........ ...T......  240
MDCK    .......... .C........ .......... .......... .G........ ...C...... 1740

Cons.   GCCAYCAACC AYRTCATTCC CAAYGGGAAG TCKGARGAYA GCATCAAGGG CCTKCAGCSR
BLEC    ....T..... .TG....-.. ...C--.... ..T..G..C. .......... ...G....GG  297
MDCK    ....C..... .CA....... ...T...... ..G..A..T. .......... ...T....CA 1800

Cons.   GAAGATGTKA ATCTGTTGGT GACCTGCAGA GARGAGGGCA AYCCAGTGGC TTCSTTAGGK
BLEC    ........G. .......... .......... ..G....... .C........ ...C.....T  357
MDCK    ........T. .......... .......... ..A....... .T........ ...G.....G 1860

Cons.   CATTCRGAGG CAGAAACACC MGTRGATGCK TATTCCAAYG GGCARGCAGC TCTYATGGGT
BLEC    .....G.... .......... C..G.....G ........C. ....G..... ...C......  417
MDCK    .....A.... .......... A..A.....T ........T. ....A..... ...T...... 1920

Cons.   GAGAAAGAGA GAAAGAARGA ARCAGAGGAY GGARGCCGGT ACTGGAAGTT CATMGATTGG
BLEC    .......... .......A.. .G.......C ...A...... .......... ...C......  477
MDCK    .......... .......G.. .A.......T ...G...... .......... ...A...... 1980

Cons.   TTYTGTGGCT TTAAAAGTAA GAGCCTCAGC AAGAGGAGTC TCAGAGACCT GATGGAGGAG
BLEC    ..T....... .......... .......... .......... .......... ..........  537
MDCK    ..C....... .......... .......... .......... .......... .......... 2040

Cons.   GAGGCTGTTT GTTTACAAAT GTTGGAAGAR CCTCCACAAG TTAAASTAAT ACTMAATATY
BLEC    .......... .......... .........A .......... .....G.... ...C.....T  597
MDCK    .......... .......... .........G .......... .....C.... ...A.....C 2100

Cons.   GGACTTTTTG CTGTGTGTTC ACTTGGAAT
BLEC    ......... ......... .........                                      626
MDCK    ......... ......... .........                                     2129
```

B

```
Cons.   NRPGFIKDIH YMYVATALFW .TGLITVIVS LLTPPPTKEQ IRTTTFWSKK
BLEC    .......... .......... I......... .......... ..........   50
MDCK    .......... .......... V......... .......... ..........  550

Cons.   SLVVKESCSP KDEPYKMQEK SILRCSENSE A.NH.IP..K SEDSIKGLQ.
BLEC    .......... .......... .......... .I..V..-T. .........R   99
MDCK    .......... .......... .......... .T..I..NG. .........P  600

Cons.   EDVNLLVTCR EEGNPVASLG HSEAETPVDA YSNGQAALMG EKERKKE.ED
BLEC    .......... .......... .......... .......... .......A..  149
MDCK    .......... .......... .......... .......... .......T..  650

Cons.   G.RYWKFIDW FCGFKSKSLS KRSLRDLMEE EAVCLQMLEE PPQVK.ILNI
BLEC    .S........ .......... .......... .......... .....V....  199
MDCK    .G........ .......... .......... .......... .....L....  700

