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The Role of Protein Kinases in the Regulation of the Na+/H+ Antiporter in Xenopus Oocytes

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THE ROLE OF PROTEIN KINASES IN THE REGULATION OF THE Na^+/H^+ ANTIPORTER IN *Xenopus* OOCYTES

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CHAPTER I

INTRODUCTION

Purpose

The Na^+/H^+ exchanger (NHE) was first studied in renal systems and was thought to play a role in renal acidification as well as transepithelial water and salt transport. It has been shown since, however, that the Na^+/H^+ exchanger (or antiporter) is found not only in epithelial cells (Grinstein and Rothstein, 1986), but also is found in almost all cells, displaying a role in key cellular functions such as control of cell volume, regulation of intracellular pH, and initiation of cell growth and proliferation (Grinstein *et al.*, 1992; Sardet *et al.*, 1990).

In pH regulation, the antiporter is responsible for allowing one Na^+ ion into the cell for every H^+ ion that is released from the cell. It has been demonstrated that a correlation exists between an increase in intracellular pH (pH_i) and an increase in the rate of protein synthesis in sea urchin eggs (Grainger *et al.*, 1979). It has further been demonstrated that an increase in pH_i plays an important role in the regulation of synthesis of proteins that are key factors in *Xenopus laevis* oocyte meiotic maturation (Houle and Wasserman, 1983). As the pH_i increases, the rate of protein synthesis also increases, and the rate of *Xenopus* oocyte maturation is dependent on the pH_i (Wasserman *et al.*, 1986). Due to the importance of pH_i during oocyte meiotic maturation, the purpose of my study was to investigate the regulation of the Na^+/H^+ antiporter in *Xenopus laevis* oocytes.

My working hypothesis was that the rise in pH_i that occurs during oocyte maturation is the result of the phosphorylation of the Na^+/H^+ antiporter caused by one or more protein kinases. During oocyte meiotic maturation, there exists a protein kinase

cascade, activated by progesterone, which ultimately leads to germinal vesicle breakdown (GVBD) (Sagata *et al.*, 1989). The following five protein kinases within this cascade were investigated in this study: c-mos kinase, raf-1 kinase, MAP kinase kinase, MAP kinase, and S6 kinase. The goal of my study was to determine which one of these protein kinases, if any, plays a role in the activation of the Na^+/H^+ antiporter during oocyte maturation. It has been noted that elevated levels of cAMP block the progesterone-induced increase in pH_i during oocyte meiotic maturation (Rezai, 1994). It is unknown where within the protein kinase cascade cAMP blocks the increase in pH_i. In order to answer these questions I utilized the following strategies:

1) I attempted to activate the Na^+/H^+ antiporter by microinjecting one of the five active protein kinases into *Xenopus laevis* oocytes.
2) I attempted to determine if this kinase is the only protein that is needed to activate the antiporter.
3) I attempted to determine where in the kinase cascade cAMP blocks the pHi increase by artificially altering cAMP levels within the oocyte prior to the microinjection of each kinase.
4) Propose which of the five kinases, if any, is responsible for the activation of the Na^+/H^+ antiporter during *Xenopus* oocyte maturation.

Literature Review

Oocyte meiotic maturation is the process responsible for preparing the egg for fertilization (Eckberg, 1988). Oocyte maturation has been studied in a variety of organisms, both vertebrate and invertebrate, but the most thorough research in this area has been achieved utilizing the amphibian as a model system (Smith, 1989).

In *Xenopus laevis*, oogenesis is an asynchronous process having oocytes in different stages of growth all present in the ovary at the same time. The growth period of

oocytes has been divided into six stages, I-VI (Dumont, 1972), with stage VI being fully-grown oocytes (1200-1300 μm in diameter). At this final stage, oocytes are arrested in late G_2 phase of meiosis I until they are triggered by hormones to progress to the second meiotic metaphase, where they are capable of participating in fertilization (Smith, 1989). Only the stage VI oocytes are capable of responding to hormonal stimulation and resuming meiosis from prophase arrest (Wasserman *et al.*, 1986).