Cons.   GLFAVCSLG
BLEC    .........                                              208
MDCK    .........                                              709
```

Figure 3. Kyte-Doolittle hydropathy analysis on 626 bp lens epithelial cDNA and established MDCK Na^+/myo-inositol cotransporter sequence.

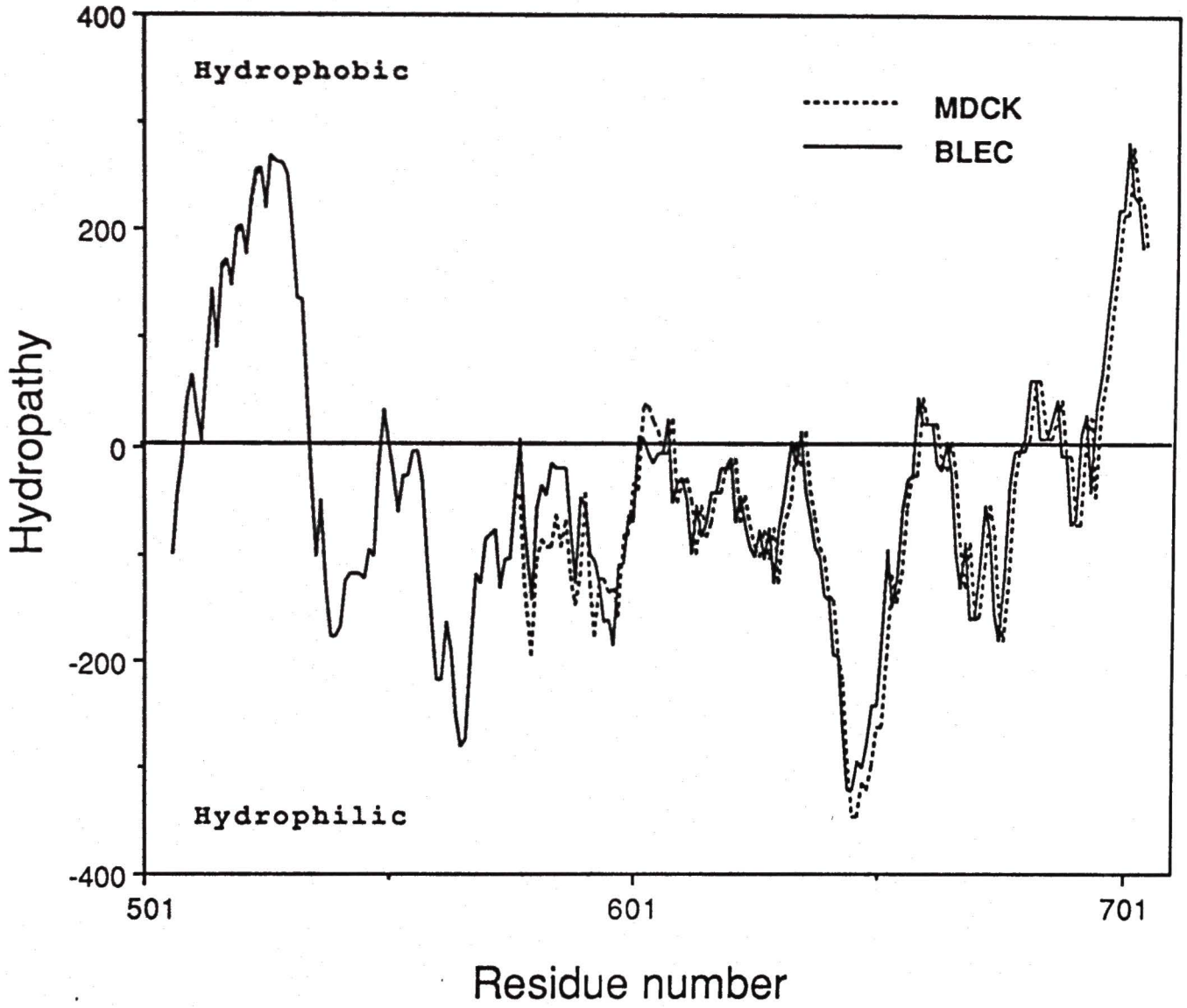
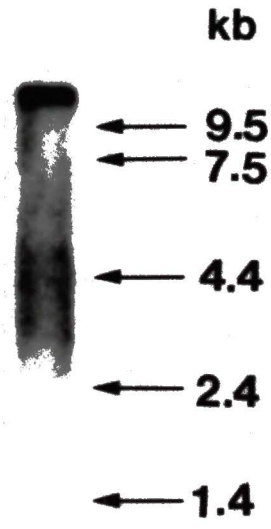

400
200
0
-200
-400
Hydrophobic
Hydrophilic
MDCK
BLEC
Hydropathy
501
601
701
Residue number

Figure 4. Northern analysis of bovine lens epithelial poly $(A)^+$ RNA. The probes were the 626 bp lens epithelial cDNA and β-actin genomic DNA as a control for determining the consistency in loading RNA. (A) Cells maintained for 12 hr in high ambient sodium chloride medium. The whole blot shows a range of very minor bands and a consistently prominent band at 10.5 kb probing with the 626 bp cDNA fragment. (B) The cells were maintained for 20 hr in (1) physiological medium or medium containing (2) 40 mM galactose, (3) 40 mM galactose and 150 mM sorbitol, (4) 40 mM galactose, 150 mM sorbitol and 0.1 mM zopolrestat and (5) 116 mM NaCl (in excess to the sodium level normally in physiological medium). The blot was probed with the 626 bp cDNA fragment and then reprobed with β-actin. Five μg of poly $(A)^+$ RNA (measured at 260 nm) was applied to each lane. 10.5 kb = predominant lens cell Na^+/myo-inositol cotransporter mRNA.

A



B

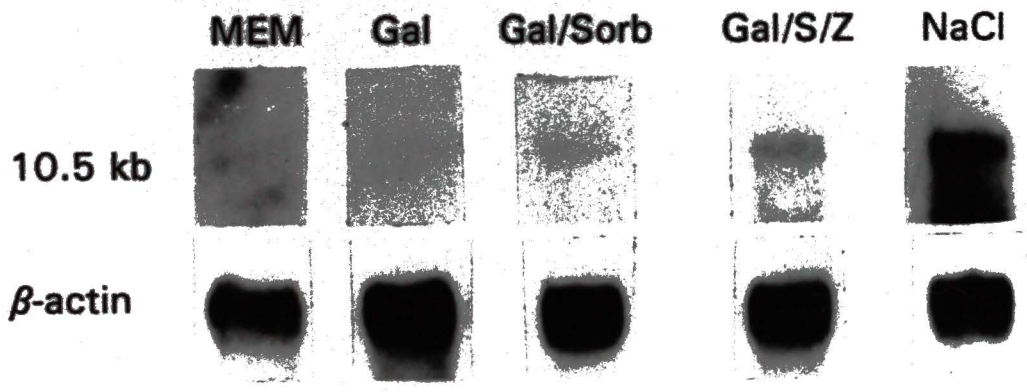


TABLE 1. **Induction of Na^+/Myo-inositol Cotransporter mRNA of Bovine Lens Epithelial Cells Cultured in 5 Different Media for 1 Day**

	Physiological Medium	40 mmol/l Galactose	40 mmol/l Galactose +150 mmol/l Sorbitol	40 mmol/l Galactose +150 mmol/l Sorbitol +0.1 mmol/l Zopolrestat	116 mmol/l NaCl
Na^+/MI Cotransporter	X 1	X 0.88	X 1.5	X 2.4	X 8

The demonstration of the expression of Na^+/myo-inositol cotransporter gene and the hypertonic-induced Na^+/myo-inositol cotransporter mRNA abundance in lens epithelial cells leads to the following study. The effects of hypertonic stress on Na^+/myo-inositol cotransporter and aldose reductase gene expression were investigated by Northern blot analysis over a time course of bovine lens epithelial poly(A)$^+$ RNA abundance. The interaction between the Na^+/myo-inositol cotransporter and aldose reductase activity was also studied by carbohydrate analysis of myo-inositol and sorbitol accumulation. Furthermore, the effects of decreased medium tonicity on Na^+/myo-inositol cotransporter mRNA levels from hypertonic-cells were investigated.

Chapter III

The following manuscript was published in **Investigative Ophthalmology & Visual Science,** 1994; **35:** 4118-4125.

Osmoregulatory Alterations in Myo-inositol Uptake by Bovine Lens Epithelial Cells. IV. Induction Pattern of Na^+/Myo-inositol Cotransporter mRNA Under Hypertonic Conditions Denotes an Early-Onset, Interactive, Protective Mechanism Against Water Stress.

Short Title: Osmoregulatory Alterations in Myo-inositol Uptake

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Proprietary interest category: N

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Key Words: hypertonicity, induction, Na^+/myo-inositol cotransporter mRNA, osmoregulation, cultured lens epithelial cells.

Purpose. **To examine the effect of hypertonicity on the induction of the Na^+/Myo-inositol (Na^+/MI) cotransporter(s) in cultured bovine lens epithelial cells (BLECs).**

Methods. **A Na^+/MI cotransporter 626 bp reverse transcription-polymerase chain reaction product amplified from lens cell RNA and aldose reductase (AR) cDNA probes were used to measure their respective mRNA content by Northern blot analysis.**

Results. **Northern blot analysis of BLEC mRNA hybridized to a Na^+/MI cotransporter cDNA showed that Na^+/MI cotransporter mRNA increased when secondary cultures of BLECs were exposed to physiological medium supplemented with 116 mmol/l NaCl. A time course further revealed a maximal increase in Na^+/MI cotransporter mRNA by 8 h. Thereafter, the level of Na^+/MI cotransporter mRNA steadily declined for the duration of the 72 h incubation period despite continuous exposure of BLECs to hypertonicity. AR mRNA levels maximally increased by 24 h of cell exposure to hypertonic condition. Unlike Na^+/MI cotransporter mRNA, AR mRNA remained elevated throughout the remaining duration of the experiment. Hypertonic exposure resulted in a steady-state accumulation of myo-inositol and sorbitol over six days. Inhibition of sorbitol formation prompted the intracellular myo-inositol content to a higher level.**

Conclusions. **These data suggest that *enhanced* MI transport and accumulation, as an adaptive osmoregulatory response to hypertonicity in cultured BLECs, is a primary, early-onset, protective mechanism against water stress, succeeded by, *enhanced* sorbitol formation and accumulation, a secondary, late-onset protective**

mechanism. The lens appears to respond to the preliminary stages of hyperosmotic stress by induction of Na^+/MI cotransporter mRNA, indicating that the myo-inositol carrier protein(s) play an initial responsive role in the management of osmotic stress. Lens water stress management is interactive as myo-inositol and sorbitol levels are regulated in concert.

The lens must be able to withstand variations in extralenticular osmolality, as with, for instance, glucose-derived osmotic stress. As part of this adaptive response, the lens must compensate for water stress by accumulating osmotically active, but chemically inert, nonperturbing, organic solutes termed, osmolytes.[1] The compatible organic osmolytes comprise three classes of compounds: (1) sugar alcohols or polyols, (2) neutral free amino acids and related solutes, and (3) methylamines plus urea. At least three putative compatible osmolytes have been identified in cultured lens epithelial cells, namely sorbitol, a polyol produced by the cell,[2] myo-inositol[3,4] and taurine,[5] which are a polyol and an amino acid derivative, respectively. Doubtless, other nonperturbing lens osmolytes will be characterized. The intracellular accumulation of these compatible organic osmolytes operate to maintain osmotic balance and protect the cell against the perturbing effects of high intracellular concentrations of electrolytes, which might otherwise adversely affect protein structure and function.

Osmotically stressed cultured *dog* lens epithelial cells reportedly respond with an increase in aldose reductase (AR) mRNA of approximately twofold by 24 h with a maximum increase of between four- and eightfold by 48 h.[2] As it appears to take several days for the activity of AR to reach a maximum, the lens cell must manifest an early-onset response to protect itself from acute hypertonic stress. *Enhanced* myo-inositol uptake activity is that early adaptive mechanism in cultured *bovine* lens epithelial cells.The accumulation of myo-inositol in cultured BLECs, resulting from exposure to hypertonic medium, has been attributed to increased uptake activity from the extracellular medium to

the cell,[6] originating from increased maximal velocity (Vmax) of both the high- and low-affinity Na^+/MI cotransporters without significant change in Michaelis constant (Km). Those data suggested that hypertonicity increased the number of cotransporter carrier proteins in the plasma membrane. Indeed, we have demonstrated the necessity for protein synthesis as an integral component of the adaptive response to hypertonicity.[6] The present study extends that observation by demonstrating that Na^+/MI cotransporter mRNA is also elevated under hypertonic conditions. The pattern of Na^+/MI cotransporter mRNA induction indicates a rapid, early-onset response to acute osmotic insult, in contrast to the later induction of AR mRNA.

MATERIALS and METHODS

Cell culture

Bovine *(Bos taurus)* eyes obtained from a local slaughterhouse were brought on ice to the laboratory where the lenses were removed aseptically. Bovine lens epithelial cells were isolated and cultured as previously described.[7] The studies were performed with confluent monolayers in 150-cm^2 culture flasks (representing 2nd-passage cells).

Determination of intracellular myo-inositol and sorbitol

Intracellular MI and sorbitol were identified by anion exchange chromatography and pulsed electrochemical detection using a Dionex BioLC chromatographic system (Dionex Corp., Sunnnyvale, CA) as previously described.[6] Confluent monolayers of bovine lens epithelial cells were maintained in either physiological medium (Eagle's minimal essential medium containing approximately 135 mmol/l NaCl, **257 ± 2 mosm**) or physiological medium made hypertonic by the additional supplementation of 116 mmol/l NaCl (**473 ± 6 mosm**, hereafter referred to as high ambient sodium chloride medium) for up to six days in the presence and absence of 0.1 mmol/l Sorbinil (Pfizer, Groton,CT) prior to being dispersed with trypsin, suspended in their respective Ca^{2+}-Mg^{2+}-free, serum-free, physiological or high ambient sodium chloride medium and centrifuged at 2500 x g at 4°C for 8 min. The cells were resuspended in 0.9 ml of 0.3 N zinc sulphate (Sigma Chemical Co., St. Louis, MO) and cell disruption accomplished by rapid freezing in liquid nitrogen and thawing at 37°C, the process being repeated three times. Thereafter, the samples were transferred to a 5.0 ml Dounce homogenizer and subjected to five strokes while being maintained in an ice-bath. The homogenate was centrifuged at 18,000 x g at 4°C for 20

min and the cell pellet saved for protein determination.[8] The supernatant was adjusted to 1 ml with 0.3 N zinc sulphate and 1.0 ml of 0.3 N barium hydroxide (Sigma Chemical Co., St. Louis, MO) was added followed by centrifugation at 2,500 x g at 4°C for 8 min and the supernatant stored at -20°C for subsequent myo-inositol analysis without further modification.

Extraction of lens epithelial cell RNA

The cells from three subconfluent 150 cm^2 flasks exposed for 12, 24 and 48 h under hypertonic conditions as described above were dispersed in Ca^2-Mg^{2+}-free, serum-free, high ambient sodium chloride medium containing 0.125% trypsin/0.05% EDTA, washed 1x with the same medium and pelleted by centrifugation at 1500 x g for 7 min. Total lens epithelial cell RNA was extracted using RNAzol B kit (Biotex Laboratories, Houston, TX). Poly $(A)^+$RNA from ten subconfluent 150 cm^2 flasks of cultured bovine lens epithelial cells exposed from 0 - 72 h under the same experimental conditions as described above was isolated by using the Fast Track mRNA isolation kit (Invitrogen, San Diego, CA).

Northern blot analysis

Purified lens epithelial cell total RNA or poly $(A)^+$ RNA was separated by electrophoresis in 1.0 % agarose-formaldehyde denaturing gels as described[9] and blotted to a nylon membrane (Schleicher and Schuell, Inc., Keene, NH) for a period of 16 hr in 20 x SSC. The gel containing total RNA was supplemented with two µg per lane of ethidium bromide (GIBCO BRL, Gaithersburg, MD).

Following the blotting, the nylon membrane was baked at 80°C for a period of 1 1/2 hr

under vacuum. The poly $(A)^+$ blot was then subjected to hybridization with ^{32}P-oligolabelled 626 bp lens cell Na^+/MI cotransporter cDNA using Random Primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, IN) or aldose reductase cDNA or β-actin genomic DNA[10] probes using previously described procedures.[11] The total RNA blot was subjected to hybridization with ^{32}P-labelled β-actin genomic DNA or an 18S ribosomal RNA oligo probe, again using previously employed standard procedures.[11] The characterization of the 626 bp cDNA portion of a Na^+/myo-inositol cotransporter amplified from lens epithelial RNA was recently reported.[12] After hybridization, the blot was subjected to a stringent wash procedure. The blot was first washed twice with 2x SSC with 0.1% SDS at room temperature for a period of 15 min and subsequently washed in 0.1x SSC with 0.1% SDS at 42°C for a period of 30 min with shaking. Afterwards, the blot was subjected to fluorography utilizing an intensifying screen and Hyperfilm (Amersham, Arlington Heights, IL) at -70°C. The density of the lens cell Na^+/myo-inositol cotransporter mRNA, aldose reductase mRNA and β-actin mRNA was determined by use of Quantity One, an image-processing and computer analysis program for the Unix SparcStation as developed by Protein Data Imaging (Huntington Station, New York).

For hypertonicity-reversal studies, the cultured cells were maintained in high ambient sodium chloride medium for 24 h and thereafter, conditioned high ambient sodium chloride medium was replaced with fresh physiological medium. Poly $(A)^+$ RNA was isolated as described above after 4, 8, 12 and 24 h of medium reversal and subsequently subjected to Northern blot analysis using the Na^+/MI cotransporter and β-actin probes as described earlier. In parallel experiments, myo-inositol and sorbitol levels were

determined.

RESULTS

Measurement of Na^+/myo-inositol cotransporter mRNA and aldose reductase mRNA

Utilizing the reverse transcription-polymerase chain reaction (RT-PCR), we amplified a 626 bp cDNA portion for a Na^+/MI cotransporter from cultured lens epithelial cell RNA.[12] Our chosen PCR primers were designed to permit the amplification of a PCR product from an established Na^+/MI cotransporter from Madin-Darby canine kidney cell cDNA.[13] The cDNA probe aided in the identification and calculation of the relative content of Na^+/MI cotransporter mRNA in hypertonically stressed lens epithelial cells.

Northern blot analysis of poly $(A)^+$ RNA from BLECs showed some faint minor bands and one prominent band; the size of the predominant BLEC Na^+/MI cotransporter mRNA being approximately 10.5 Kb (Fig. 1). The size of the Na^+/MI cotransporter mRNA agreed with that previously reported for the MDCK Na^+/MI cotransporter mRNA transcript.[13] Cells transferred to hypertonic medium responded with a transient inductive profile of Na^+/MI cotransporter mRNA as demonstrated by Northern blot (Fig. 2A). The Na^+/MI cotransporter mRNA was *maximally* expressed around 8 h. Thereafter, the cotransporter mRNA steadily declined to near control levels (Fig. 2) over the duration of the 72 h exposure to hyperosmotic insult. Consistent results were repeatedly observed in several similar experiments. After hybridization using the 626 bp Na^+/MI cDNA probe, the blot was stripped and reprobed with the AR cDNA (Fig. 2B). Unlike the early-onset pattern of induction of the Na^+/MI cotransporter mRNA, the level of AR mRNA was not

appreciably elevated above physiological control until 24 h of incubation under hypertonic condition. However, once induced, unlike the Na^+/MI cotransporter mRNA, the level of AR mRNA remained elevated for the duration of the 72 h experiment. Our data for AR expression in hypertonically-treated *bovine* lens epithelial cells is entirely consistent with previously reported data on AR induction in *dog* lens epithelial cultures.[2]

In order to determine that amounts of poly $(A)^+$ RNA loaded in each lane were comparable, the blots were again stripped and subsequently hybridized with a genomic probe for β-actin. The relative level of β-actin was elevated with increasing duration of exposure to hypertonic condition, most clearly evident by 24 h. The levels of β-actin remained elevated for the duration of the experiment, 72 h (Fig. 2C), suggesting induction of β-actin. This, however, did not compromise the interpretation of the results as it was evident from figure 2A that Na^+/MI cotransporter mRNA levels increased and declined during that period of time wherein AR mRNA was not induced, i.e. the increase in Na^+/myo-inositol cotransporter mRNA preceded the increase in aldose reductase mRNA. Nor can the data be the result of a lane loading problem or else one would expect that figures 2A and 2B would have increased in parallel. A similar mRNA inductive profile was observed with a cDNA probe of a second "housekeeping gene"; glyceraldehyde-3-phosphate dehydrogenase, indicative of the difficulty we encountered in finding an appropriate "control" gene that does not respond to osmotic stress. Other workers, utilizing rat liver perfused with hypotonic medium[14] or isolated rat hepatocytes exposed to hypotonic and hypertonic incubation media[15] have shown that actin mRNA levels can be unstable, attributed to cellular response to the persistent mechanical pressures of water

stress.

It remained, however, necessary to ascertain whether the temporal escalation of synthesis of the β-actin transcript observed in Fig. 2C was, in fact, due to a variation of loading of poly $(A)^+$ RNA in each lane of the gel. Re-examination of the expression of β-actin was repeated with lens cell cultures incubated for a 12, 24 and 48 h time-course of sodium hypertonic exposure with the exception that *total* lens epithelial cell RNA was collected. The difficulty with normalization on the basis of β-actin mRNA levels in figure 2, was thus overcome in figure 3, by correlation with rRNA levels, as determined by ethidium bromide staining and 18S ribosomal RNA probe. A consistent level of 18 S ribosomal RNA, as detected by ethidium bromide staining, demonstrated the invariability of our lane loading (Fig. 3A). Likewise, consistency in lane loading was apparent by the 18S ribosomal RNA bands (Fig. 3B) as demonstrated by hybridization of the 18S ribosomal probe.The β-actin transcript was elevated with increasing duration of exposure to hypertonic condition (Fig. 3C). Northern blot analysis of *total* RNA from lens epithelial cells using the lens Na^+/myo-inositol cotransporter 626 bp cDNA as probe failed to detect any transcripts (data not shown), in all likelihood due to an insufficient amount of MI cotransporter message relative to total RNA. These data further verified the validity of the temporal discrimination in the observed induction profile of the Na^+/MI cotransporter and AR transcripts obtained by Northern blot analysis of the poly $(A)^+$ RNA (Fig. 2).

BLECs maintained in high ambient NaCl medium (473 ± 6 mosm) for 24 h later switched to physiological medium (257 ± 2 mosm), showed that Na^+/MI cotransporter mRNA returned to near control physiological levels by 4 h, not detectable above control

levels by 8 h (Fig. 4A). Medium reversal likewise responded with a 24% drop in β-actin mRNA levels after 4 h in control medium but, thereafter, remained consistent at approximately 50% (of β-actin levels in hypertonic medium) for the duration of the experiment, 8 - 24 h (Fig. 4B).

Measurement of intracellular myo-inositol and sorbitol

Carbohydrate analysis showed that myo-inositol and sorbitol (Fig. 5, left panel) accumulated in hypertonically stressed cells. The intracellular free myo-inositol pool appeared to be regulated in accordance with sorbitol levels. When the lens cell cultures were exposed to hyperosmotic insult, myo-inositol accumulated, rapidly approaching its steady-state. However, as the intracellular level of sorbitol accumulated and approached its steady-state, myo-inositol concentration was maintained, but never exceeded, its plateau level (Fig. 5, left panel). Inhibition of sorbitol formation with the aldose reductase inhibitor, Sorbinil, prompted the intracellular myo-inositol content to reach a higher steady-state (Fig. 5, right panel), in compensation for the lack of sorbitol. When cells hypertonically stressed for 24 h were switched to physiological medium, both myo-inositol and sorbitol were dramatically reduced within 24 h of medium reversal (Fig. 6).

DISCUSSION

It was recently reported that primary cultures of *human* lens epithelium exposed to hypertonic medium containing NaCl (600 mosm) or cellobiose (500 mosm) for 72 h, displayed an increased myo-inositol concentration of 218% and 147% of control, respectively.[5] Secondary cultures of *bovine* lens epithelium, likewise, respond to