In vivo, meiotic maturation is initiated by luteinizing hormone (LH), a gonadotropin released from the anterior pituitary into the circulatory system. Upon reaching the ovary, LH stimulates the follicle cells surrounding the stage VI oocytes, causing the follicles to synthesize and release progesterone, a steroid hormone. Progesterone then acts on the oocytes to induce meiotic resumption (Masui and Markert, 1971). The interaction between progesterone and the oocyte does not follow the typical steroid-target cell model. In other cell systems, steroids are usually translocated to the nucleus where they alter specific gene transcription (Beato, 1989). In oocytes, progesterone acts at the plasma membrane of the oocyte. The signal is then transduced across the plasma membrane into the cytoplasm of the cell where it is responsible for triggering a complex series of reactions ultimately leading to meiotic maturation of the oocyte. Experiments have been performed to confirm that progesterone has to act at the plasma membrane of the oocyte to induce meiotic maturation, and that progesterone does not induce maturation from within the cytoplasm of the oocyte. Microinjection of progesterone into the cytoplasm of stage VI oocytes of *Rana pipiens* and *Xenopus laevis* does not induce meiotic maturation (Masui and Markert, 1971; Wasserman *et al.*, 1986). Progesterone that is covalently bound to polystyrene beads, so that it cannot pass through the plasma membrane, is able to induce meiotic maturation when applied to the oocyte surface (Godeau *et al.*, 1978). Investigators have also identified a steroid receptor on the plasma membrane of *Xenopus laevis* oocytes via photoaffinity labeling (Sadler and

Maller, 1982; Blondeau and Baulieu, 1984). More recently, the steroid receptors on the plasma membrane were characterized utilizing radiolabeled progesterone to determine their binding affinity (Liu and Patino, 1993).

During meiotic maturation, the oocyte nucleus (germinal vesicle, GV) migrates to the animal pole of the cell, the nuclear envelope undergoes dissolution (germinal vesicle breakdown, GVBD), the chromosomes condense, the spindle forms, and homologous chromosomes are segregated. During the migration of the germinal vesicle toward the animal pole, the pigment granules that are located at the pole are displaced. This displacement results in the formation of a white spot surrounded by a dark band made up of the displaced pigment granules, the first visible indication of oocyte maturation.

Concurrent with these morphological events are several physiological events that take place within the oocyte during meiotic maturation. These events include an increase in intracellular Ca^{2+}, a decrease in cAMP levels, an increase in intracellular pH, an increase in protein synthesis, an increase in total protein phosphorylation, and activation of maturation promoting factor (MPF), a cytoplasmic factor responsible for GVBD and chromosome condensation (Wasserman and Smith, 1978).

The mechanism by which progesterone activates these morphological and physiological events that constitute oocyte maturation has been compared to the transmembrane signalling events of peptide hormones. It is thought that by changing the intracellular concentrations of second messengers, progesterone indirectly regulates the intracellular events. Two second messengers, inositol triphosphate (IP_3) and 1,2-diacylglycerol (DAG), are generated when membrane-bound phosphotidylinositol 4,5-bisphosphate (PIP_2) undergoes hydrolysis as a result of the activation of phospholipase C. IP_3 triggers the release of Ca^{2+} from the endoplasmic reticulum, resulting in elevated levels of Ca^{2+} which activate calmodulin-dependent kinase (CAM), while DAG is responsible for activating protein kinase C (PKC) (reviewed by Eckberg, 1988; Smith,

1989). Cyclic AMP, another second messenger and known regulator of protein kinase A (PKA), decreases during oocyte maturation. This decrease in cAMP is due to progesterone inhibition of the cell's adenylate cyclase activity by inhibiting guanine nucleotide exchange on the associated G_s protein (Sadler and Maller, 1981; Sadler and Maller, 1983).

One of the physiological events occurring during oocyte maturation is an increase in intracellular pH (pH_i). Studies indicate that the 0.3-0.4 unit increase in pH_i may directly or indirectly be responsible for the increase in the rate of protein synthesis during oocyte maturation. Stage VI *Xenopus laevis* oocytes have a pH_i of 7.2-7.3 and a rate of protein synthesis of about 20 ng/hr, while mature oocytes have a pH_i of 7.6-7.7 and a rate of protein synthesis of about 40 ng/hr. If a stage VI oocyte is subjected to artificial alkalinization, an increase in the rate of protein synthesis occurs (Houle and Wasserman, 1983). These data suggest that pH_i is in some way responsible for the regulation of protein synthesis during maturation of the oocyte.

The regulation of pH_i has been investigated in several cell systems where pH_i acts as a regulator for various cellular functions. The one similarity that appears to hold throughout most of the cell systems is that the increase in pH_i seems to be the result of the stimulation of Na^+/H^+ antiporters. In *Xenopus* oocytes, if the extracellular sodium is replaced with choline chloride, or if amiloride (a Na^+/H^+ antiporter inhibitor) is used, the increase in pH_i in response to progesterone is completely inhibited (Wasserman and Houle, 1984; Stith and Maller, 1985; Towle *et al.*, 1991; Rezai, 1994).