hypertonic insult with an increase in Na^+/MI cotransporter uptake activity and accumulate intracellular myo-inositol.[6] In a recent report, we documented that BLECs also upregulate Na^+/MI cotransporter mRNA when exposed to high ambient NaCl.[12] In the present study, we extend that preliminary observation by demonstrating that cellular synthesis of the Na^+/MI cotransporter transcript vacillates with increasing duration of hypertonic exposure. As a result of 72 h of continuous osmotic insult, the Na^+/MI cotransporter mRNA was maximally induced by 8 h but thereafter, steadily declined to physiological levels over the next 64 h (Fig. 2). The transient induction profile of the Na^+/MI cotransporter mRNA suggests that myo-inositol accumulation in the bovine lens cell must function in concert with other protective osmolytes in defense of sustained, chronic osmotic stress. Induction of AR mRNA was maximally induced by 24 hr, and thereafter, the level of AR mRNA remained in this steady-state plateau for the duration of the experiment (Fig. 2). *Enhanced* MI accumulation in cultured lens cells, as an adaptive osmoregulatory response to hypertonicity, appears to be an *early-onset* protective mechanism against the effects of *acute* water stress, and is closely followed by *an enhanced capacity* for sorbitol accumulation (i.e. increased aldose reductase activity resulting from AR mRNA induction), which represents a *late-onset,* secondary protective mechanism against the effects of *chronic* hypertonicity.

As first pointed out by Hohman and coworkers,[16] myo-inositol uptake and accretion and sorbitol formation and accumulation are not redundant mechanisms acting superfluously in response to osmotic insult. Utilizing cultured glomerular endothelial cells, they recognized the existing relationship between "early-onset" accumulation of myo-inositol followed by

sorbitol in response to osmotic stress. Our data advance their initial observation by demonstrating the temporal variation in the early inductive profile of the Na^+/MI cotransporter mRNA relative to the "late-onset" synthesis of the AR transcript. Moreover, Hohman and coworkers,[16] further identified an important association between myo-inositol and sorbitol accumulation: namely, that myo-inositol concentrations decreased once the aldose reductase transcript was induced and sorbitol accumulation reached its maximal level, suggesting that these two mechanisms were acting in concert. Data reported in the current study, likewise, confirm the contention that myo-inositol uptake and sorbitol accumulation in cultured bovine lens cells function in accordance. Na^+/MI cotransporter mRNA was *maximally* expressed approximately 16 h prior to *maximal* expression of AR mRNA under identical treatment conditions (Fig. 2). However, Na^+/MI cotransporter mRNA returned to near physiological levels at a point in time when AR mRNA continued to be maximally induced by hypertonic treatment. Medium reversibility experiments indicated that hypertonically-induced Na^+/MI cotransporter mRNA levels returned to control levels by 4 - 8 h (Fig. 4A). The rapid turnover (decreased rate of synthesis and/or increased rate of degradation) of the Na^+/MI cotransporter mRNA transcript suggests an amazing capacity by the lens cell to osmoregulate in response to medium tonicity. Similar medium reversal experiments with rat mesangial cells and Chinese hamster ovary cells have previously shown that AR mRNA did not return to control levels until 48 h.[17] Moreover, myo-inositol reached a steady-state plateau in hypertonically stressed BLECs and did not increase as intracellular sorbitol accumulation reached its steady-state (Fig. 5, left panel), yet myo-inositol content compensated for

osmotic stress by attaining higher intracellular levels in the presence of an aldose reductase inhibitor (Fig. 5, right panel). Lastly, both myo-inositol and sorbitol decreased in parallel to near physiological concentrations when the cells were switched from hypertonic to isotonic medium (Fig. 6). Lens osmoregulatory systems appear to be designed to regulate the intracellular levels of myo-inositol and sorbitol *in concert*, intracellular concentration being linked to the steady-state equilibrium of each active osmolyte. The data further suggest that *enhanced* myo-inositol uptake in defense of *acute* osmotic stress is an *early-onset protective mechanism*, followed by *enhanced* aldose reductase activity, a *late-onset protective mechanism*, responding to *chronic* osmotic stress.

That myo-inositol uptake and accretion and sorbitol formation and accumulation are *interactive* osmoregulatory mechanisms is further evident from our past studies. We previously reported that myo-inositol accumulation in cultured BLECs functions by a mechanism that includes at least two processes: a sodium-dependent, high-affinity transport system; and a sodium-dependent, low-affinity transport system.[4] Glucose competitively inhibits the high-affinity transport site and has no apparent adverse effect on the low-affinity transport site; sorbitol inhibits the low-affinity transport site and has little apparent effect on the high-affinity transport site.[3] Neither the high- nor the low-affinity transport sites are affected by galactose; whereas galactitol noncompetitively inhibits both the high-affinity transport site and the low-affinity transport site.[6] In addition, intracellular galactitol formation and accumulation downregulates Na^+/MI cotransporter mRNA expression.[12] It would thus appear that sorbitol (or galactitol) and myo-inositol are active, nonperturbing, organic osmolytes in cultured BLECs and that the increased enzymatic

formation of the former obviates a necessity to limit transport activity and accumulation of the latter (why accrue two osmolytes serving similar function?) and accomplishes this via multiple mechanisms; (1) modulation of myo-inositol uptake by polyol-mediated (partial) suppression of high- and low-affinity transport sites, (2) modulation by polyol-mediated downregulation of Na^+/myo-inositol cotransporter mRNA and (3) by an as yet poorly defined mechanism, polyol-mediated modulation of myo-inositol efflux.

The direct impairment of the myo-inositol transport system and downregulation of Na^+/MI cotransporter mRNA that results from lens cell exposure to high ambient glucose (and galactose) or extracellular hypertonicity, therefore, both represent plausible mechanisms that could, at least in part, account for the depletion of intracellular myo-inositol associated with hyperglycemia or hyperosmotic insult. When extracellular glucose rises, as with the diabetic state, animal models of hyperglycemia, or introduction of high ambient glucose in physiologic surrogates like the BLEC culture system, myo-inositol uptake through the high-affinity transport system is reduced and myo-inositol uptake via the low-affinity transport system likely proceeds normally only if an adequate concentration of extracellular myo-inositol is available. The situation is further exacerbated as glucose is converted to sorbitol, suppressing the activity of the low-affinity transport system. Polyol accumulation further impacts on myo-inositol uptake by suppression of Na^+/MI cotransporter mRNA levels and by polyol-driven efflux of myo-inositol from cell to medium.

While hyperosmotic enhancement of myo-inositol accumulation results from increased uptake activity due to upregulation of Na^+/myo-inositol cotransporter mRNA[12] and

ensuing *de novo* synthesis of the myo-inositol carrier protein(s),[6,18] clearly, the early genetic response to hyperosmotic stress in eukaryotic cells remains to be identified. It is conceivable that immediate early gene (IEG) expression encodes DNA binding proteins that can modify the rate at which other genes are transcribed.[19] Distinct IEG profiles may, at least in part, form the underlying basis which governs the diverse programs of gene expression leading to osmolyte accumulation in NaCl.

Hypertonicity then (by virtue of induction of AR mRNA), like the diabetic state (by virtue of increased substrate availability), elicits the increase of intracellular sugar alcohols, and in analogous fashion impacts on the myo-inositol uptake system in the lens. While it may be unlikely that the lens, the tissue from which these cells are derived from, would be exposed to hypertonic conditions *in situ*, it is nevertheless intriguing to observe that in the cultured BLEC model, the act of concentrating one organic osmolyte (i.e. sorbitol) in the face of chronic osmotic insult, an evolutionarily ancient mechanism to protect against hypertonic stress, may itself promote depletion of another osmolyte (i.e. myo-inositol). The eventual loss of intracellular myo-inositol might be an underlying causative factor leading to diabetic complications. *This may be particularly relevant for the case of sugar cataract development in the lens, as the high concentration of intralenticular myo-inositol is likely derived primarily, if not exclusively, by active transport from aqueous humor via anterior lens epithelium.* That is, little to no ouabain-resistant (i.e. sodium independent) passive uptake of myo-inositol occurs in cultured lens epithelium[20] and there is no appreciable synthesis of this metabolite in the lens.[21]

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Figure 1. Northern blot analysis of Na^+/MI cotransporter(s) using bovine lens epithelial poly $(A)^+$ RNA. RNA was extracted from lens cells maintained for 12 h in high ambient sodium chloride medium. The blot was probed with the lens 626 bp Na^+/MI cotransporter cDNA and shows the prominent 10.5 kb band and other minor bands. Five μg of poly $(A)^+$ RNA (measured at 260 nm) was applied to the lane.

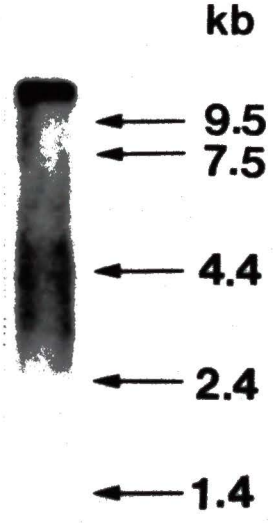

kb
9.5
7.5
4.4
2.4
1.4

Figure 2. Time course of expression of Na^+/MI cotransporter mRNA by Northern blot analysis of bovine lens epithelial poly $(A)^+$ RNA. (A) RNA from lens cells in physiological medium (lane 1) later switched to high ambient sodium chloride medium for 2 (lane 2), 4 (lane 3), 6 (lane 4), 8 (lane 5), 10 (lane 6), 12 (lane 7), 24 (lane 8), 48 (lane 9) and 72 h (lane 10). (B) The blot was stripped and subsequently probed with AR cDNA. (C) The same blot reprobed with genomic β-actin. Five μg of poly $(A)^+$ RNA was applied to each lane.

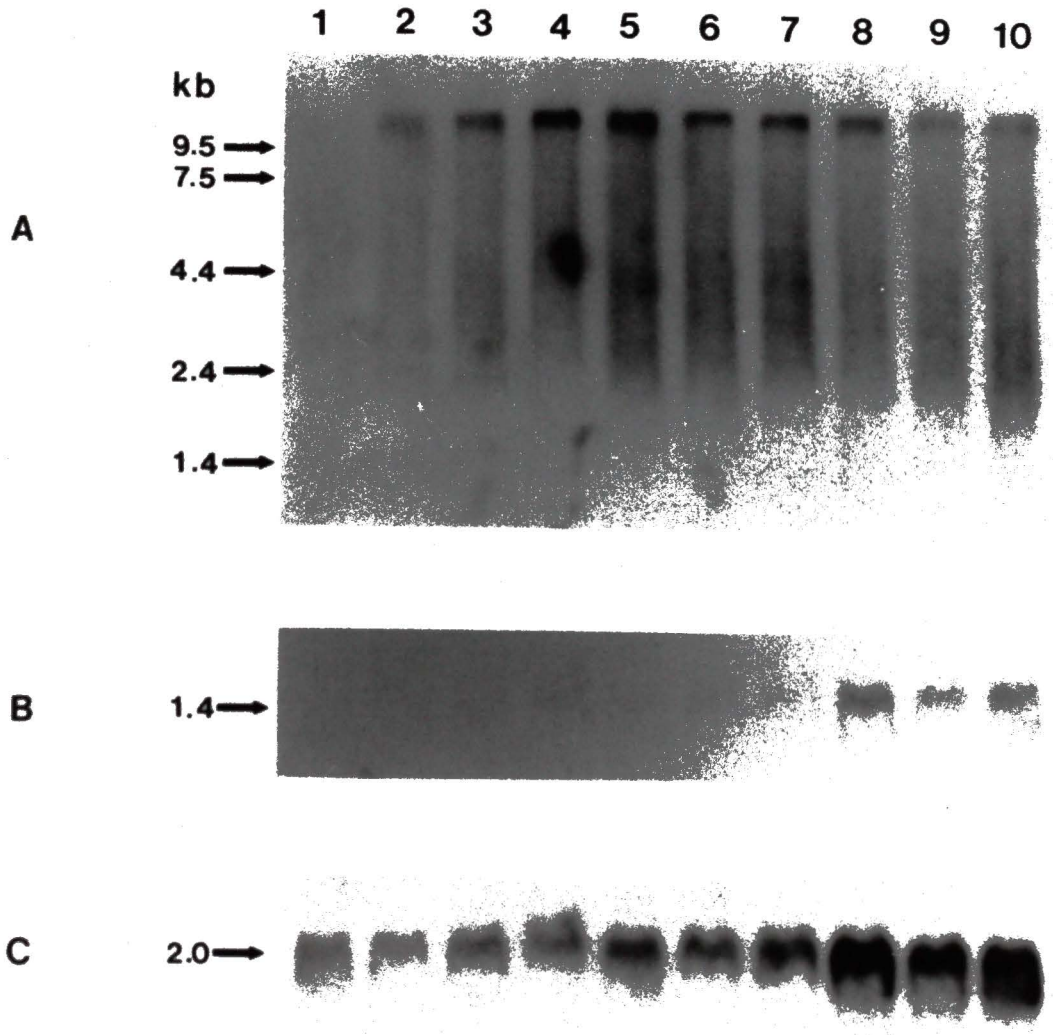

1
2
3
4
5
6
7
8
9
10
kb
9.5
7.5
4.4
2.4
1.4
A
1.4
B
2.0
C

Figure 3. Northern blot of *total* bovine lens epithelial cell RNA. Total RNA was extracted from lens cells exposed to high ambient sodium chloride medium for 12 (lane 1), 24 (lane 2) and 48 h (lane 3). (A) The gel stained with ethidium bromide showing the prominent 18S ribosomal RNA bands (arrow) as visualized with ultraviolet light. (B) The blot of the same gel with 18S ribosomal RNA oligo probe. (C) Northern analysis of the blot probed with genomic β-actin. Twenty μg of total RNA was applied to each lane.

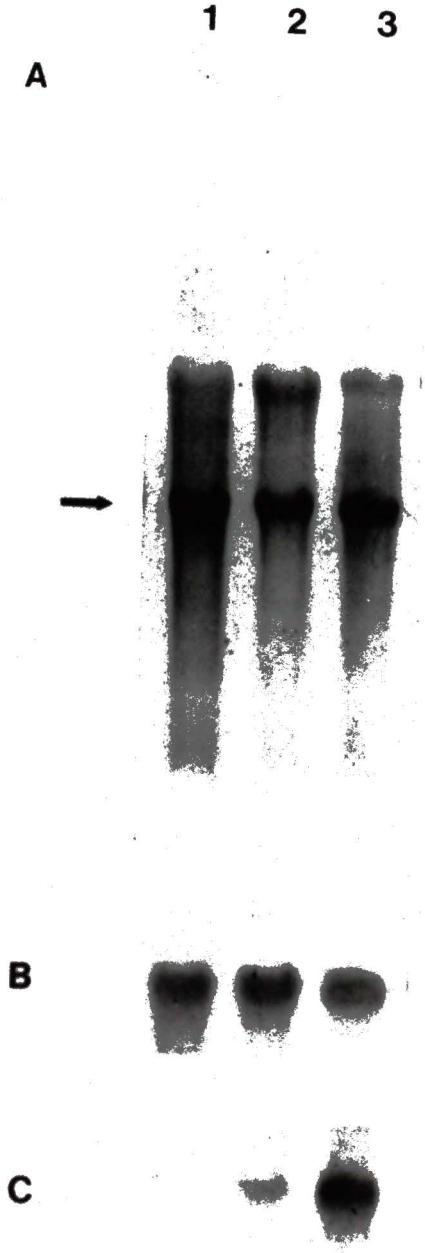

1
2
3
A
B
C

Figure 4. Time course of turnover of Na^+/MI cotransporter(s) by Northern blot analysis of bovine lens epithelial poly $(A)^+$ RNA. (A) RNA from lens cells incubated in high ambient sodium chloride medium for 24 h (lane 1) and switched to physiological medium for 4 (lane 2), 8 (lane 3), 12 (lane 4) and 24 h (lane 5) and probed with a Na^+/MI cotransporter 626 bp cDNA. (B) The same blot reprobed with genomic β-actin. Five μg of poly $(A)^+$ RNA was applied to each lane. The operational term "turnover" does not attempt to make a distinction between degradation of mRNA and changes in transcription rate.

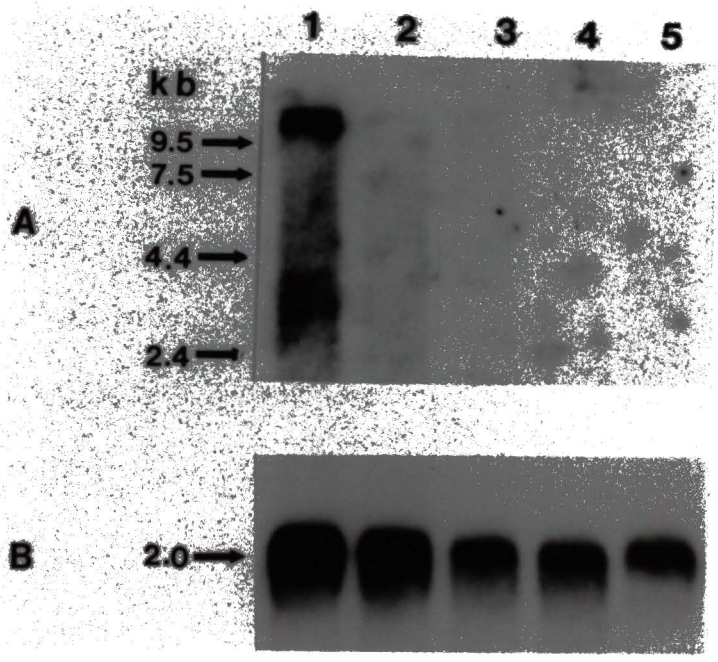
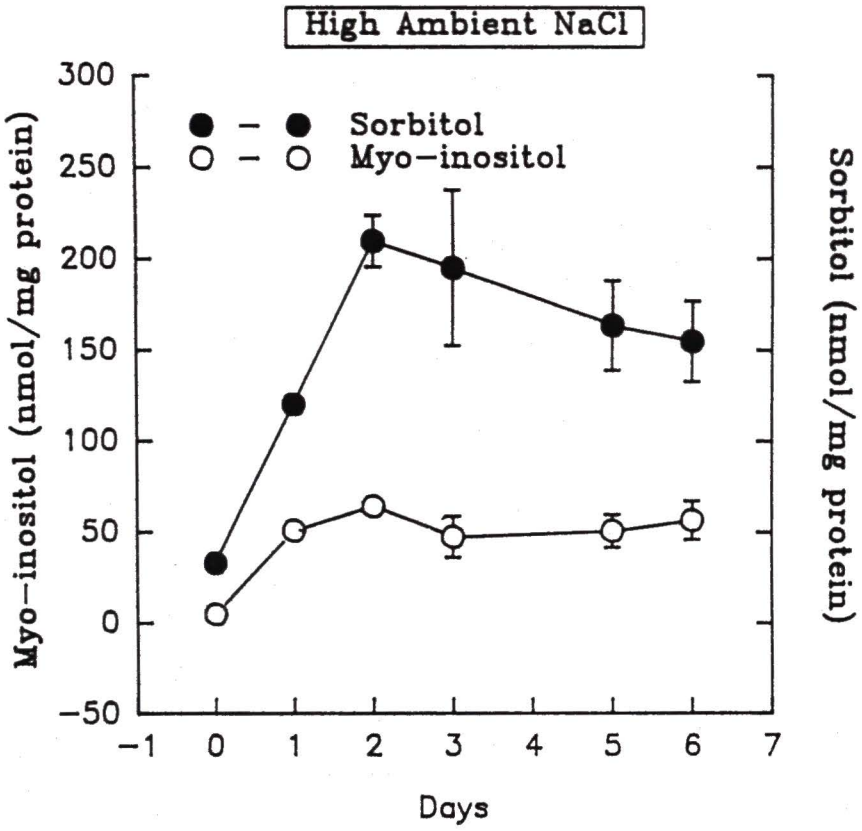

1
2
3
4
5
kb
9.5
7.5
A
4.4
2.4
B
2.0

Figure 5. Sorbitol and myo-inositol intracellular content in cultured lens cells incubated in high ambient NaCl medium (473 ± 6 mosm) without (left panel) or with (right panel) 0.1 mmol/l Sorbinil for 1 - 6 days. Data represents mean ± SE taken from replicate determinations from individual flasks. When the SEM is not visible it is due to the error bars being smaller than the symbol. *Significant from the myo-inositol determinations by NaCl without sorbinil treatment using analysis of covariance ($p < 0.05$).

Sorbitol and Myo-inositol Intracellular Content in Cultured Lens Cells Incubated in Hypertonic Medium



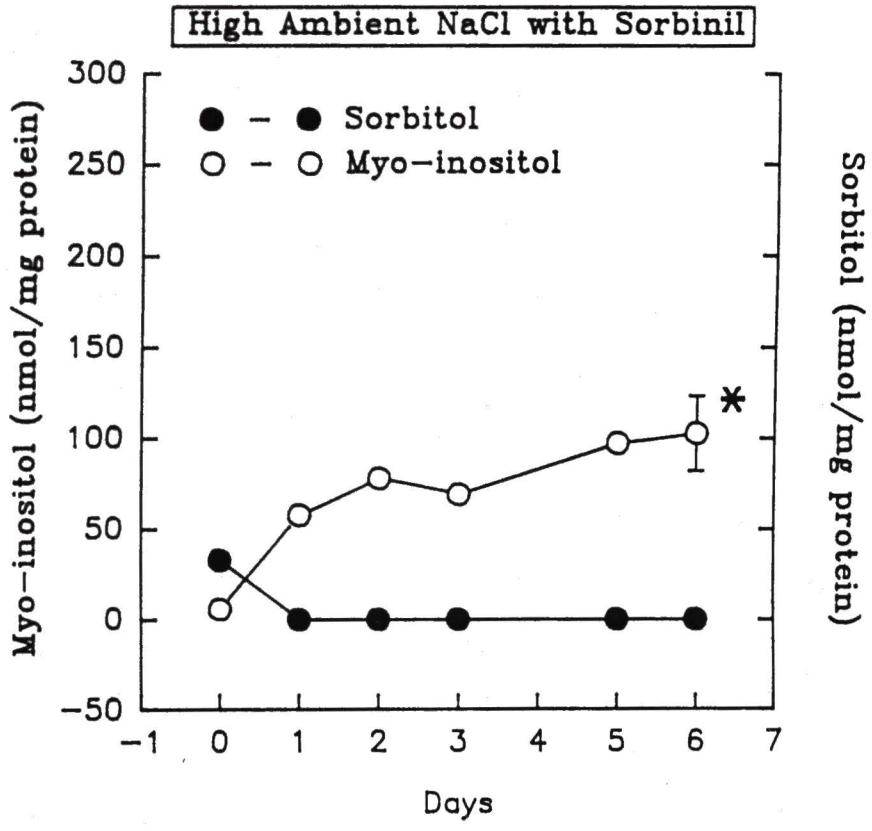
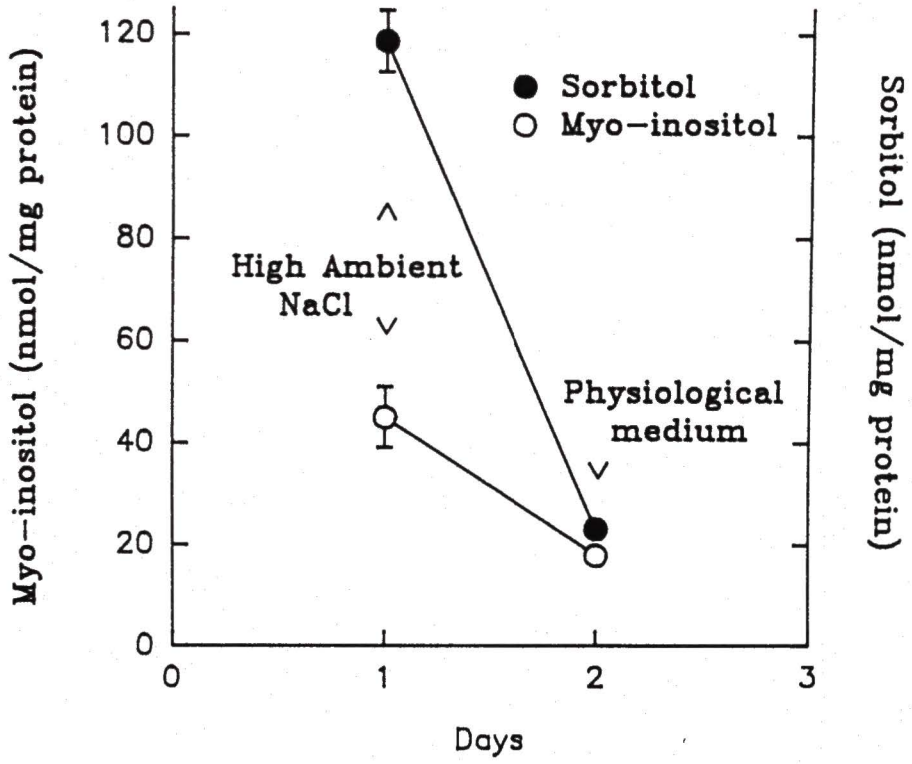


Figure 6. Sorbitol and myo-inositol intracellular content in cultured lens cells incubated in high ambient NaCl medium (473 ± 6 mosm) for 24 h and then returned to physiological (isotonic) medium (257 ± 2 mosm) for 24 h. Data represents mean ± SE taken from triplicate determinations from individual flasks.

Effect on Intracellular Myo-inositol and Sorbitol after Reversal from Hypertonic to Isotonic Medium



The previous two chapters established the osmoregulation of the Na^+/myo-inositol cotransporter gene expression in lens epithelial cells. However, the molecular mechanism of transcriptional regulation of this gene in lens cells in physiological or hypertonic conditions is unknown due to the lack of genomic information. In this study, the effects of osmotic insult on the transcription initiation of the Na^+/myo-inositol cotransporter gene in lens epithelial cells was investigated. The bovine Na^+/myo-inositol cotransporter gene was cloned and its promoters were functionally analyzed by transient transfection assays.

Chapter IV

The following manuscript will be submitted to ***Experimental Eye Research***.

Cloning the Bovine Na^+/Myo-inositol Cotransporter Gene and Characterization of an Osmotic-Responsive Promoter

Running Title: Hypertonicity Initiates Preferential Promoter Utilization

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Hyperosmotic-induced enhancement of myo-inositol accumulation in cultured bovine lens epithelial cells stems from increased uptake activity due to upregulation of the Na^+/myo-inositol cotransporter mRNA and *de novo* synthesis of myo-inositol carrier protein(s). The molecular mechanism of transcriptional regulation of the Na^+/myo-inositol cotransporter was investigated. The effects of hypertonicity on the 5'-ends of the Na^+/myo-inositol cotransporter mRNA was examined by use of the rapid amplification of cDNA ends (RACE) technique. 5'-RACE analysis revealed that two isotonic (i.e. physiologic) and one additional hypertonic transcription start sites were present in the Na^+/myo-inositol cotransporter gene in cultured bovine lens epithelial cells. Further, the bovine Na^+/myo-inositol cotransporter gene was cloned and its promoters characterized by transient transfection assays using luciferase reporter constructs of various fragments of the 5'-flanking regions upstream of the individual transcription start sites. Among the promoters, only the hypertonic promoter was osmotically responsive which showed a *2.7-fold* induction of activity subsequent to hypertonic insult. The region between -536 to -300 bp upstream of the hypertonic transcription start site contains the osmotic response element. Preferential utilization of the osmotically-responsive promoter contributes to the elevation of Na^+/MI cotransporter mRNA abundance and increased myo-inositol uptake activity as initiated by osmotic stress.

1. Introduction

When extracellular tonicity increases, cells shrink because of water loss. Cells subjected to hypertonicity initially regulate their volume by electrolyte uptake, a transitory event. Thereafter, the accumulated electrolyte is replaced with the concentration of compatible "organic osmolytes," in order to counterbalance elevated extracellular tonicity and prevent water loss (Yancey *et al.*, 1982). Osmoregulation via the Na^+/myo-inositol cotransporter has been demonstrated in renal cells (Handler and Kwon, 1993), brain glial cells (Paredes *et al.*, 1992) and lens epithelial cells. In cultured bovine lens epithelial cells (BLECs), myo-inositol (MI) is accumulated to concentrations 1000 times its extracellular concentration because of an increase in V_{max} of a sodium-coupled myo-inositol transport protein in response to hypertonicity (Cammarata and Chen, 1994). The hypertonic-induced enhancement of myo-inositol uptake activity is preceded by the augmentation of transcription and abundance of Na^+/MI cotransporter mRNA (Zhou, Agarwal and Cammarata, 1994; Zhou *et al.*, 1994) and *de novo* protein synthesis (Cammarata *et al.*, 1994). The molecular mechanism governing transcriptional regulation by hypertonicity of the Na^+/MI cotransporter was investigated in the current study. 5'-RACE analysis of Na^+/MI cotransporter cDNA indicated that multiple transcription start sites were utilized by the cultured lens epithelial cells. Besides two *isotonic* (i.e. physiologic) transcription start sites, osmotic insult elicits preferential utilization of a third, *hypertonic* transcription start site, located further upstream. The preferential utilization of the hypertonic transcription start site correlates with the induction of the Na^+/MI cotransporter mRNA abundance and enhancement of MI uptake activity. This was further confirmed by cloning

the bovine Na^+/myo-inositol cotransporter gene and characterizing its promoters. Transient transfection assay verified multiple functional promoters for the Na^+/myo-inositol cotransporter, of which, one was osmotically-responsive. Deletion of the sequence between -536 to -300 bp upstream of the hypertonic transcription start site caused the *loss* of the osmotic responsiveness of this promoter, indicative of an osmotic response element. These data provided evidence that preferential utilization of this osmotically-responsive promoter contributes to the hyperosmotic-induced upregulation of the Na^+/MI cotransporter gene expression in cultured bovine lens epithelial cells.

2. Materials and Methods

Cell culture

Bovine eyes obtained from a local slaughterhouse were brought on ice to the laboratory and the lenses removed aseptically. Lens epithelial cells were isolated and cultured as previously described (Cammarata, Jackson and Yorio, 1988).

Cloning the 5'-untranslated regions of the Na^+/myo-inositol cotransporter gene

The RACE (rapid amplification of cDNA ends) method (Forman, Dush and Martin, 1988) was used to amplify 5' untranslated regions of the Na^+/myo-inositol cotransporter utilizing a 5'-AmpliFINDER RACE kit (Clontech, Palo Alto, CA). The primer extension reaction was performed in a total volume of 30 µl containing: 2 µg of poly $(A)^+$ RNA obtained from cultured BLECs incubated in physiologic medium (PM, 266±2 mosm) or PM further supplemented with 200 mM urea (451±2 mosm), 150 mM D-mannitol (414±2 mosm) or 116 mM NaCl (467±2 mosm) ± the aldose reductase inhibitor, sorbinil (Pfizer, Groton, CT) for 10 hours, respectively, 10 pmol of a gene-specific primer (p1) complimentary to nucleotides +101 to +120 relative to the ATG translation initiation codon of the Na^+/MI transporter cDNA (Kwon *et al.*, 1992, 5'-GAAGTATCCACTCAC GGTGC-3') and 20 units of AMV reverse transcriptase (Clontech, Palo Alto, CA). The reaction mixture was incubated at 52°C for 30 min. A single strand nucleotide anchor 5'-CACGAATTCACTATCTGATTCTGGAACCTTCA GAGG-3' was ligated to the 3'-end of the primer extension product by using T4 RNA ligase (Edwards, Delort and Mallet,

1991). One percent of the total ligation product was used as the template for PCR amplification. The PCR antisense primer was a nested gene-specific primer (p2) designed to be complimentary to nucleotides +17 to + 46 relative to the ATG of the MDCK Na^+/MI cotransporter cDNA (5'-ACAGGGCCACTATGGCAAT GTCTGCTGTCT-3'). The anchor primer, 5'-CTGGTTCGGCCCACCTCTGAAGGTTC CAGAATCGATAG-3', was the sense primer. Ten pmol of each primer was used for the PCR reaction in a total volume of 50 µl containing: 0.2 mM of each dNTPs, 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$ and 2.5 units of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). The PCR program was as follows: 94°C 2 min., 35X [94°C 1 min, 57°C 1 min, and 72°C 2 min], 72°C 7 min. The PCR products were electrophoresed on a 1.2% agarose gel. It should be pointed out that the size of RACE products are the sum of 5'-untranslated region and primers (anchor primer and p2) plus a 16 bp coding region (about 93 bp total). The RACE cDNAs were isolated and subcloned into vector pCRII (TA cloning system kit, Invitrogen, San Diego, CA). The nucleic acid sequence of the 5'-RACE cDNAs (*a-d*) were sequenced by using the dideoxynucleotide method (Sequenase kit, version 2.0, United States Biochemical, Cleveland, OH). The DNA sequences were determined by use of DNA Scan, an image-processing and analysis computer program for the Unix SparcStation as developed by Protein Data Imaging (Huntington Station, New York).

Northern blot analysis

Poly $(A)^+$ RNA was extracted from cultured BLECs incubated in physiologic medium or PM further supplemented with 200 mM urea, 150 mM D-mannitol or 116 mM NaCl