In mammalian cell systems, the Na^+/H^+ antiporter is proposed to be regulated via a phosphorylation event. It has been suggested that phosphorylation induces a conformational change within the Na^+/H^+ antiporter, causing it to acquire an increased affinity for cytoplasmic H^+ (Wakabayashi *et al.*, 1992; Borgese *et al.*, 1992). In support of this proposal, investigators have demonstrated that concurrent with the increase in

antiporter activity, there is an increase in the phosphorylation of NHE1 (Na^+/H^+ Exchanger 1), a transmembrane glycoprotein of 110 kDa in fibroblasts, epithelial cells, and blood platelets (Livne *et al.*, 1991; Sardet *et al.*, 1990; Wakabayashi *et al.*, 1992; Grinstein *et al.*, 1992; Guizouarn *et al.*, 1993). With the general consensus being that the Na^+/H^+ antiporter is activated via phosphorylation, investigations have been performed using mammalian cell systems to determine which protein kinase(s) is involved in this event (Guizouarn *et al.*, 1993; Wakabayashi *et al.*, 1992).

In *Xenopus* oocytes, protein kinases regulated by secondary messengers following progesterone stimulation were studied and it was found that neither Protein Kinase C, Protein Kinase A, nor Calcium-Calmodulin Kinase play a role in the activation of the Na^+/H^+ antiporter (Rezai, 1994). In addition, maturation promoting factor (MPF) H-1 kinase, a cytoplasmic kinase which is responsible for GVBD and chromosome condensation, does not appear to play a role in regulating oocyte pH_i (Rezai, 1994). Another series of kinases within a cascade are known to be activated in response to progesterone stimulation during oocyte meiotic maturation. This progesterone-induced kinase cascade is: c-mos kinase ----> raf-1 kinase ----> MAP kinase kinase ----> MAP kinase ----> S6 kinase. I have concentrated on the protein kinases within this cascade to determine which one, if any, is responsible for activation of the Na^+/H^+ antiporter in *Xenopus laevis* oocytes.

The proto-oncogene protein product p39 c-mos kinase has been proposed to be the "initiator" of oocyte maturation (Sagata *et al.*, 1989). Within one to two hours following progesterone stimulation, c-mos kinase is newly synthesized from stored mRNA and phosphorylated within the oocyte. c-mos kinase mRNA is found in only three types of tissues in adult *Xenopus laevis*; brain, testis, and ovary, with the ovary containing the highest concentrations (Sagata *et al.*, 1988). Within the ovary, c-mos kinase mRNA expression is restricted to the oocytes, that is the germ line, not the somatic cells. On a per

oocyte level, the amount of c-mos kinase mRNA remains constant throughout oocyte growth and maturation, with the protein product appearing only in stage VI oocytes that have been stimulated by progesterone (Sagata *et al.*, 1988). Injection of either c-mos kinase mRNA or c-mos kinase protein into stage VI oocytes, in the absence of progesterone stimulation, was sufficient to induce GVBD. In addition, injection of c-mos kinase antisense oligonucleotides into stage VI oocytes inhibited progesterone-induced GVBD (Sagata *et al.*, 1988; Daar *et al.*, 1993). Injection of c-mos kinase protein into stage VI oocytes that had been pre-treated with cycloheximide (an inhibitor of protein synthesis) was still capable of initiating GVBD in the absence of progesterone stimulation, whereas oocytes pre-treated with cycloheximide followed by progesterone treatment did not undergo GVBD (Yew *et al.*, 1992). These results suggest that c-mos kinase is the only newly synthesized protein needed to initiate GVBD.

Preliminary experiments have been performed that examined the role of c-mos kinase in the activation of the Na^+/H^+ antiporter (Rezai, 1994). A high dose of Maltose Binding Protein (MBP)-c-mos kinase protein fusion protein was capable of increasing the intracellular pH of stage VI oocytes that were not treated with progesterone. Since c-mos kinase rapidly activates other protein kinases within the kinase cascade, however, this study was unable to conclude if c-mos kinase directly regulates the Na^+/H^+ antiporter (Rezai, 1994).

Further observations have been made concerning other protein kinases becoming phosphorylated and activated following c-mos kinase activation. It has been shown that MAP kinase (Mitogen Activated Protein kinase formerly known as Microtubule Associated Protein kinase) becomes activated both in cell-free extracts as well as in fully-grown *Xenopus* oocytes subsequent to c-mos kinase stimulation, and that active c-mos kinase is capable of phosphorylating and activating purified MAP kinase kinase *in vitro* (Nebreda *et al.*, 1993; Posada *et al.*, 1993). Thus, c-mos kinase is an upstream activator

of the MAP kinase pathway within the cascade, and may possibly function directly through the activation of MAP kinase kinase.