medium for 10 hours using a Fast Track mRNA isolation kit (Invitrogen, San Diego, CA). Two μg of poly(A)$^+$ RNA from each condition was utilized for the Northern analysis as previously described (Zhou, Agarwal and Cammarata, 1994). The blot was hybridized with a ^{32}P-labeled **626 bp** Na$^+$/myo-inositol cotransporter cDNA probe (7 x 10^8 cpm/μg) from cultured bovine lens epithelial cells (Zhou, Agarwal and Cammarata, 1994) using a Random Primed DNA Labeling kit (Boehringer-Mannheim, Indianapolis, IN), stripped according to previously described procedures (Agarwal, Nir and Papermaster, 1990) and rehybridized with a β-actin genomic DNA probe.

A 109 bp cDNA with a sequence encoding a *distinctive* portion of the hypertonically-responsive RACE product ***a*** was amplified by PCR using primers (**p3 and p4**, see Fig. 2 for positions) designed to permit the isolation of a PCR product that included a cDNA sequence not replicated in the other three RACE products ***b-d*** (refer to Results). The PCR primers had the following sequences; 5'-AGTGGAGCCCGTCAGCTTCT-3' (p3) and 5'-ACTCGAGGCATGCCCGAATGGATGGCG-3' (p4). The PCR template was the longest obtained RACE product ***a*** (containing the **522 bp** 5'-UTR) subcloned into pCRII vector. The **109 bp** cDNA was then subcloned into the pCRII vector and the nucleotide sequence confirmed. The blot was hybridized with the ^{32}P-labeled 109 bp cDNA probe (7 x 10^7 cpm/μg), prepared by a modified PCR method (Sturzl *et al.*, 1991).

Myo-inositol accumulation

The cultured cells, initially maintained in serum-supplemented physiologic medium, were divided into four groups, and the medium replaced with fresh physiologic medium or PM

augmented with 200 mM urea, 150 mM mannitol or 116 mM NaCl for a 20 hour incubation period. All culture medium contained 15 μM myo-inositol. Thereafter, the cells were transferred to their appropriate (serum-free) medium (half of each group going into medium consisting of 25 μM myo-inositol and the other half switched to medium containing 400 μM myo-inositol). All media contained a trace amount of myo-[^{3}H]inositol (0.25 μCi/ml, 94 Ci/mmol, Amersham, Arlington Heights, IL). BLECs were subjected to a three hour uptake period at 37°C. Afterwards, the cell monolayer was prepared for liquid scintillation counting as previously described (Cammarata *et al.*, 1992a). Replicate 1.0 ml aliquots were taken for liquid scintillation counting. Triplicate 25 μl aliquots were taken for protein determination as previously described (Bradford, 1976).

Isolation and characterization of the bovine Na^+/myo-inositol cotransporter gene

A bovine liver genomic library in EMBL-3 SP6/T7 vector (Clontech, Palo Alto, CA) was screened. Approximately 2 x 10^6 bacteriophage plaques were screened with the [^{32}P]-labeled 626 bp cDNA probe (located near the 3'-end of the open reading frame, ORF, Zhou, Agarwal, Cammarata, 1994) to isolate genomic clones that contained the entire ORF, part of the 5'-untranslated region, UTR (including transcription start sites b and c), and the 3'-end of the intron. The 109 bp RACE cDNA *a* specific probe was also used to screen and isolate additional clones with exon 1 (the remainder of the 5'-UTR) and 5'-end of the intron. After screening, positive clones were further characterized by Southern hybridization. Appropriate restriction fragments were subcloned into pBluescript KS (-) (Stratagene, La Jolla, CA) for further analysis. Collectively, restriction mapping, Southern

blots analysis, and sequence analysis were combined to characterize the genomic structure. The nucleic acid sequences were determined by using the dideoxynucleotide method (Sequenase kit, version 2.0, United States Biochemical, Cleveland, OH). The DNA sequences were determined by use of DNA Scan, an image-processing and analysis computer program for the Unix SparcStation as developed by Protein Data Imaging (Huntington Station, New York).

Promoter-Luciferase constructs

Figure 5B is a schematic representation of the promoter-luciferase constructs. For promoter a (where the transcription start site of *a* is designated as +1), the DNA fragments summarized and listed as -3.8kb/+600, -2.8kb/+600, and -1043/+600, respectively, were digested by appropriate restriction enzymes and subcloned upstream of the luciferase reporter gene in pGL3-basic vector (Promega, Madison, WI). Fragment -2.8kb/+600 was also inserted into pGL3-basic in the reverse orientation to make the p(-2.8kb/+600)asLuc construct. DNA fragments -769/+52, -536/+52, and -300/+52 were amplified by PCR and subcloned into pCRII vector. The sequences of the sense oligonucleotide PCR primers are summarized and listed as follows: 769/+52, 5'-AATAGGGGAGTCCTCCCA-3'; -536/+52, 5'-GGAAGGAGAACCCCGTGT-3'; and -300/+52, 5'-TCTGAGGGAGGCTATCGGT-3', respectively. The anti-sense primer is 5'-AGAAGCTGACGGGCTCCACT-3'. These DNA fragments were verified by sequencing, isolated by digestion with *Kpn I + Xho I* or *Hind III + Xho I*, depending on the orientation of the fragments in pCR II, and subcloned into pGL3-basic vector.

For promoters *b* and *c* (where the transcription start site *b* is designed as +1'), DNA fragments -1180'/+37' (containing isotonic promoter *b* only) and -1180'/+279' (containing isotonic promoters *b* and *c*) were PCR amplified and subcloned into pGL3-basic vector as described above. The sense PCR primer is 5'-CCTGTCATATTCACAT-3'. The anti-sense primers are -1180'/+37', 5'-GTTGCAGCTAAGTCTAGTGA-3'; and -1180'/+279', 5'-TGAGTAACCCCAGCCAA-3'. Fragment -1180'/+279' was also inserted into pGL3-basic in the reverse orientation to make the p(-1180'/+279')asLuc construct. Plasmid DNA was purified using QIAGEN (Chatsworth, CA) plasmid purification kit.

Transient transfection, luciferase, and β-galactosidase assays

Twenty-four hours prior to transfection, primary cultures of bovine lens epithelial cells were trypsinized and seeded at 2 x 10^5 cells per 60 mm tissue culture dish. The culture medium was replaced with fresh medium two to four hours before transfection. Transient transfection was carried out using the calcium phosphate method with BES buffer (Chen and Okayama, 1987). For each 60-mm dish, 0.1 ug/kb of the construct DNAs, 0.5 ug of pCMV-lac DNA, and pUC19 DNA to 5.5 ug total DNA were added. Plasmid pCMV-lac, β-galactosidase gene driven by the cytomegalovirus promoter, was used to assess the transfection efficiency. After adding the DNA:$CaPO_4$ precipitates into the dish, cells were maintained at 37°C, 3% CO_2 for 16 hours. Transfected cells were washed twice with PBS and maintained in physiologic medium for 24 hours at 37°C, 5% CO_2, then either replaced with fresh physiologic medium or switched to hypertonic medium (physiologic medium

supplemented with an additional 116 mM NaCl) for an additional 24 hours. Approximately 64 hours after the transfection, cells were lysed. Both the luciferase and β-galactosidase activities were measured. Each construct was assessed by transfection assay using duplicate determinations from a minumun of four individual cell populations.

Enhanced luciferase assay kit (Analytical Luminescence Laboratory, San Diego, CA) was utilized to determine luciferase activity. Cells were lysed with 300 ul of 1% Triton X-100, 0.1 M phosphate buffer, pH 7.8, 2 mM EDTA, and 1 mM DTT. Fifty ul of lysate was added to 100 ul of substrate A, and the luciferase reaction initiated by the rapid injection of 100 ul of substrate B. Light output was measured for 10 seconds at room temperature using a monolight luminometer (model 2010, Analytical Luminescence Laboratory, San Diego, CA).

β-Galactosidase activity was measured using a chemiluminescent reporter assay kit (TROPIX, Bedford, MA). Twenty ul of lysate was added to 200 ul of reaction buffer and incubated at room temperature for 60 min. After addition of 300 ul of light emission accelerator, light output was measured for 5 seconds using a monolight luminometer (Analytical Luminescence Laboratory, San Diego, CA).

Luciferase activity of each construct was first normalized to the β-galactosidase activity. Relative luciferase activities were expressed as relative units compared to luciferase activity of *hypertonically*-treated cells that transfected with p(-536/+52)Luc (luciferase activity of p(-536/+52)Luc was designated as 100).

3. Results

Identification of the bovine Na^+/myo-inositol cotransporter gene transcription start sites

The rapid amplification of cDNA ends technique was performed to amplify the 5'-end of the Na^+/myo-inositol cotransporter from cultured bovine lens epithelial cells (Fig. 1). The positions of the primers (**p1**, **p2**, and anchor primer) and anchor used for RACE is shown in Fig. 1A. An antisense oligonucleotide primer **p1** complementary to nucleotides +101 to +120 relative to the ATG translation initiation codon of the Na^+/MI cotransporter gene was used to extend poly(A)$^+$ RNA. An anchor was ligated to the 3'-end of the primer extension cDNAs. These cDNAs were PCR amplified using a nested gene-specific primer, **p2**, complementary to nucleotides +17 to +46 and an anchor primer. The RACE products from cultured BLECs maintained in physiologic medium ± sorbinil or PM further supplemented with 200 mM urea, 150 mM D-mannitol, or 116 mM NaCl is shown in Fig. 1B. Two RACE products ***b*** and ***c***, which included **339** and **225 bp** of 5'-UTR, respectively, were cloned from BLECs cultured in physiologic medium (266±2 mosm). The RACE products ***b*** and ***c*** were observed under all the experimental conditions, irrespective of medium tonicity. An *unique* RACE product ***a***, which included **522 bp** of 5'-UTR, was obtained with BLECs exposed to NaCl hypertonic medium (467 ± 2 mosm), irrespective of whether sorbinil was present or absent. The RACE product ***a*** was, likewise, observed with cells exposed to PM made hyperosmotic by the further supplementation of 150 mM D-mannitol (414 ± 2 mosm) or 200 mM urea (451 ± 2 mosm). These results established that the RACE product ***a*** was the direct result of the hyperosmotic insult.

RACE product *d* (90 bp 5'-UTR) was obtained as a result of the urea, D-mannitol and sorbinil treatments, but not from BLECs cultured in physiologic medium or the hypertonic NaCl medium in the absence of sorbinil. The manifestation of RACE cDNA *d* appeared not to be associated with extracellular tonicity. The possible function, if any, of RACE product *d*, is presently ambiguous and was not further investigated other than that it was included in the sequence analysis (see below).

The nucleotide sequence of the four RACE products was determined (Fig. 2). Nucleic acid sequence analysis of the four RACE products indicated homologous, overlapping sequences with different 5' initiating ends. The sequences of the overlapping regions were identical among the four RACE products. These results suggest that the 5'-RACE cDNAs represent transcripts resulting from *multiple* transcription start sites. These data also implicate that hypertonicity as a modulating impetus for Na^+/MI cotransporter gene expression regulates Na^+/MI cotransporter gene transcription via an hypertonically-sensitive transcription start site.

Northern blot analysis

Northern blot analysis of poly $(A)^+$ RNA extracted from BLECs cultured in physiologic medium or PM supplemented with 200 mM urea, 150 mM D-mannitol or 116 mM NaCl and hybridized with the 626 bp bovine lens Na^+/MI cotransporter cDNA probe (Zhou, Agarwal and Cammarata, 1994) is shown in Fig. 3A. The 626 bp probe includes the cDNA sequence encoding the polypeptide of a putative major extracellular loop (Kwon *et al.*, 1992) of the Na^+/MI cotransporter, and as such represents a part of the mRNA

transcript encoded for by the open reading frame. As previously demonstrated (Zhou, Agarwal and Cammarata, 1994; Zhou *et al.*, 1994) and reconfirmed in figure 3A, the blot probed with the **626 bp** cDNA typically shows the predominant 10.5 kb band. The bovine lens Na^+/MI cotransporter mRNA content extracted from epithelial cells maintained in physiologic medium is normally very low (although a band can be visualized by overexposure). A higher level of Na^+/MI cotransporter mRNA was observed with BLECs exposed to PM supplemented with 200 mM urea (Fig. 3A, lane 2) and was increasingly more evident with the 150 mM D-mannitol treatment (Fig. 3A, lane 3). The highest level of induction of the Na^+/myo-inositol cotransporter mRNA was observed when BLECs were exposed to hypertonic NaCl medium (Fig. 3A, lane 4). After hybridization with the **626 bp** Na^+/MI cotransporter cDNA probe, the blot was stripped and reprobed with a genomic probe for β-actin (Fig. 3B). The relative level of β-actin was similar in all lanes.

To determine whether the transcript from the hypertonic transcription start site represents at least a portion of the hyperosmotically-induced upregulated Na^+/MI cotransporter mRNA, the blot was again stripped and subsequently hybridized with the **109 bp** RACE cDNA ***a*** specific probe. The **109 bp** cDNA probe includes cDNA sequence encoding a portion of the hypertonic RACE cDNA ***a*** specific region (from -489 bp to -380 bp upstream of ATG, between primers **p3** and **p4** as shown in Fig. 2). In other words, the **109 bp** cDNA probe is specific to RACE product ***a*** and, as such, is not duplicated in any of the other three RACE products. The **109 bp** cDNA probe (Fig. 3C), like the **626 bp** cDNA probe (Fig. 3A), hybridized to the 10.5 kb mRNA. The pattern of

abundance of Na^+/MI cotransporter mRNA observed with the **109 bp** cDNA was identical to that paradigm observed with the **626 bp** cDNA probe, i.e. the highest level resulted from hypertonic NaCl > hypertonic D-mannitol > hypertonic urea > physiologic medium. Hybridization with the **109 bp** cDNA probe revealed, not only, the 10.5 kb band, but two other bands as well, 2.7 kb and 1.1 kb (Fig. 3C).

Myo-inositol accumulation

Myo-[^{3}H]inositol uptake was markedly enhanced in cultured BLECs exposed to hyperosmotic medium by the addition of extracellular urea, D-mannitol or NaCl for 20 hours (Fig. 4). Myo-[^{3}H]inositol accumulation exhibited stimulation at both 25 μM and 400 μM myo-inositol, conditions shown to be optimal for the conjectured high- and low-affinity MI transport sites (Cammarata, *et al.*, 1992b), suggesting that the high- and low-affinity Na^+/MI cotransporter activities are coregulated; both of which being induced by hypertonic stress. The degree of enhanced MI uptake activity was as follows; hypertonic NaCl > hypertonic D-mannitol > hypertonic urea > physiologic medium. The pattern of enhanced MI uptake activity (Fig. 4) was consistent with the order of increased Na^+/MI cotransporter mRNA abundance (Fig. 3), suggesting that the enhancement of Na^+/MI cotransporter uptake activity in cultured BLECs is primarily controlled at the level of transcription.

Isolation and characterization of the bovine Na^+/MI cotransporter gene

A bovine liver genomic library was initially screened by use of the **626 bp** cDNA probe

(Zhou, Agarwal, Cammarata, 1994). After several positive clones were isolated, Southern hybridization, restriction mapping, and sequence analysis revealed that those clones contained the entire 2154 bp ORF, as well as part of the 5'-UTR (340 bp) which included transcription start sites ***b*** and ***c***, and the 3' portion of the intron. One of these clones, λBSMIT2, was further analyzed. By again screening the library, but this time using the **109 bp** RACE cDNA ***a*** specific cDNA probe, several clones were isolated which contained the remain 5'-end of the 5'-UTR (from -522 bp to -341 bp upstream of the translation start codon ATG) which included transcription start site ***a***, the 5'-flanking region upstream of the transcription start site ***a***, and the 5' portion of the intron. A clone, designated as λBSMIT11, was further studied. A schematic diagram of the bovine Na^+/MI cotransporter gene structure is shown in Fig. 5A. It has an intron free ORF (2154 bp) and an intron, of as yet undetermined size, located in the 5'-UTR which separates transcription start site ***a*** from ***b***. There are consensus splicing donor and acceptor sequences 5'gt....ag3' at the intron-exon junction.

Partial nucleotide sequence and the deduced amino acid sequence of the bovine Na^+/MI cotransporter gene are shown in Fig. 6. Fig. 6A represents the partial nucleotide sequence of clone λBSMIT11 from -1043 to + 231 bp (transcription start site ***a*** is designated as +1). There is a TATA-like sequence, $_{-30}$TTTAAG$_{-25}$, located 30 bp upstream of the transcription start site ***a***. A transcription activator Sp1 binding element, $_{-76}$CCCGCC$_{-71}$, is located 41 bp away from the TATA-like box, which is regarded as a functional location (30-70 bp away from TATA box) for Sp1 (Dynan and Tjian, 1985). For the sequence

from -1180' to +2856' bp of the clone designated λBSMIT2 (transcription start site ***b*** is designated as +1'), two TATA-like sequences are located 38 and 24 bp upstream of the transcription start sites ***b*** and ***c***, respectively (Fig. 6B). The sequence surrounding transcription start site ***c***, CTCAATCC, differs by only one nucleotide from the consensus TdT initiator motif $_{-3}$CTCANTCT$_{+5}$ (Smale *et al.*, 1990). Although this initiatior was reported to be capable of directing basal transcription in TATA-less promoters, it has been shown that the initiator can also direct transcription in the presence of a TATA box and that the initiator and the TATA box can function synergistically (Concino *et al.*, 1984; Roeder, 1991). The positions of the transcription start sites ***a-d*** in the genomic sequence were determined by alignment of genomic sequence with the RACE cDNA sequences as shown in figure 2.

Characterization of the Na^+/MI cotransporter promoters

Transient transfection assays in BLECs maintained in physiologic or hypertonic medium were used with luciferase reporter constructs of various fragments of the 5'-flanking regions upstream of individual transcription start sites and promoter activities determined (Fig. 7). The promoter-luciferase constructs p(-2.8kb/+600)Luc, p(-1180'/+37')Luc and p(-1180'/+279')Luc (refer to Fig. 5B for a schematic description of the constructs), which constitute the 5'-flanking regions upstream of transcription start sites ***a***, ***b***, and ***b+c***, respectively, were transiently transfected into separate BLEC cultures. The additional constructs, p(-2.8kb/+600)asLuc and p(-1180'/+279')asLuc, which contain 5'-flanking

regions of transcription start sites ***a*** and ***b+c***, respectively, inserted upstream of the luciferase reporter gene in the *anti-sense orientation*, were also transiently transfected, as was the empty vector. The pCMV-lac construct was cotransfected, in every case, to ascertain transfection efficiency.

One-half of each set of transfected cells was maintained in physiologic medium (266±2 mosm) prior to the cells being lysed for subsequent luciferase and β-galactosidase assays. As indicated in Fig. 7, the 5'-flanking regions upstream of transcription start sites ***a***, ***b***, and ***b+c*** resulted in luciferase activities 15 to 33-fold greater than the activity observed with the empty vector (*promoterless* pGL3-basic). The reverse orientation constructs showed promoter activity equivalent to the empty vector. These results confirm that promoters ***a*** and ***b***, and possibly ***c*** are functional in cultured BLECs. Moreover, the results of the promoter analysis supports the 5'-RACE analysis in that the generation of multiple 5'-RACE cDNAs are likely due to utilization of different promoters rather than to an artificial termination of the reverse transcription reaction.

In order to verify whether any of the promoter regions were osmotically-responsive, the other half of each set of transfected cells was exposed to hypertonic medium (467±2 mosm, physiologic medium supplemented with 116 mM NaCl) for 24 hours, and thereafter, assessed for luciferase and β-galactosidase activity. The promoter activity of constructs p(-1180'/+37')Luc (isotonic promoter ***b***) and p(-1180'/+279')Luc (isotonic promoters ***b*** + ***c***) *did not* significantly increase after cell exposure to hypertonic medium when compared to the physiologic condition (Hypertonic/Isotonic= 1.2 and 1.1,