The protein kinase that immediately follows c-mos kinase within the cascade has not been determined at this point, but researchers have found that raf-1 kinase is required for progesterone-induced oocyte meiotic maturation and that it functions downstream of c-mos kinase (Muslin *et al.*, 1993). The gene encoding raf-1 kinase was first isolated from murine sarcoma retrovirus 3611 (Rapp, 1992). The protein product of the transforming gene of this retrovirus, v-raf protein, was compared to known amino acids sequences of tyrosine kinases as well as a phosphoamino acid analysis of the protein product itself, both results showing that the v-raf protein product is a serine/threonine kinase. Three genes have since been identified that code for the cellular homologs of v-raf in vertebrates, c-raf, A-raf, and B-raf. All three are classified as proto-oncogenes in that certain amino acid sequence mutations can cause oncogenic characteristics. All of the raf-1 proteins share three highly conserved regions (CR1, CR2, CR3) which make up the primary structure of these proteins. CR1, which consists of amino acid residues 62-196, contains a binding domain for the ras molecule which is followed by a cysteine zinc finger motif. CR2, consisting of amino acid residues 255-268, is rich in serine and threonine residues which act as a major phosphorylation site for regulating kinase activity. CR3 consists of amino acid residues 331-625, is the conserved kinase domain of the protein. The protein itself can be subdivided into two regions, the regulatory region which consists of CR1 and CR2 at the amino-terminus, and the catalytic region consisting of CR3 at the carboxy-terminus. In order for raf-1 kinase to become activated, it has to be phosphorylated on serine residues, namely Ser43, Ser259, and Ser621 (Morrison *et al.*, 1993). It has been hypothesized that the regulatory region acts as an autoinhibitor, with the mechanism not yet fully understood. It has been suggested that the regulatory region folds over the catalytic region, thereby inhibiting any enzymatic activity. Consistent with this hypothesis

were the observations from several investigators that excess expression of the regulatory region of raf-1 kinase blocked the activation of the kinase domain in a dominant negative manner, and that if the regulatory region in the whole enzyme was mutated or deleted altogether, a constitutively active raf-1 kinase was produced (Morrison *et al.*, 1993; Fabian *et al.*, 1993).

In other cell systems, raf-1 kinases are located in the cytoplasm and play an important role in transmission of signals from cell surface receptors to transcription factors within the nucleus (Williams and Roberts, 1994). It has been shown that raf-1 kinase plays a role in the signal cascade shared by epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) (Kizaka-Kondoh *et al.*, 1992). The data indicated biochemical links among activated EGF and PDGF receptors, the $p21^{ras}$-GTPase-activating protein mitogenic signalling system, and raf-1 kinase. Ras, like all known GTP-binding proteins, cycles between an active and inactive form, stimulated by several extracellular signals. Activated ras appears to act as a regulator of a protein kinase cascade (Hall, 1994). The following model has been proposed for the role of ras in raf-1 kinase activation. Activated ras recruits inactive raf-1 kinase to the plasma membrane by binding to the amino-terminal domain, but ras is not responsible for anchoring raf-1 kinase to the membrane. Raf-1 kinase appears to be attached to the membrane mainly via membrane cytoskeletal elements. A ras-independent event occurs by an unknown factor at the membrane level which unmasks the catalytic domain, which was previously covered by the amino-terminal regulatory domain, allowing phosphorylation at serine residues 259 and 621, thereby activating raf-1 kinase (Stokoe *et al.*, 1994; Daum *et al.*, 1994; Williams *et al.*, 1994).

Raf-1 kinase may share common regulatory cofactors with PKC because of the similarity between amino acid motifs in the regulatory domains of the two kinases. This appears to be unlikely however, since diacylglycerol and phosphatidylserine, two lipid

modulators that function as regulatory cofactors for PKC, did not activate purified raf-1 kinase (Bruder and Rapp, unpublished results). Furthermore, it has been stated that phorbol esters do not bind to the regulatory domain of raf-1 kinase, but bind strongly to PKC (Bruder and Rapp, unpublished results). Although phorbol esters do not bind raf-1 kinase directly, it has been demonstrated in somatic cells that treatment with the phorbol ester 12-O-tetradecanoyl-phorbol-13-acetate (TPA, an activator of PKC) does induce activation of raf-1 kinase. Researchers investigated the possible role of PKCα in the activation of raf-1 kinase, reaching the conclusion that phosphorylation of Ser499 by PKCα is essential, and may be a prerequisite for autophosphorylation of Ser259 and subsequent activation of raf-1 kinase (Kolch *et al.*, 1993).