respectively). However, a *2.7-fold* induction of promoter activity was observed in cells transfected with p(-2.8kb/+600)Luc which contains the osmotically-responsive promoter ***a*** (Isotonic: 7.7±1.3 vs Hypertonic: 20.8±1.8). These data indicate that, among the Na^+/MI cotransporter promoters, only the hypertonic promoter ***a*** is osmotically responsive.

The osmotic-responsiveness of promoter ***a*** was further characterized. A series of promoter-luciferase constructs containing various lengths of the 5'-flanking region upstream of hypertonic transcription start site ***a*** were prepared (Fig. 5B). Cultured lens epithelial cells maintained in physiologic medium and subsequently transfected with each of the following constructs: p(-3.8kb/+600)Luc, p(-2.8kb/+600)Luc, or p(-1043/+600) Luc, all showed similar promoter activity. Moreover, hypertonically-treated cells displayed similar fold-induction of promoter activity with 3.0, 2.7, and 2.6-fold induction relative to those in physiologic medium. These data demonstrate that the sequence from -3.8 kb to -1043 bp does not significantly influence the promoter activity as well as the osmotic responsiveness of the promoter ***a***. Transient transfection with construct p(-769/+52)Luc resulted in elevated promoter activity for both physiologic-treated and hypertonic-treated cells, suggesting that the sequence between -1043 to -769 bp contains a negative regulatory cis-element. Since the construct p(-769/+52)Luc still had the osmotic-response (Hypertonic/Isotonic= 2.0), neither this negative cis-element nor the sequence between -1043 to -769 bp is regulated by the tonicity. Both promoter activity and the ensuing effect by osmotic insult on construct p(-536/+52)Luc was identical toconstruct p(-769/ +52)Luc, suggesting that the sequence between -769 to -536 bp does not bear any influence on promoter activity. However, further deletion to -300 bp abolished the

osmotic response of the promoter (Hypertonic/isotonic= 1.2). Therefore, a putative *osmotic response element is located between -536 to -300 bp*. Moreover, the promoter activity of construct p(-300/+52)Luc, irrespective of medium tonicity, was dramatically reduced relative to p(-536/+52)Luc, suggesting a putative *positive cis-element* exists between *-536 to -300 bp*.

4. Discussion

To better understand the molecular mechanisms of osmoregulation which regulates Na^+/MI cotransporter gene expression, we investigated the effect of hypertonicity on transcription initiation of the Na^+/MI cotransporter gene in cultured BLECs. The bovine Na^+/MI cotransporter gene was cloned and its promoters characterized. An osmotically-responsive promoter was identified and the genomic position of its osmotic response element localized.

The 5'-RACE experiments revealed multiple transcription start sites for the bovine lens Na^+/MI cotransporter gene. Two RACE cDNAs ***b*** and ***c*** (with 339 and 225 bp of 5'-UTR) were uncovered from cultured cells maintained in physiologic medium; hypertonic insult resulted in the generation of a novel RACE cDNA ***a*** (with 522 bp of 5'-UTR) (Fig. 1). Nucleotide sequence analysis of the RACE cDNAs (Fig. 2) displayed identical overlapping sequences starting from different 5'-ends, suggesting that these RACE cDNAs were the products of different transcription start sites (i.e. alternative promoters) within the Na^+/MI cotransporter gene. Multiple transcription start sites have previously been reported for the canine betaine γ-amino-*n*-butyric acid transporter gene (Takenaka *et al.*, 1995), another

example of an osmotically-responsive gene.

Preferential utilization of the hypertonic transcription start site ***a*** resulted in an increase of the Na^+/MI cotransporter mRNA abundance as evident by Northern blot analysis using the 109 bp cDNA probe (specific to the hypertonically-sensitive RACE cDNA ***a***, Fig. 3C). In other words, under hyperosmotic conditions, at least some portion of the 10.5 kb transcript *must* have been generated from this transcription start site. Because of the difference in the specific activity of the two cDNA probes, it was not possible to determine the precise portion the transcript from the hypertonically-sensitive start site ***a*** represented relative to the total Na^+/myo-inositol cotransporter mRNA. However, a positive correlation could be established with regards to the relative abundance of Na^+/myo-inositol cotransporter mRNA (Fig. 3) and enhanced myo-inositol carrier protein activity, as demonstrated by the increased intracellular accumulation of myo-[^{3}H]inositol (Fig. 4).

To lend further support to these conclusions, the bovine Na^+/MI cotransporter gene was cloned by screening a bovine genomic library. The Na^+/MI cotransporter gene contains an intron free open reading frame, but an intron (of undetermined size) is located in the 5'-UTR between transcription start sites ***a*** and ***b*** (Fig. 5A). The cloning of both the human and bovine Na^+/myo-inositol cotransporter genes have previously been reported (Berry *et al.*, 1995, Mallee *et al.*, 1996), but those reports were restricted to the genomic sequence of the open reading frame only. Ours is the first report of cloning the bovine Na^+/myo-inositol cotransporter gene to include not only the open reading frame, *but the 5'-flanking regions as well as the entire 5'-UTR.*

We cannot definitively rule out by 5'-RACE analysis alone the possibility that some of the multiple transcription start sites may be due to an artificial termination of the reverse transcription reaction by secondary structure. However, transient transfection of BLECs utilizing promoter-luciferase constructs containing 5'-flanking regions upstream of transcription start sites ***a***, ***b*** and ***b+c*** of the bovine Na^+/MI cotransporter gene supports the functionality of promoter ***a*** and promoter ***b*** and possible promoter ***c***. In accord with this is the fact that anti-sense orientation constructs p(-2.8kb/+600)asLuc and p(-1180'/+279')as Luc lost promoter activity.

Exposure of transfected cells to hypertonic insult revealed that only promoter ***a*** was osmotically-responsive (Fig. 7). Osmotic stress resulted in a 2.7-fold induction of this promoter activity. The two isotonic promoters, ***b*** and ***c***, appear not to be regulated by changes in extracellular tonicity. Since only promoter ***a*** is induced under hypertonic stress, it follows that while as many as three promoters may be functional in the bovine Na^+/MI cotransporter gene, only one promoter is *osmotically-responsive*. The isotonic promoter ***b*** (and possibly c) are, by all indications (Fig. 1B), nonpreferentially utilized regardless of tonicity. Although promoter ***a*** is likely also utilized at a low level under normal physiologic conditions, hyperosmotic insult potentiates preferential transcription initiation via the osmotically-responsive promoter ***a***, which, as discussed above, positively correlated with the induction of Na^+/MI cotransporter mRNA abundance (Fig. 3) and enhanced transporter activity (Fig. 4).

Two other eukaryotic osmotic response elements (tonicity-sensitive elements) have

been identified to date; one for the aldose reductase gene (Ferraris *et al.*, 1994) and the other for the betaine-γ-amino-n-butyric acid transporter gene (Tekenaka *et al.*, 1994). To the best of our knowledge, the current study is the first to characterize multiple Na^+/MI cotransporter promoters and identify an osmotic response element in promoter ***a***. Multiple cis-elements, including both positive and negative regulatory cis-elements, also appear to function in promoter ***a***. The putative osmotic response element, which apparently mediates the hypertonic induction of promoter ***a*** activity, is located between -536 to -300 bp upstream of transcription start site ***a***. Experiments are in progress to further localize the osmotic response element of the Na^+/MI cotransporter gene.

Acknowledgments

This work represents partial fulfillment of the requirements for the doctoral degree for Cheng Zhou.

FOOTNOTES

The abbreviations used are: BLECs, bovine lens epithelial cells; MI, myo-inositol; 5'-UTRs, 5'-untranslated regions; RACE, rapid amplification of cDNA ends; PM, physiological medium; MDCK, Madin-Darby canine kidney; PCR, polymerase chain reaction; bp, base pairs; ORF, open reading frame.

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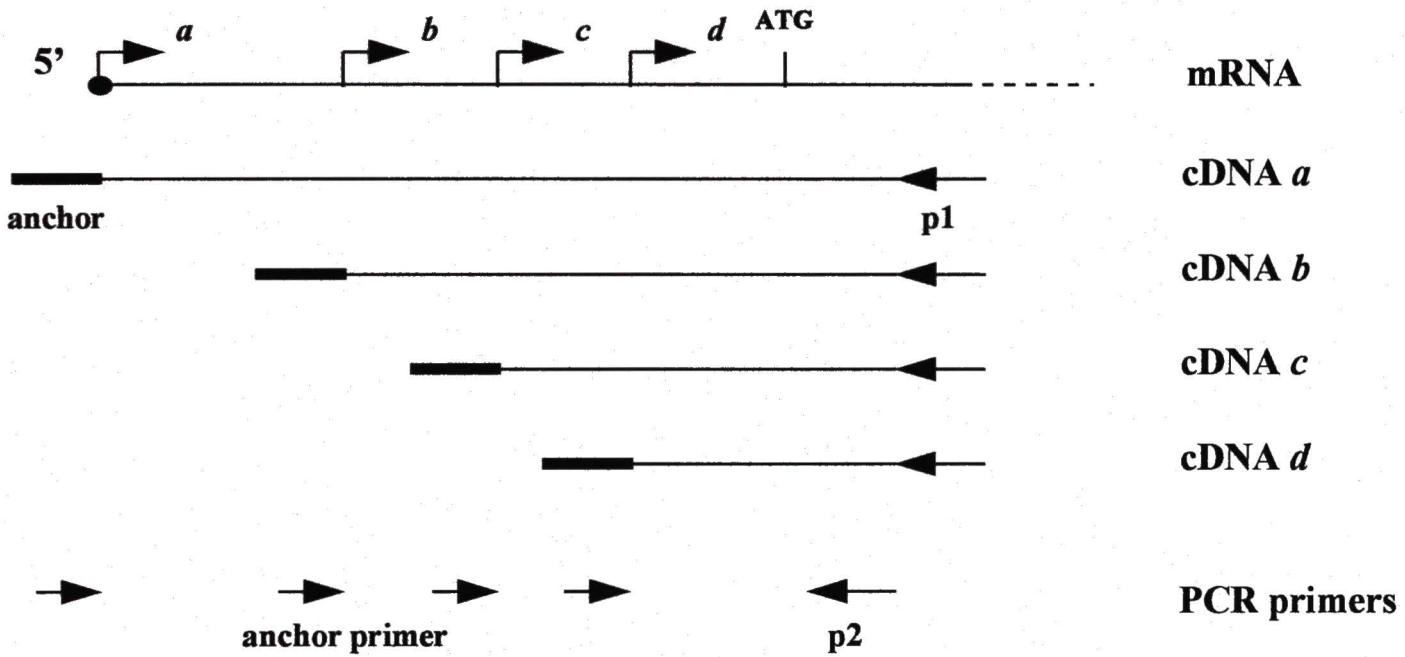
Figure 1. Identification of the Na^+/myo-inositol cotransporter gene transcription start sites in cultured bovine lens epithelial cell. *A*: schematic diagram of 5'-RACE analysis. The mRNA and first-strand cDNAs are indicated by *thin lines*. The anchor is indicated by *the thick line*. The positions and directions of primers used for primer extension (p1) and PCR (anchor primer and p2) are indicated by *thin lines with arrows*. Transcription start sites ***a-d*** are indicated by *arrows*. cDNAs ***a-d*** correspond to RACE products in panel B. *B*: the 5'-RACE products from BLECs maintained in physiological medium (lane 1), or PM made hypertonic by additional supplementation with 116 mM NaCl (lane 2), 116 mM NaCl + sorbinil (lane 3), PM + sorbinil (lane 4), 150 mM D-mannitol (lane 5), 200 mM urea (lane 6) and a 1 kb DNA ladder (lane 7) are shown. The arrows indicate the RACE cDNAs, ***a-d***, with the 5'-UTR of 522 bp, 339 bp, 225 bp and 90 bp, respectively. The size of RACE products are the sum of 5'-UTR and primers (anchor primer and p2) plus a 16 bp coding region (about 93 bp total). The band pattern shown was a typical representation of three separate preparations of RNA from cultured lens epithelial cell populations.

A


5'
a
b
c
d
ATG
mRNA
cDNA a
anchor
p1
cDNA b
cDNA c
cDNA d
PCR primers
anchor primer
p2

B

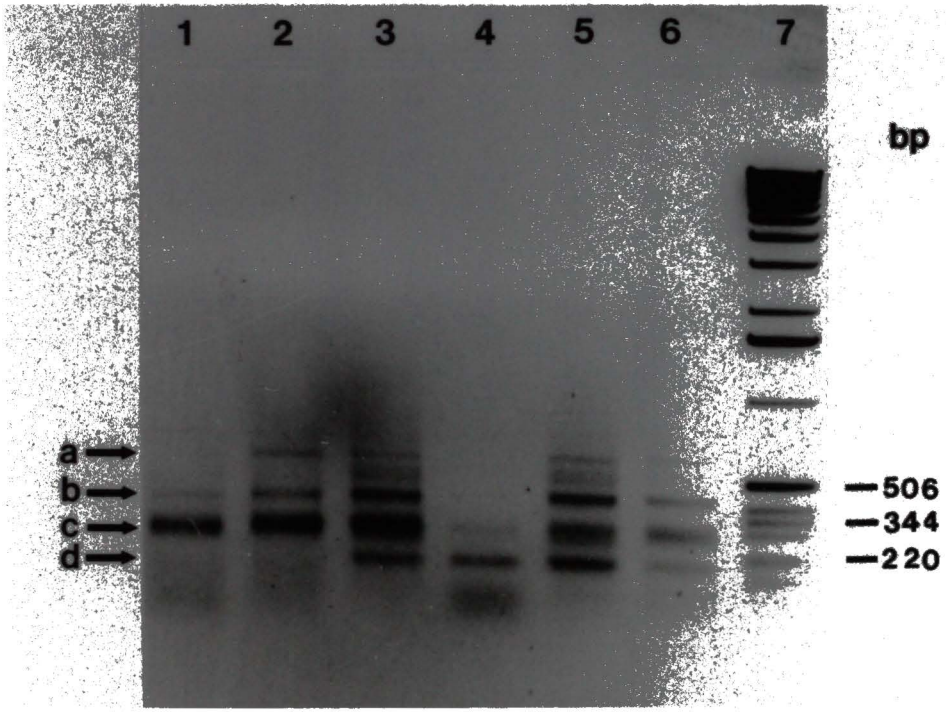


Figure 2. Nucleotide sequences of the four 5'-RACE cDNAs. The **ATG** transcription initiation codon is *boldfaced* and is indicated as +1. The *arrows* represent the four transcription start sites, ***a-d***, and correspond to the RACE cDNAs ***a-d***. The positions of the primers (p1 and p2) used for 5'-RACE are *underlined with arrows*. The *dotted lines with arrows* (p3 and p4) show the positions of the PCR primers used to amplify the 109 bp RACE product ***a*** specific probe.

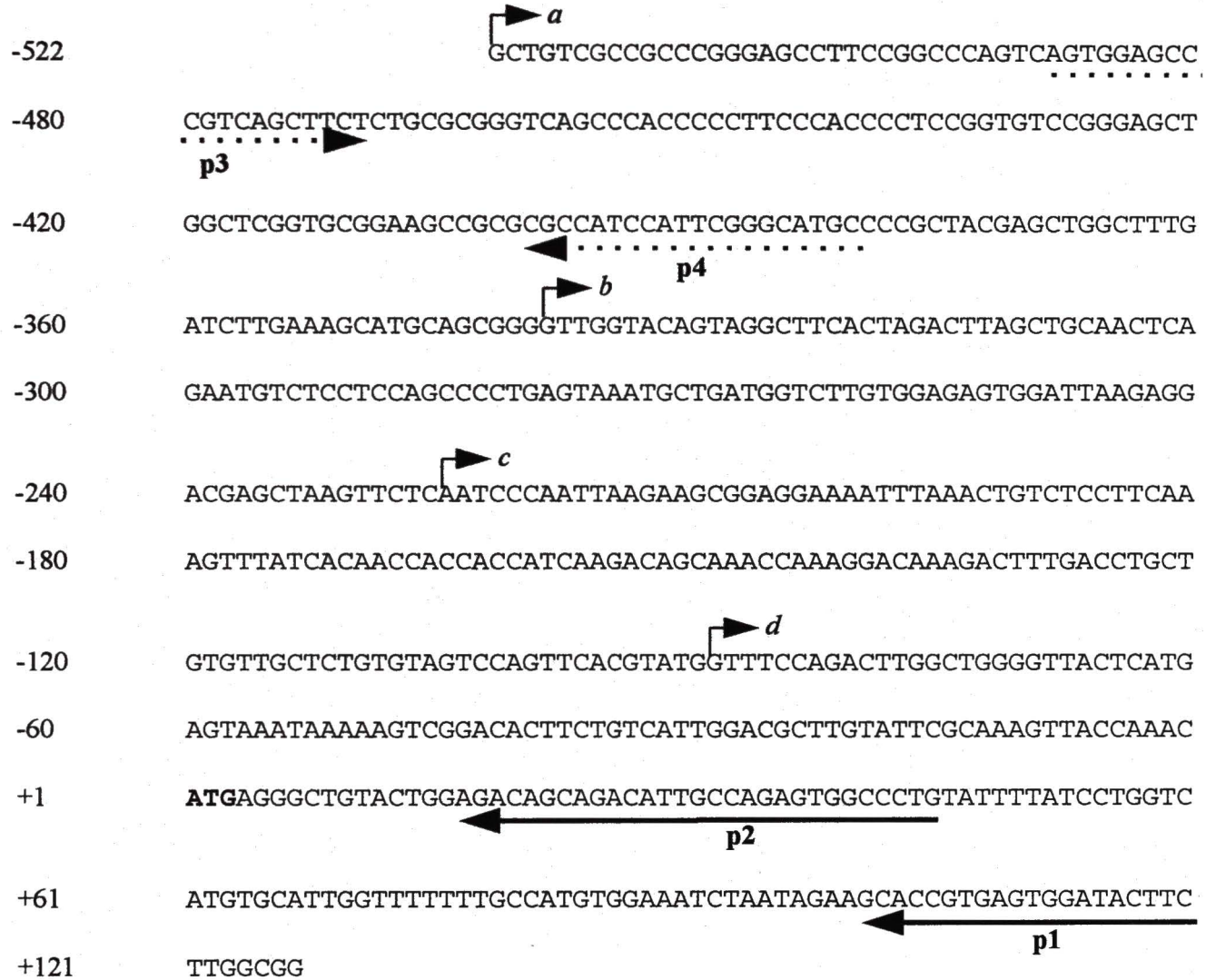
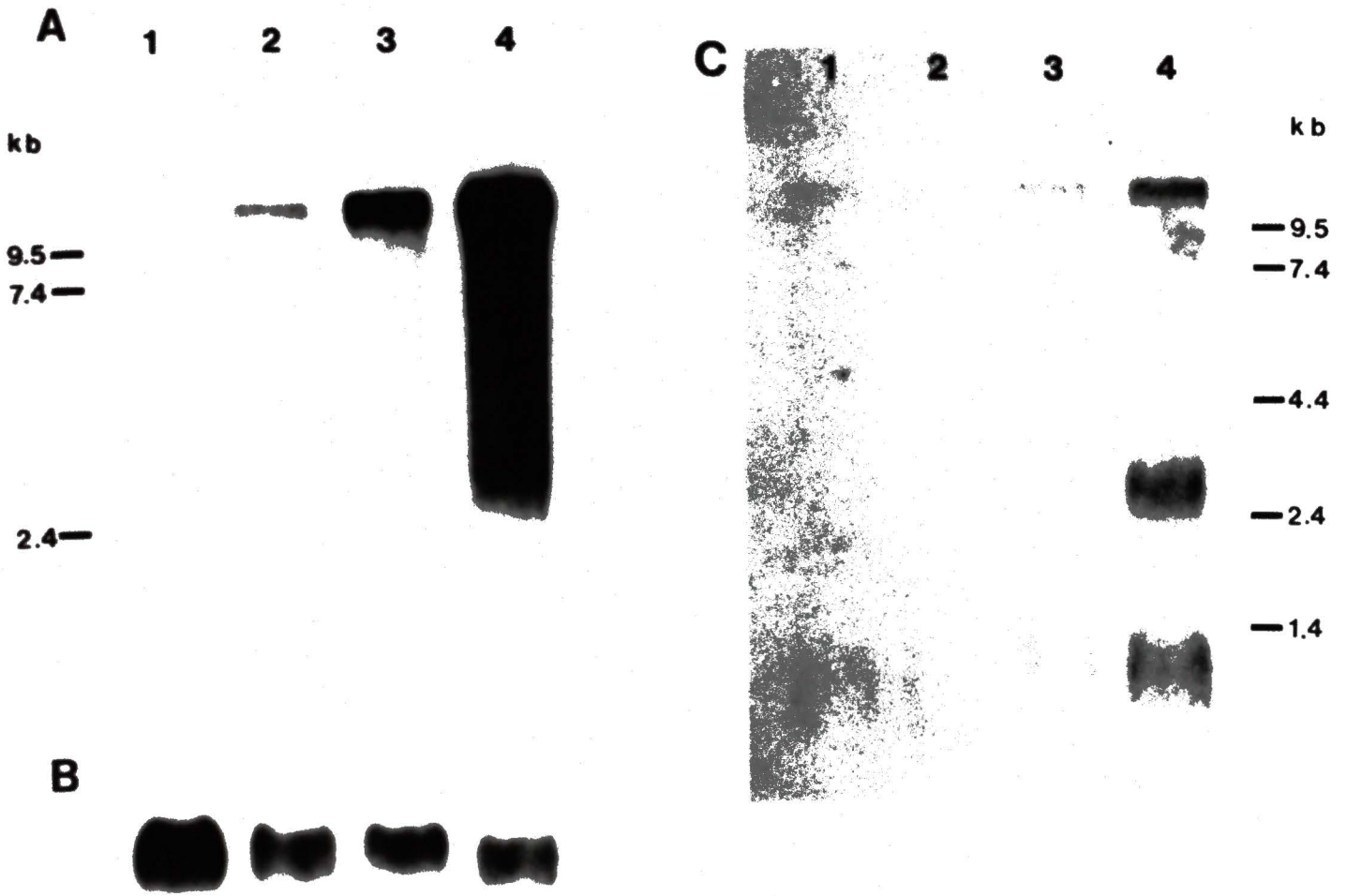

a
-522
GCTGTCGCCGCCCGGGAGCCTTCCGGCCCAGTCAGTGGAGCC
-480
CGTCAGCTTCTCTGCGCGGGTCAGCCCACCCCCTTCCCACCCCTCCGGTGTCCGGGAGCT
p3
-420
GGCTCGGTGCGGAAGCCGCGCGCCATCCATTCGGGCATGCCCCGCTACGAGCTGGCTTTG
p4
b
-360
ATCTTGAAAGCATGCAGCGGGGTTGGTACAGTAGGCTTCACTAGACTTAGCTGCAACTCA
-300
GAATGTCTCCTCCAGCCCCTGAGTAAATGCTGATGGTCTTGTGGAGAGTGGATTAAGAGG
c
-240
ACGAGCTAAGTTCTCAATCCCAATTAAGAAGCGGAGGAAAATTTAAACTGTCTCCTTCAA
-180
AGTTTATCACAACCACCACCATCAAGACAGCAAACCAAAGGACAAAGACTTTGACCTGCT
d
-120
GTGTTGCTCTGTGTAGTCCAGTTCACGTATGGTTTCCAGACTTGGCTGGGGTTACTCATG
-60
AGTAAATAAAAAGTCGGACACTTCTGTCATTGGACGCTTGTATTCGCAAAGTTACCAAAC
+1
ATGAGGGCTGTACTGGAGACAGCAGACATTGCCAGAGTGGCCCTGTATTTTATCCTGGTC
p2
+61
ATGTGCATTGGTTTTTTTGCCATGTGGAAATCTAATAGAAGCACCGTGAGTGGATACTTC
p1
+121
TTGGCGG

Figure 3. Northern blot analysis of poly(A)$^+$ RNA from cultured BLECs. The blot was hybridized with a 626 bp bovine lens Na^+/myo-inositol cotransporter cDNA probe *(A)*, stripped and reprobed with a β-actin genomic DNA probe *(B)*, stripped again and subsequently reprobed with a 109 bp cDNA probe that represents a distinct portion of the RACE product ***a*** *(C), not* present in the other three RACE products. Cells were maintained for 10 hours in physiologic medium (lane 1), or PM made hypertonic by additional supplementation with 200 mM urea (lane 2), 150 mM D-mannitol (lane 3) or 116 mM NaCl (lane 4). Two μg of poly(A)$^+$ RNA was applied to each lane. *(D)*: the positions of the two probes in the Na^+/MI cotransporter cDNA. Arrows ***a-d*** indicate the four transcription start sites. *ATG* and *TGA* are the translation initiation and stop codons, respectively.


A
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3
4
kb
9.5
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2.4
B
C
1
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3
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kb
9.5
7.4
4.4
2.4
1.4

D

Figure 4. Effect of osmotic insult on myo-inositol accumulation. Bovine lens epithelial cells were maintained in physiologic medium (PM) or PM made hypertonic by additional supplementation with 200 mM urea, 150 mM D-mannitol or 116 mM NaCl for 20 hours. The cultures were transferred to the appropriate serum-free medium including a trace amount of myo-[^{3}H]inositol for a 3 hour uptake period at 37°C containing either 25 μM or 400 μM myo-inositol. Data points represent the mean ± standard error taken from triplicate determinations from individual flasks.

Effect of Osmotic Insult on Myo-inositol Accumulation

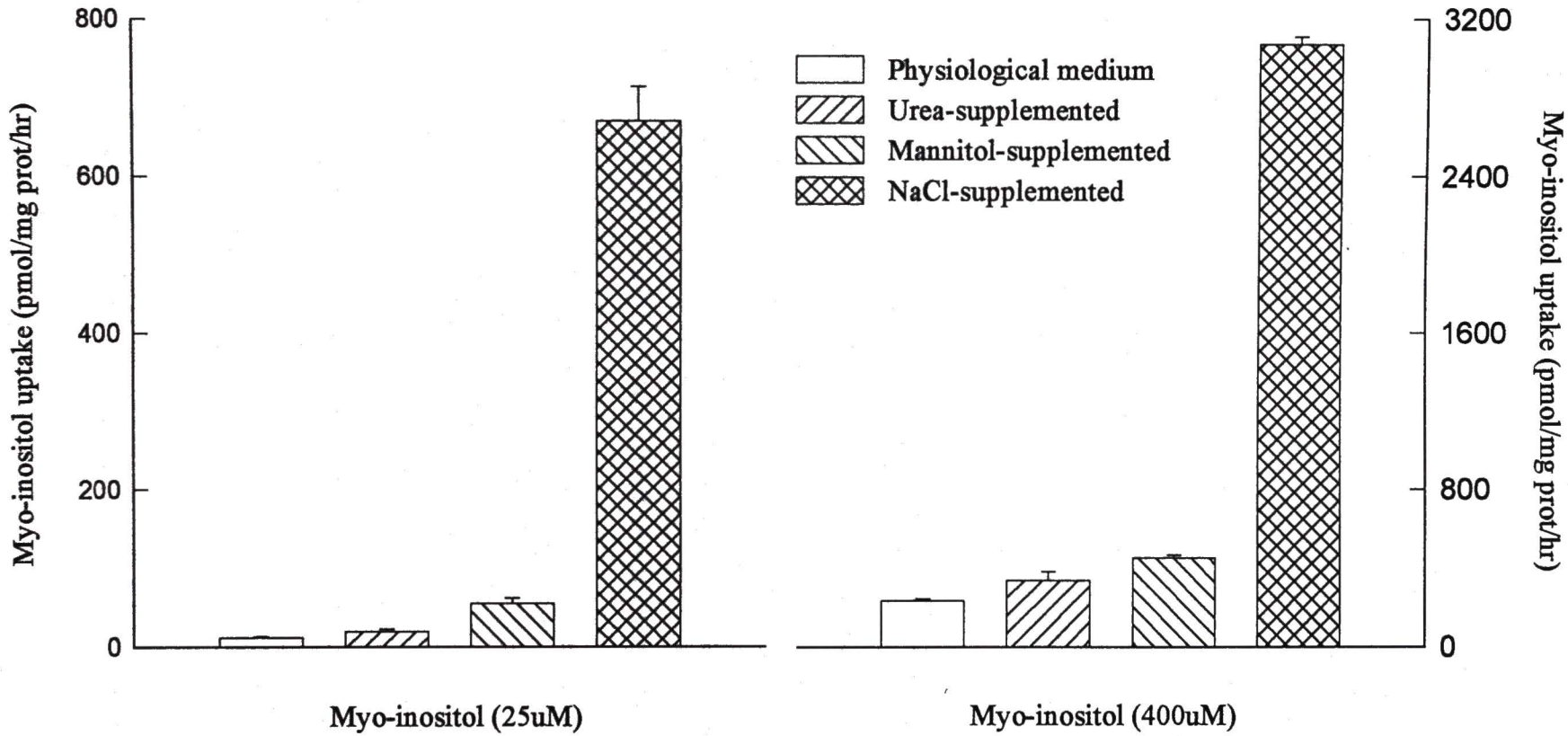

Figure 5. Schematic diagram of the bovine Na^+/MI cotransporter gene. *(A)*:organization of the gene. *Open* and *hatched boxes* represent the noncoding and coding regions, respectively. *ATG* and *TGA* are the translation initiation and stop codons. Arrows *a-c* indicate the locations of the transcription start sites. B: BamHI; H: HindIII; K: KpnI; N: NotI; Nc: NcoI; P: PstI; Pv: PvuII; S: SmaI; X: XbaI. *(B)*:schematic presentation of the promoter-luciferase constructs. Transcription start sites ***a*** and ***b*** are designated as +1 and +1', respectively.

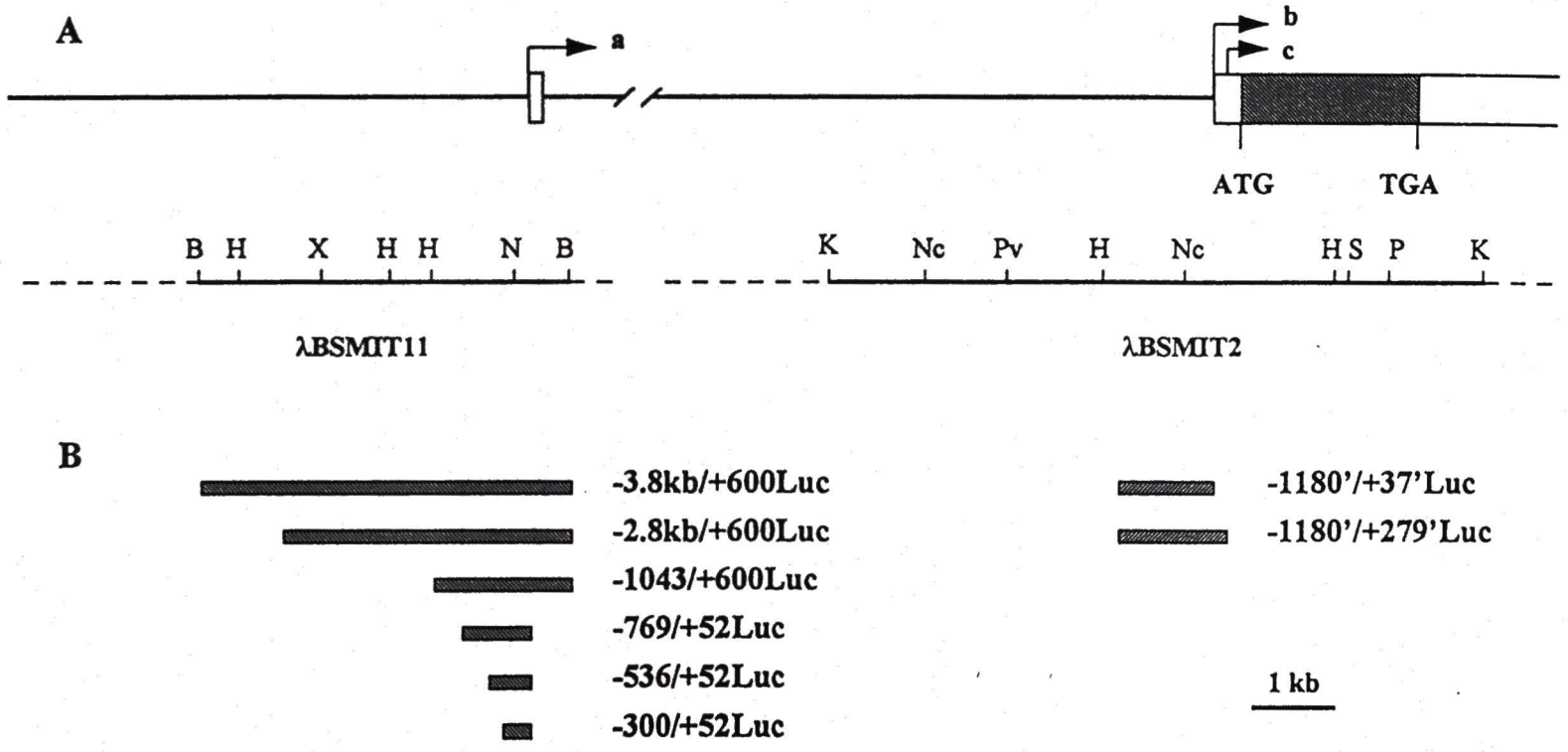
A
a
b
c
ATG
TGA
B H X H H N B
K Nc Pv H Nc H S P K
λBSMIT11
λBSMIT2
B
-3.8kb/+600Luc
-2.8kb/+600Luc
-1043/+600Luc
-769/+52Luc
-536/+52Luc
-300/+52Luc
-1180'/+37'Luc
-1180'/+279'Luc
1 kb

Figure 6. Nucleotide and deduced amino acid sequences of the bovine Na^+/MI cotransporter gene. Small case letters represent the intron and 5'-flanking sequence. *Arrows **a-d*** indicate the four transcription start sites. The TATA-like sequences are *boxed.* The consensus RNA splicing donor and acceptor sequences 5'gt.....ag3' are *underlined* at the intron-exon junction. *(A):* partial nucleotide sequence of clone λBSMIT11 including exon 1, the 5'-flanking region of the hypertonic transcription start site ***a*** (+1), and the 5'-end of the intron. The Sp1 binding site is *boxed with a dotted line. (B):* partial nucleotide sequence of clone λBSMIT2 including exon 2 and a part of the intron. The isotonic trsncription start site ***b*** is designed as +1'. The initiator motif around the transcription start site ***c*** is *underlined with a dotted line.* Translation initiation (*ATG*) and stop (*TGA*) codons are *bold-faced.*

A

-1043 aagctttcagcttggttctctaa

-1020 gtgaacgcccagtaaacattcaaagaatatcggtttgagccggagtattagcctgtcctt

-960 agcaacaacacgtgggtcaggctatgcgacacaacaacaaatgccagtgtgtgccaggca

-900 tcgttctcagcggcttatgagcatcaagtcactcgcaggttatcacggcccctagaaagt

-840 aggtgcctttcacatccccctttatgcaggagccggccccaaggtcaggcagcccgcga

-780 gggatggggcgaataggggagtcctcccaccactgccggcgccgggcgcacttttcccgg

-720 cgaaggggtcccgggaagccgcccccgggcagccacgctggaaaaggagagcttgtgggc

-660 aagggcgcttagggcagcccaccctggtagcacccccctgcggagtcgggggtggtggtgt

-600 gtggaggggggcgcaaatccaagccgtagaacgttccagaacctgggtggggcgggggggt

-540 gcggggaaggagaaccccgtgtcccgcagacattcccgcgcggagaagcgtggccgtgga

-480 ccaggcccctcgcgctccggagtcgcggagtagggacgcgcgcgcgcgcgcgcacggacc

-420 gcatcgcaccgcaccgcacgcacgcgcacacgggggcaggatcgccccgccgcccaggac

-360 cgcgacgggaggcctggaaagttccagctggttcgagaagcgtacgtagccagcgcaggg

-300 tctgagggaggctatcggtggctgccggcggctgctgcgctttccggccggagagacccg

-240 ctgcggctctccgctccgtcgcgccggcccggccaggggctgggggctggggcggggcg

-180 gggaaggggccccagtgcgcgcgccgcgagaaccgggcttttcgatgggggcgcgcggcc

-120 gccttaaggaggggggcgtggctggcgcccgtgtgggggggtgttgtccgccgaggggggcgt

-60 ggcagctccgtctgcgcagctgccagcgcctttaagcccgggctcgcgcggccagagcgt

+1 GCTGTCGCCGCCCGGGAGCCTTCCGGCCCAGTCAGTGGAGCCCGTCAGCTTCTCTGCGCG

+61 GGTCAGCCCACCCCCTTCCCACCCCTCCGGTGTCCGGGAGCTGGCTCGGTGCGGAAGCCG

+121 CGCGCCATCCATTCGGGCATGCCCCGCTACGAGCTGGCTTTGATCTTGAAAGCATGCAGC

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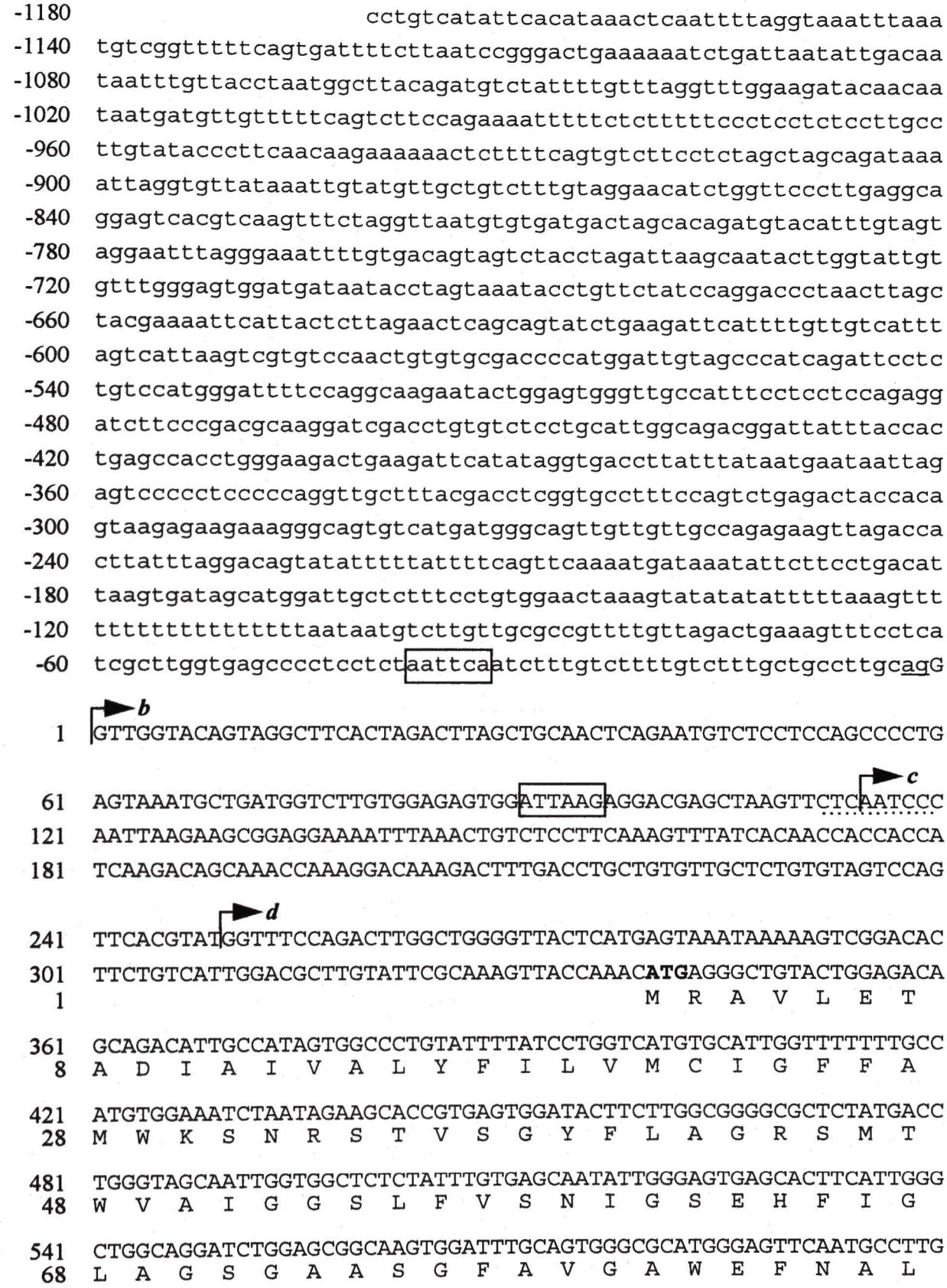
B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-180 taagtgatagcatggattgctctttcctgtggaactaaagtatatatatttttaaagttt
-120 ttttttttttttttaataatgtcttgttgcgccgttttgttagactgaaagtttcctca
-60 tcgcttggtgagcccctcctctaattcaatctttgtcttttgtctttgctgccttgcagG
b
1 GTTGGTACAGTAGGCTTCACTAGACTTAGCTGCAACTCAGAATGTCTCCTCCAGCCCCTG
c
61 AGTAAATGCTGATGGTCTTGTGGAGAGTGGATTAAGAGGACGAGCTAAGTTCTCAATCCC
121 AATTAAGAAGCGGAGGAAAATTTAAACTGTCTCCTTCAAAGTTTATCACAACCACCACCA
181 TCAAGACAGCAAACCAAAGGACAAAGACTTTGACCTGCTGTGTTGCTCTGTGTAGTCCAG
d
241 TTCACGTATGGTTTCCAGACTTGGCTGGGGTTACTCATGAGTAAATAAAAAGTCGGACAC
301 TTCTGTCATTGGACGCTTGTATTCGCAAAGTTACCAAACATGAGGGCTGTACTGGAGACA
1 M R A V L E T
361 GCAGACATTGCCATAGTGGCCCTGTATTTTATCCTGGTCATGTGCATTGGTTTTTTTGCC
8 A D I A I V A L Y F I L V M C I G F F A
421 ATGTGGAAATCTAATAGAAGCACCGTGAGTGGATACTTCTTGGCGGGGCGCTCTATGACC
28 M W K S N R S T V S G Y F L A G R S M T
481 TGGGTAGCAATTGGTGGCTCTCTATTTGTGAGCAATATTGGGAGTGAGCACTTCATTGGG
48 W V A I G G S L F V S N I G S E H F I G
541 CTGGCAGGATCTGGAGCGGCAAGTGGATTTGCAGTGGGCGCATGGGAGTTCAATGCCTTG
68 L A G S G A A S G F A V G A W E F N A L

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601  CTGCTTTTGCAACTTCTGGGATCGGTTTTCATCCCGATTTACATCCGGTCAGGGGTATAC
 88  L  L  L  Q  L  L  G  S  V  F  I  P  I  Y  I  R  S  G  V  Y

661  ACCATGCCTGAATACTTGTCCAAGCGATTTGGTGGCCATAGGATTCAGGTCTATTTTGCA
108  T  M  P  E  Y  L  S  K  R  F  G  G  H  R  I  Q  V  Y  F  A

721  GCCTTGTCTCTGATTCTCTATATCTTCACCAAGCTCTCTGTGGATCTGTATTCGGGTGCC
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781  CTCTTTATCCAGGAGTCTTTGGGTTGGAACCTCTATGTGTCTGTCATCCTGCTCATTGGC
148  L  F  I  Q  E  S  L  G  W  N  L  Y  V  S  V  I  L  L  I  G

841  ATGACCGCTTTGTTGACCGTCACCGGAGGCCTTGTCGCAGTAATCTACACAGACACCTTG
168  M  T  A  L  L  T  V  T  G  G  L  V  A  V  I  Y  T  D  T  L

901  CAGGCTCTGCTCATGATCGTTGGGGCACTCACACTTATGGTGATTAGCATGATGGAGATT
188  Q  A  L  L  M  I  V  G  A  L  T  L  M  V  I  S  M  M  E  I

961  GGCGGGTTTGAGGAAGTTAAGAGAAGGTACATGTTGGCCTCACCCAATGTCACTTCCATC
208  G  G  F  E  E  V  K  R  R  Y  M  L  A  S  P  N  V  T  S  I

1021 CTGCTGACGTACAACCTTTCCAACACAAATTCTTGTAATGTCCACCCTAAGAAAGATGCA
228  L  L  T  Y  N  L  S  N  T  N  S  C  N  V  H  P  K  K  D  A

1081 CTGAAAATGTTGCGGAACCCAACAGCTGAAGATGTTCCTTGGCCTGGATTCATCCTTGGG
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1141 CAGACCCCGGCTTCAGTCTGGTACTGGTGTGCCGACCAGGTCATCGTGCAGAGGGTCCTA
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348  N  I  A  Y  P  R  L  V  M  K  L  V  P  V  G  L  R  G  L  M

1441 ATGGCCGTGATGATTGCCGCTCTGATGAGTGACTTGGACTCCATTTTCAACAGCGCCCAA
368  M  A  V  M  I  A  A  L  M  S  D  L  D  S  I  F  N  S  A  Q

1501 GCCATATTCACCCTCGATGTGTACAAACTCATCCGCAAGAGGCCCAGTTCCCGGGAACTG
388  A  I  F  T  L  D  V  Y  K  L  I  R  K  R  P  S  S  R  E  L

1561 ATGATCGTGGGCAGGATATTTGCGGCCTTCATGGTGGTGATCAGCATCGCTGAGGTGCCC
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1621 ATCATCGTGGAGATGCAAGGAGGCCAGATGTACCTTTACATCCAGGAGGTAGCTGACTAC
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1681 CTGACACCCCCAGTTGCGACCCTGTTCCTTCTGGCCATTTTCTGGAAGAACTGCAATGAA
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1981 TCTAAGAAGAGCCTGGTGGTCAAGGAGAGCTGCTCCCCGAAAGATGAACCATACAAAATG
 548 S  K  K  S  L  V  V  K  E  S  C  S  P  K  D  E  P  Y  K  M

2041 CAAGAGAAGAGCATTCTGAGATGCAGTGAGAATAGTGAGGCCATCAACCACGTCATCCCC
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2101 AACGGGAAGTCTGAGGACAGCATCAAGGGCCTGCAGCCGGAAGATGTGAATCTGTTGGTG
 588 N  G  K  S  E  D  S  I  K  G  L  Q  P  E  D  V  N  L  L  V

2161 ACCTGCAGAGAGGAGGGCAACCCAGTGGCTTCCTTAGGTCATTCGGAGGCAGAAACACCC
 608 T  C  R  E  E  G  N  P  V  A  S  L  G  H  S  E  A  E  T  P

2221 GTGGATGCGTATTCCAACGGGCAGGCAGCTCTCATGGGTGAGAAAGAGAGAAAGAAAGAA
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2281 GCAGAGGACGGAAGCCGGTACTGGAAGTTCATCGATTGGTTTTATGGCTTTAAAAGTAAG
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2341 AGCCTCAGCAAGAGGAGTCTCAGAGACCTGATGGAGGAGGAGGCTGTTTGTTTACAAATG
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 688 L  E  E  P  P  Q  V  K  V  I  L  N  I  G  L  F  A  V  C  S

2461 CTTGGAATTTTCATGTTTGTTTATTTCTCCTTATGAACTTTTAAGGATATGATGAGACAC
 708 L  G  I  F  M  F  V  Y  F  S  L  *

2521 TAACTTAAGAAAATACTGGTCTTTGGAAAACAACAACAAAAAAAACCCTGCAAACTTATG
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2761 ATACCAAGTGAGGAGAGACCAATTATTCTCATAGAGCACTTAGAGCAGAGTTATATGCTA
2821 GGTATGTGAGTAGATACACTGTCTGCACTGCAATCT
```