Based on the results indicating the importance of raf-1 kinase in somatic cell lines, researchers have speculated that it may play an important role in germ cells as well. Specifically, researchers are investigating the possible role of raf-1 kinase during progesterone-induced meiotic maturation in *Xenopus* oocytes. After progesterone stimulation, an increase in raf-1 kinase phosphorylation and enzymatic activity was observed concurrently with an elevation in MAP kinase activity. Upon injection of mRNA encoding oncogenic raf-1 into nonsteroid-treated oocytes, MAP kinase phosphorylation, H1 histone phosphorylation, and germinal vesicle breakdown all took place. When oocytes were injected with a dominant negative mutant of raf-1 to block endogenous raf-1 kinase activity and then treated with progesterone, none of the events mentioned above occurred. mRNA encoding c-mos kinase injected into unstimulated oocytes led to phosphorylation of both raf-1 and MAP kinases. Together, all of these results lead to the conclusion that raf-1 kinase activity is necessary for progesterone-induced oocyte meiotic maturation, and that raf-1 kinase is located downstream of c-mos kinase within the cascade (Muslin *et al.*, 1993; Fabian *et al.*, 1993).

It is believed that the only physiological substrate for active raf-1 kinase is MAP

kinase kinase. It was shown that MAP kinase kinase was phosphorylated both *in vitro* as well as *in vivo* by raf-1 kinase (Dent *et al.*, 1992; Huang *et al.*, 1993). Unlike most other kinase interactions, raf-1 kinase seems to form a stable complex with MAP kinase kinase, with MAP kinase kinase binding to the carboxy-terminal (catalytic) domain of raf-1 kinase. It has been shown that raf-1 kinase activates MAP kinase kinase by phosphorylating two serine residues, Ser217 and Ser221, within the catalytic domain of MAP kinase kinase (Alessi *et al.*, 1994). Recently, a group of investigators have shown that Cdc25 phosphatase, the activator of Maturation Promoting Factor (MPF, a complex of $p34^{cdc2}$ kinase and cyclin B), associates with raf-1 kinase in somatic mammalian cells and in frog meiotic oocytes (Galaktionov *et al.*, 1995). The observed interaction between Cdc25 and raf-1 kinase occurred at the carboxy-terminal region of raf-1 kinase *in vitro*, suggesting a strong and specific interaction between the two proteins.

MAP kinase kinase is a 45 kD protein which was first purified from unfertilized *Xenopus* oocytes (Matsuda *et al.*, 1992). It was named MAP kinase kinase for its ability to induce phosphorylation and activation of inactive MAP kinases *in vitro*. MAP kinase kinase is a dual specificity kinase in that it can undergo autophosphorylation on serine, threonine, and tyrosine residues (Kosako *et al.*, 1992; Nakielny *et al.*,1992) and phosphorylate MAP kinase on threonine and tyrosine residues (Crews and Erikson, 1992; Kosako et al., 1994). No other protein or peptide substrate has yet been identified for MAP kinase kinase other than p42/p44 MAP kinases (Wu *et al.*, 1993). Recently, an anti-*Xenopus* MAP kinase kinase antibody that specifically inhibits *Xenopus* MAP kinase kinase activity was produced . Microinjection of this antibody inhibited activation of MAP kinase by both progesterone stimulation and microinjection of active c-mos kinase in *Xenopus* oocytes. It was also observed that in addition to blocking MAP kinase activation, microinjection of the antibody blocked the activation of MPF as judged by inhibition of GVBD and histone H1 kinase activation (Kosako *et al.*, 1994). These

results strengthen the argument that MAP kinase kinase is responsible for activating MAP kinase *in vivo*, and acts downstream of c-mos kinase within the cascade.

MAP kinase is a 42kDa serine/threonine protein kinase that is activated in several cell types under different conditions. These include initiation of intracellular signaling by receptor tyrosine kinases, protein kinase C, or G proteins, which may activate MAP kinase by a common pathway, or by pathways that are distinct for each condition (Guan, 1994; Matsuda *et al.*, 1994). Upon stimulation by the above mentioned agents, MAP kinase becomes phosphorylated on both tyrosine and threonine residues and becomes activated (Payne *et al.*, 1991). It has further been shown that MAP kinase is activated during progesterone-induced oocyte meiotic maturation in *Xenopus*, and that a kinase activated in response to steroid stimulation is capable of phosphorylating MAP kinase on tyrosine and threonine residues (Posada and Cooper, 1991). It has been more recently shown that this dual phosphorylation event is the result of MAP kinase kinase phosphorylating these tyrosine and threonine residues on MAP kinase (Her *et al.*, 1993). MAP kinase phosphorylates and regulates numerous cellular signalling proteins. These include cell surface proteins, cytoskeletal components, cytoplasmic kinases, and nuclear transcription factors. In *Xenopus* oocytes, MAP kinase is able to phosphorylate ribosomal S6 kinase II (Sturgill *et al.*, 1988), a protein kinase which will be discussed later, and microtubule-associated proteins (MAPs) (Shiina *et al.*, 1992). Several researchers have found protein phosphatases that specifically and efficiently dephosphorylate, and thereby inactivate, MAP kinases. *In vitro*, 3CH134, CL100, and HVH1 all appear to function as dual specificity tyrosine/threonine phosphatases that are extremely specific for MAP kinases (Nebreda, 1994; Guan, 1994). Further studies need to be performed to determine if these phosphatases are responsible for the down-regulation of MAP kinases *in vivo*.