Figure 7. Functional analysis of the bovine Na^+/MI cotransporter promoters by transient transfection in cultured BLECs. The 5'-flanking regions upstream of transcription start sites ***a***, ***b***, and ***c*** were fused to the luciferase reporter gene. A diagram of these promoter-luciferase constructs is shown in Fig. 5B. The constructs p(-1180'/+27') Luc and p(-1180'/+279')Luc contain the isotonic promoters ***b*** and ***b+c***, respectively. The constructs p(-2.8kb/+600)Luc and p(-2.8kb/+600)asLuc represent the hypertonic promoter **a** in *sense* and *anti-sense* orientation, upstream of the luciferase reporter gene, respectively. *Open* and *hatched* bars represent the relative luciferase activities of transfected cells maintained in physiologic medium or switched to hypertonic medium, respectively. Bars represent the mean values of the relative luciferase activity ± standard error from duplicate determinations of four individual experiments.

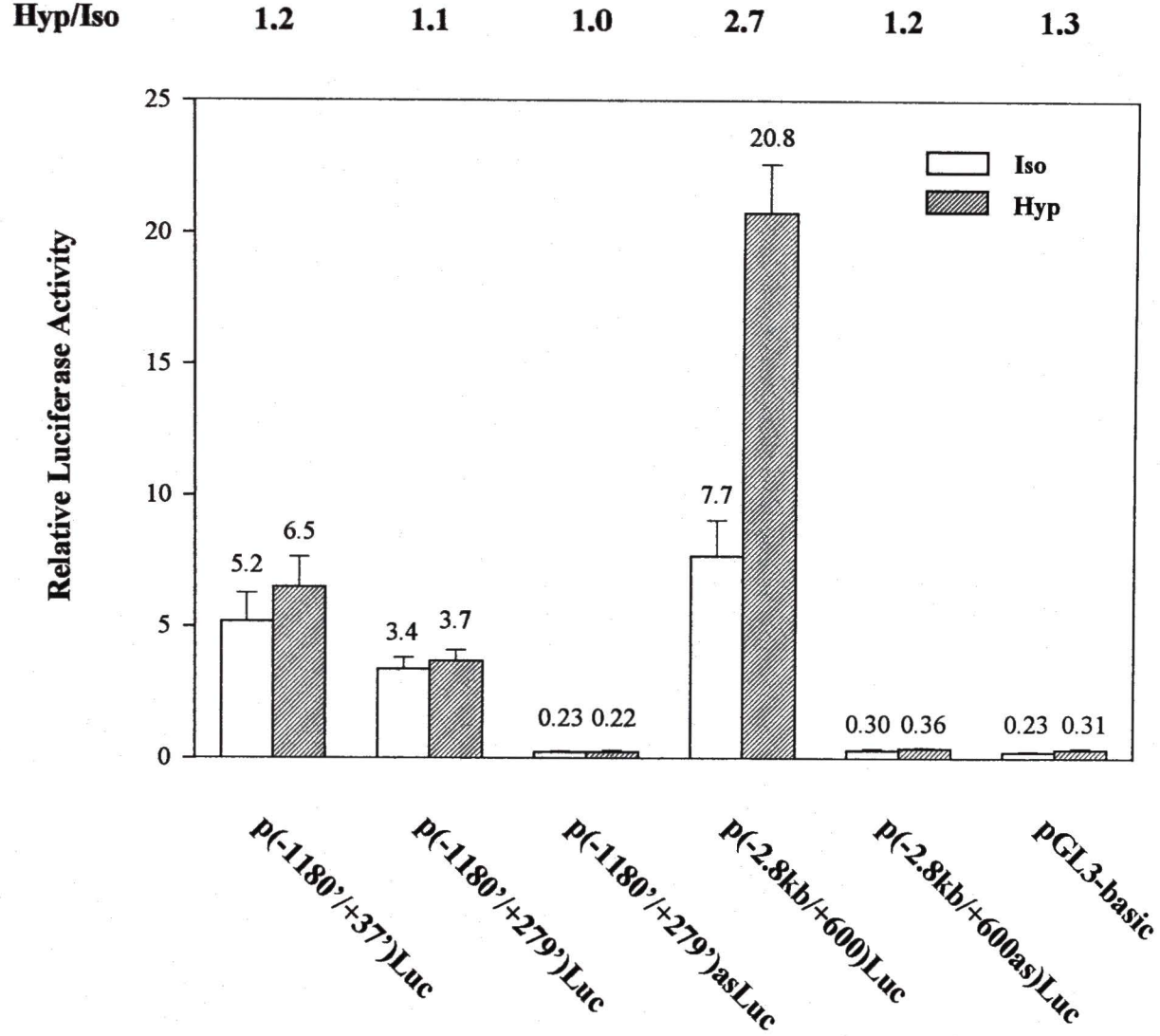
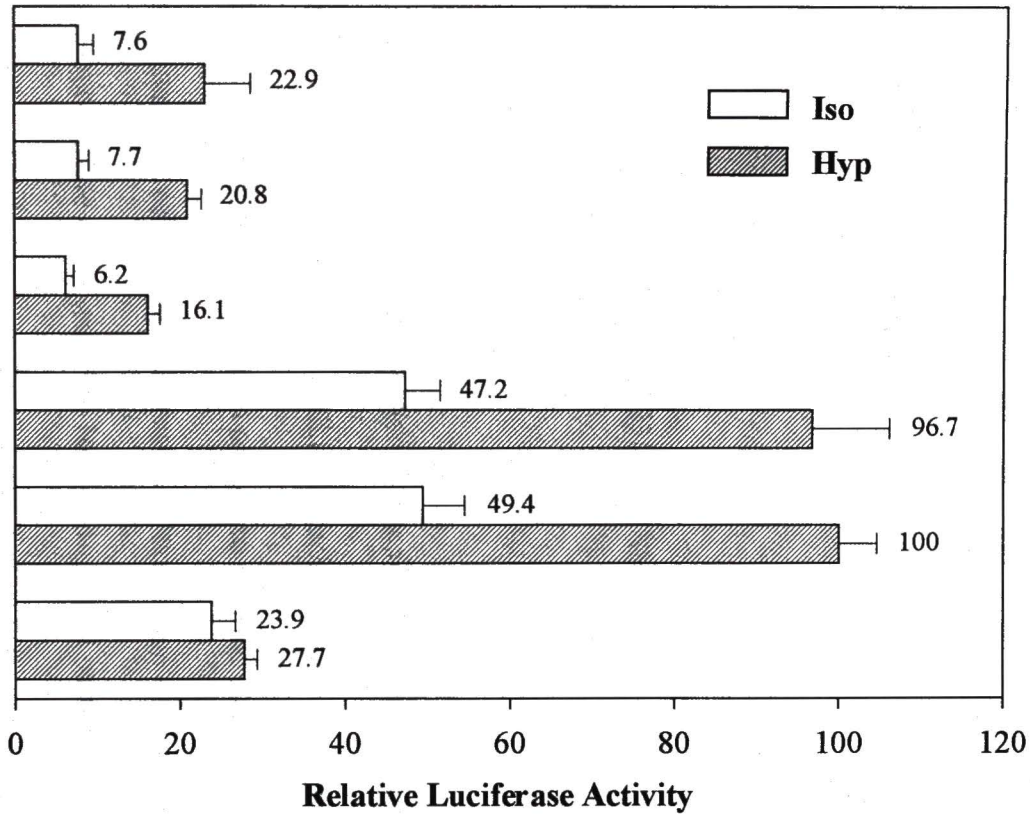