As mentioned previously, one of the physiological substrates for active MAP

kinase in *Xenopus* oocytes, as well as in other cells, is ribosomal protein S6 kinase (Pelech *et al.*, 1993). S6 kinase is a 90 kDa protein kinase which, when activated, is responsible for the serine phosphorylation of the 40S ribosomal protein S6. Observations made in various laboratories indicated that a single ribosomal protein (M_r 32,000) became phosphorylated during meiotic maturation in *Xenopus* oocytes. This protein was identified as 40S ribosomal protein S6. It was shown that the level of S6 phosphorylation increased throughout maturation, when analyzed by two-dimensional polyacrylamide gel electrophoresis (Nielsen *et al.*, 1982; Kruppa *et al.*, 1983; Wasserman and Houle, 1984). S6 kinase has been purified from *Xenopus* oocytes (Erikson and Maller, 1985) and it has further been shown that S6 kinase has an extreme substrate specificity for S6, the major phosphoprotein of 40S ribosomal subunits (Erikson and Maller, 1986). Results from other experiments suggest that the dramatic increase in S6 protein phosphorylation during oocyte meiotic maturation may not solely be due to an increase in S6 kinase activity. An 8-10 fold increase in S6 kinase activity does occur, but in conjunction with this increase, there is also a 50% decrease in S6 phosphatase activity, and possibly an increased accessibility of the ribosomal substrate to the kinase. All of these changes together may play a role in phosphorylation of S6 protein in progesterone-induced meiotic maturation in *Xenopus* oocytes (Wasserman *et al.*, 1988).

The protein kinase cascade that has been presented is only a partial explanation of what is occurring within the oocyte during meiotic maturation. Each kinase most likely phosphorylates other substrates in addition to those protein kinases which are located directly downstream within the cascade. There also exists the very good possibility that there are other kinases within this cascade that have not yet been discovered, but may play an important role during meiotic maturation of the oocyte. With regards to my study, I have investigated the roles of the five known protein kinases for their possible role in the regulation of the Na^+/H^+ antiporter during *Xenopus* oocyte meiotic maturation. In

addition, I have also tried to determine the site within the protein kinase cascade where cAMP blocks the activation of the Na^+/H^+ antiporter during oocyte meiotic maturation.

CHAPTER II

MATERIALS AND METHODS

Experimental Design

The Na+/H+ exchangers (NHE = Na+/H+ antiporter) in other cell systems appear to be regulated by phosphorylation of the NHE protein. It has already been shown that Protein Kinase C, Calcium-Calmodulin Kinase, and Protein Kinase A do not play a role in activation of the Na+/H+ antiporter in *Xenopus* oocytes (Rezai, 1994). During oocyte meiotic maturation, progesterone stimulation activates a cascade of protein kinases (Sagata *et al.*, 1989). The cascade of protein kinases is as follows. It has been shown that progesterone stimulation leads to synthesis of c-mos kinase from stored mRNA (Sagata *et al.*, 1989); c-mos kinase is located upstream of raf-1 kinase within the cascade (Muslin *et al.*, 1993); raf-1 kinase activates MAP kinase kinase (Huang *et al.*, 1993); MAP kinase kinase activates MAP kinase (Kosako *et al.*, 1994); MAP kinase activates S6 kinase (Pelech *et al.*, 1993) (see Figure 1). I have concentrated on these five protein kinases for their potential role in activating the Na+/H+ antiporter. In *Xenopus* oocytes, I hypothesize that one (or more) of these protein kinases activates the Na+/H+ antiporter by a phosphorylation event.

A) Alternative Hypotheses

Hypothesis I: c-mos kinase is responsible for activating the Na+/H+ antiporter via a phosphorylation event.

Hypothesis II: raf-1 kinase is responsible for activating the Na+/H+ antiporter via a phosphorylation event.