Hyp/Iso
1.2
1.1
1.0
2.7
1.2
1.3
Relative Luciferase Activity
25
20
15
10
5
0
Iso
Hyp
5.2
6.5
3.4
3.7
0.23 0.22
7.7
20.8
0.30 0.36
0.23 0.31
p(-1180'/+37')Luc
p(-1180'/+279')Luc
p(-1180'/+279')asLuc
p(-2.8kb/+600)Luc
p(-2.8kb/+600as)Luc
pGL3-basic

Figure 8. Characterization of the osmotically-responsive hypertonic promoter ***a***. Promoter-luciferase constructs with various length of 5'-flanking regions upstream of transcription start site ***a*** were transiently transfected into cultured BLECs. A diagram of the constructs is presented in Fig. 5B. *Open* and *hatched* bars represent the relative luciferase activities of transfected cells maintained in physiologic medium or hypertonic medium, respectively. Bars represent the mean values of the relative luciferase activity ± standard error of duplicate determinations from four individual experiments.

Constructs	Hyp/Iso
p(-3.8kb/+600)Luc	3.0
p(-2.8kb/+600)Luc	2.7
p(-1043/+600)Luc	2.6
p(-769/+52)Luc	2.0
p(-536/+52)Luc	2.0
p(-300/+52)Luc	1.2