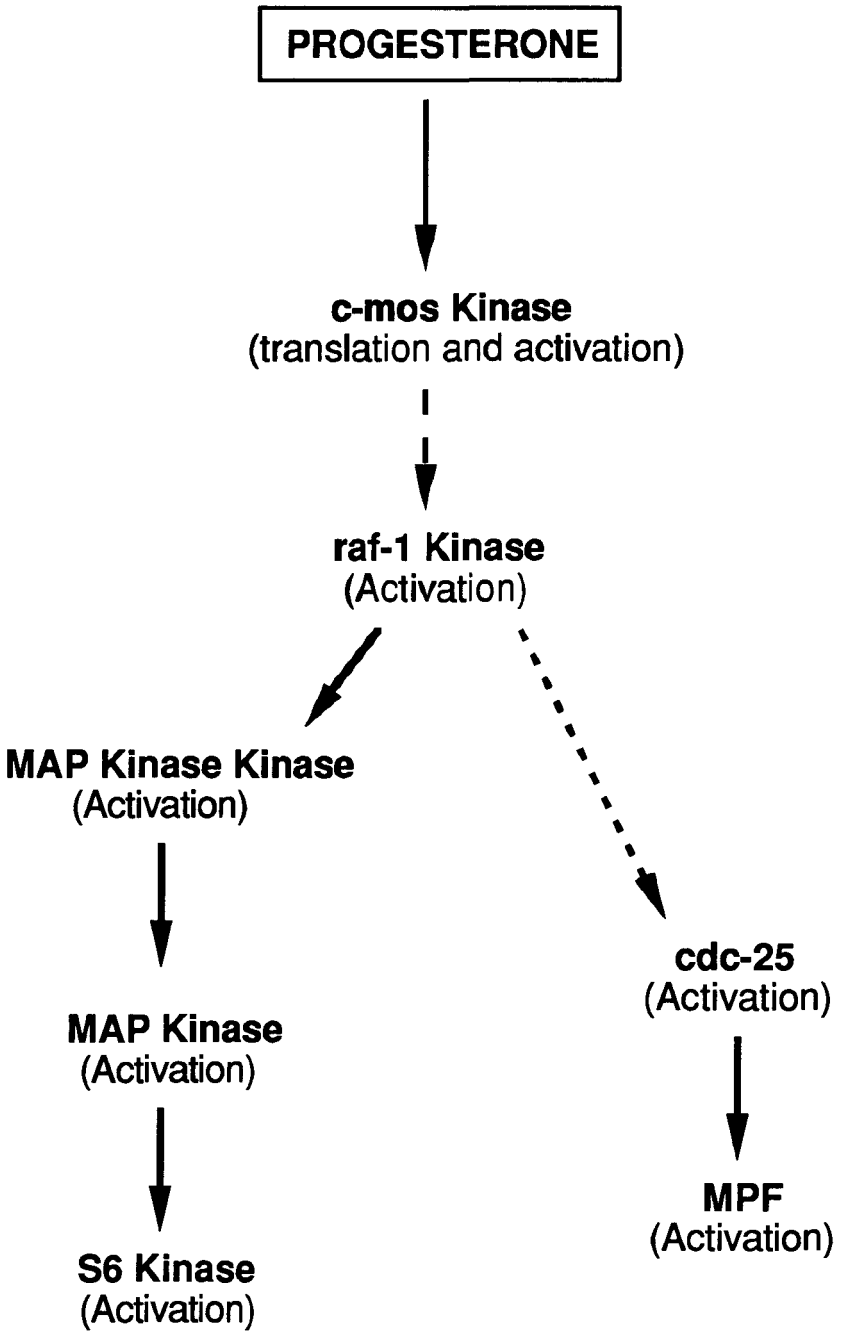

Figure 1. A proposed model of the progesterone-induced protein kinase cascade which occurs during *Xenopus* oocyte maturation. Solid arrows indicate direct activation, whereas dashed arrows indicate an indirect activation (based on results from other laboratories).

Hypothesis III: MAP kinase kinase is responsible for activating the Na+/H+ antiporter via a phosphorylation event.

Hypothesis IV: MAP kinase is responsible for activating the Na+/H+ antiporter via a phosphorylation event.

Hypothesis V: S6 kinase is responsible for activating the Na+/H+ antiporter via a phosphorylation event.

Hypothesis VI: An undetermined protein kinase within the cascade is responsible for activating the Na+/H+ antiporter via a phosphorylation event.