Chapter V

SUMMARY AND DISCUSSION

Although osmoregulation normally functions in renal cells during the urine concentrating process, in some pathological conditions, such as diabetes, congestive heart failure, renal dysfunction, non-renal cells are also subject to osmotic stress. Osmoregulation in mammalian cells includes increases of expression of a group of genes that facilitate cells to accumulate osmolytes. The Na^+/myo-inositol cotransporter is responsible for the uptake of high concentrations of myo-inositol, an osmolyte, when cells are exposed to osmotic stress.

In this dissertation, the osmoregulation of the Na^+/myo-inositol cotransporter in lens epithelial cells was first established. A 626 bp bovine lens Na^+/myo-inositol cotransporter cDNA was cloned by RT-PCR amplification. Sequence analysis revealed that the bovine Na^+/myo-inositol cotransporter cDNA is highly homologous with the canine isoform. The expression of the Na^+/myo-inositol cotransporter in lens epithelial cells was demonstrated for the first time by RT-PCR amplification as well as Northern blot

analysis. This is another example that the osmoregulation system is functional in non-renal as well as renal cells.

The effect of hypertonicity on the transporter mRNA abundance was studied by Northern blot analysis with bovine lens epithelial cells cultured in various hypertonic conditions. Hypertonicity generated by high concentrations of either sugar(sorbitol, mannitol), or urea, or NaCl was able to increase the transporter mRNA with the greatest induction by NaCl. Furthermore, the addition of aldose reductase inhibitor, zopolrestat, resulted in a higher induction of transporter mRNA which suggested that accumulation of polyol suppressed the Na^+/myo-inositol cotransporter mRNA abundance. This finding is consistent with the previous observation that inhibition of polyol formation during osmotic insult resulted in higher intracellular myo-inositol levels in cultured BLECs (Cammarata and Chen, 1994). This study indicates that hypertonic induction of the Na^+/myo-inositol cotransporter mRNA is the result of the tonicity increase regardless of the nature of the hypertonic insult. Since the Na^+/myo-inositol cotransporter and aldose reductase are the two key components that are responsible for the accumulation of the two osmolytes, myo-inositol and sorbitol, respectively, the effect of aldose reductase inhibitor on the osmotic-induced Na^+/myo-inositol cotransporter mRNA levels suggests that accumulation of myo-inositol and sorbitol in lens cells is rather interactive than independent.

The pattern of the induction of the Na^+/myo-inositol cotransporter and aldose reductase mRNA levels under osmotic stress was investigated by Northern blot analysis of poly(A)$^+$ RNA from BLECs cultured in hypertonic medium from 0 to 72 hours. The maximal induction of the Na^+/myo-inositol cotransporter mRNA was observed within 8

hours of osmotic insult. Thereafter, the level of mRNA steadily declined within 72 hours despite continuous exposure of BLECs to hypertonicity. When hypertonically stressed cells was switched to physiological medium, the hypertonic-induced Na^+/myo-inositol cotransporter mRNA levels returned to the physiological level within 4 to 8 hours suggesting that the turnover rate of the Na^+/myo-inositol cotransporter mRNA is very rapid. With such rapid induction and turnover rate, osmoregulation of the Na^+/myo-inositol cotransporter expression, at least at the mRNA level, can be achieved in a swift and precise fashion.

Unlike the Na^+/myo-inositol cotransporter, the aldose reductase mRNA level was not appreciably increased after 24 hours of hypertonic treatment and remained elevated during the 72 hour exposure period. The Na^+/myo-inositol cotransporter mRNA was maximally induced by osmotic stress about 16 hours before maximal expression of aldose reductase mRNA and declined when aldose reductase mRNA level remained induced. These results indicate that the lens osmoregulatory system regulates the intracellular levels of myo-inositol and sorbitol, two osmolytes, in concert. Lens cells respond to osmotic stress by acute enhanced myo-inositol uptake as the early-onset protective mechanism. Chronic exposure to hypertonicity results in the accumulation of another osmolyte, sorbitol, through activation of aldose reductase activity, the late-onset protective mechanism. As the results of the competition between the two osmolytes, the enhanced myo-inositol uptake is then suppressed by the increased aldose reductase activity. Accumulated sorbitol then replaces myo-inositol to become the major osmolyte that helps cells against water stress. This model was further confirmed by the carbohydrate analysis

of myo-inositol and sorbitol levels in osmotically stressed lens cells treated with or without aldose reductase inhibitors. Intracellular myo-inositol concentration was rapidly increased under osmotic insult and approached steady state levels when sorbitol accumulated. Inhibition of sorbitol accumulation resulted in higher myo-inositol levels indicating intracellular sorbitol formation via aldose reductase downregulated myo-inositol accumulation in lens epithelial cells.

The interactive nature of myo-inositol and sorbitol accumulation in lens epithelial cells can be used to account for the myo-inositol depletion in diabetic lens. In diabetic lens epithelial cells, chronic exposure to osmotic stress generated by high extralenticular glucose can lead to the elevated aldose reductase activity and suppressed Na^+/myo-inositol cotransporter. With the presence of high concentrations of glucose substrate, lens epithelial cells are capable of accumulating high intracellular sorbitol through increased aldose reductase activity. High concentration of sorbitol is then able to deplete intracellular myo-inositol by suppression of the Na^+/myo-inositol cotransporter mRNA abundance, inhibiting the Na^+/myo-inositol cotransporter protein (Cammarata et al., 1992), and accelerating the myo-inositol efflux pathway (Reeves and Cammarata, 1996). The depletion of myo-inositol may then contribute to sugar cataract formation.

The molecular mechanism of osmotic induced Na^+/myo-inositol cotransporter mRNA abundance was studied by cloning the 5'-end of the cotransporter mRNA from BLECs cultured in physiological and various hypertonic media using 5'-RACE analysis. The 5'-UTR motifs indicated that there were multiple transcription start sites in the cotransporter gene and osmotic stress resulted in preferential utilization of a hypertonic

transcription start site ***a***. Northern blot analysis and carbohydrate analysis indicate that efficient utilization of this transcription start site contributed to the induced mRNA abundance and the elevated intracellular myo-inositol level by osmotic insult.

Further investigation of the osmoregulation of the Na^+/myo-inositol cotransporter gene expression necessitates studying the Na^+/myo-inositol cotransporter gene structure and identifying the genetic element that mediates the osmotic response. One of the major contribution of this dissertation is the molecular cloning of the bovine Na^+/myo-inositol cotransporter gene and the characterization of its promoters. This is the first report of cloning the Na^+/myo-inositol cotransporter gene including the 5'-flanking regions, the entire 5'-UTR and the ORF. Alignment of the 5'-RACE cDNA sequences to the genomic sequence allows us to localize the individual transcription start sites in this gene and clone the corresponding promoter sequences. Transient transfection assays using promoter-luciferase constructs were performed in cultured BLECs to analyze the promoters of the Na^+/myo-inositol cotransporter gene. The identification of the two isotonic promoters and the osmotic-sensitive hypertonic promoter provided detailed information of how osmotic stress affects the transcription of the Na^+/myo-inositol cotransporter. It directly confirms the results of the 5'-RACE assay that activation of the osmotic-responsive hypertonic promoter ***a*** contributes to the osmotic-induced Na^+/myo-inositol cotransporter mRNA abundance and its activity.

Further characterization of the osmotic-responsive hypertonic promoter by transient transfection assays identified the osmotic-responsive element and localized it between -526 to -300 bp upstream of the transcription start site ***a***. These studies allow us

to further investigate the transcriptional regulation of the Na^+/myo-inositol cotransporter gene including identification of the sequence of this element and the transcription factor binding to this element that mediates the osmotic response in the mammalian system.

The current knowledge of the osmoregulation of myo-inositol accumulation via the Na^+/myo-inositol cotransporter is summarized in Fig. 1. Osmotic stress potentiates the hypertonic promoter ***a*** through the osmotic-responsive element which results in increased transcription rate of the Na^+/myo-inositol cotransporter. Increased transcription leads to the induction of the transporter mRNA abundance. Translation from the induced mRNA results in more transporter proteins in the cell membrane which lead to the increase of transport activity. More myo-inositol will be transported from the extracellular medium and accumulated in the cells to help maintain cell homeostasis and prevent osmotic damages.

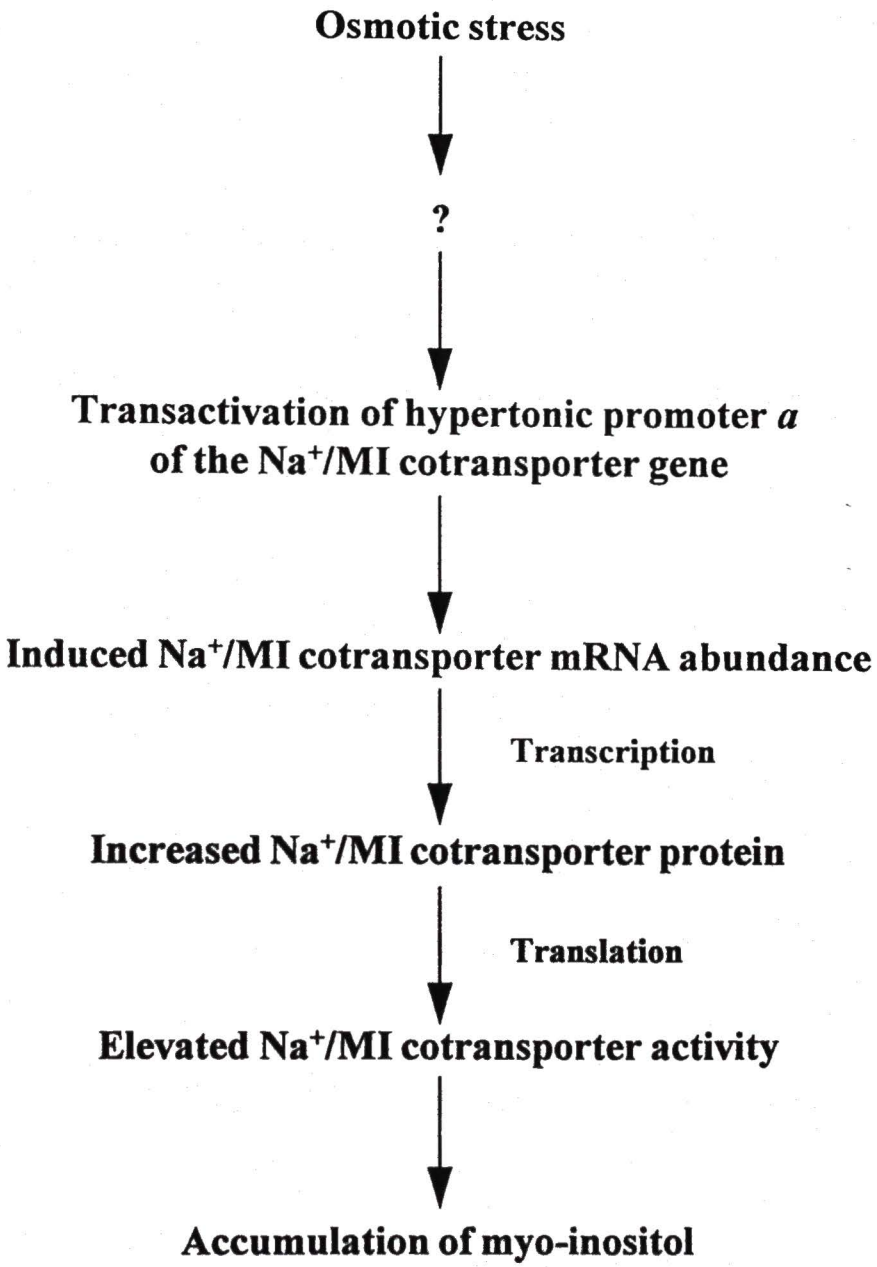


Figure 1. Diagram of osmoregulation of myo-inositol accumulation in BLECs.

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