As there is no antibody available that cross reacts with the *Xenopus* NHE peptide, I was not able to directly investigate whether phosphorylation of the antiporter occurs when oocytes were exposed to various treatments. Therefore, I utilized an indirect method to observe the involvement of the various protein kinases in regulating the antiporter. Protein kinases or mRNA encoding these protein kinases were microinjected into stage VI oocytes and the activity of the Na^+/H^+ antiporter (changes in pH_i) was measured. The six possible results are listed below.

B) Potential Outcomes

1) If c-mos kinase, in the absence of progesterone, is capable of activating the Na^+/H^+ antiporter without any of the other protein kinases downstream in the cascade also being able to do so, then Hypothesis I is supported.

2) If raf-1 kinase, in the absence of progesterone, is capable of activating the Na^+/H^+ antiporter without any of the other protein kinases downstream in the cascade also being able to do so, then Hypothesis II is supported.

3) If MAP kinase kinase, in the absence of progesterone, is capable of activating the Na^+/H^+ antiporter without any of the other protein kinases downstream in the cascade also being able to do so, then Hypothesis III is supported.

4) If MAP kinase, in the absence of progesterone, is capable of activating the Na^+/H^+ antiporter without any of the other protein kinases downstream in the cascade also being able to do so, then Hypothesis IV is supported.

5) If S6 kinase, in the absence of progesterone, is capable of activating the Na^+/H^+ antiporter without any of the other protein kinases downstream in the cascade also being able to do so, then Hypothesis V is supported.

6) If all of the above kinases, in the absence of progesterone are not capable of activating the Na^+/H^+ antiporter, then Hypothesis VI is supported.

Oocyte Isolation and Culture

Ovarian fragments were surgically removed from adult female *Xenopus laevis* (NASCO, Ft. Atkinson, Wisconsin) that had been anesthetized by hypothermia (45 minutes in ice water). The ovarian fragments were then transferred to OR-2 medium (82.3 mM NaCl, 2.5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 5.0 mM Hepes, 1.0 mM Na_2HPO_4, pH 7.6) (Wallace *et al.*, 1973), in which all further procedures were performed. Full-grown stage VI oocytes (Dumont, 1972) were removed from the ovarian lobes by manually removing the ovarian epithelium and collagen layer with watchmaker's forceps, a process known as defolliculation. Collagenase and Pronase were not used to digest the follicle cells enzymatically to prevent disruption and damage to cell surface proteins as well as the Na^+/H^+ antiporter (Towle et al., 1991).

Intracellular pH (pH_i) Measurement

The pHi of the oocytes was determined by using the weak acid, ^{14}C-Dimethyloxazoladine 2,4 dione, ^{14}C-DMO (NEN) (Wadell and Butler, 1959). In its uncharged form, ^{14}C-DMO can pass freely across the oocyte's plasma membrane. Based on both the intracellular and extracellular pH environments, a ^{14}C-DMO equilibrium will

be formed across the membrane of stage VI oocytes in approximately 2 hours (Houle and Wasserman, 1983) (see Figure 2).

^{14}C-DMO was placed in test tubes at a volume of 2.5 µl per tube (stock 50 µCi/500 µl), which was then dried under N_2 to evaporate the ethyl acetate solvent. In each of the pH_i experiments, the ^{14}C-DMO was resuspended in 1.0 ml of either OR-2 or the desired medium, vortexed, and transferred into 2 ml multiwell plates. The final concentration of ^{14}C-DMO in each well was 0.25 µCi/ml. Twenty oocytes were placed into each well per treatment, per experiment. After a minimum of 2.5 hours of incubation in ^{14}C-DMO at 20° C, drugs or hormones were added and allowed to incubate for the desired amount of time at 20° C.

Upon completion of the given incubation period, both the media and the oocyte ^{14}C-DMO DPM were determined in triplicate. For the media determination, 10 µl media from each treatment were transferred into a scintillation vial along with 10 ml Biodegradable Counting Scintillant (BCS, Amhersham) and mixed thoroughly. For the oocyte determination, 5 oocytes from each treatment were removed and quickly washed in 3 consecutive dishes containing non-radioactive OR-2 medium (approximately 5-10 seconds per dish). The oocytes were then transferred into scintillation vials where any excess medium was removed with a pasteur pipette. To each vial was added 0.5 ml NCS-II (tissue solubilizer, Amhersham) before they were placed into a 60° C incubator for 35 minutes where the oocytes were digested. The vials were then placed into a -20° C freezer for 10 minutes to cool down, after which 10 ml cold BCS-NA (Non-Aqueous Biodegradable Counting Scintillant, Amhersham) were added to each vial, mixed thoroughly, and placed into -20° C freezer for another 20 minutes. The media and oocyte samples were then counted in a Beckman LS-7000 liquid scintillation counter. The CPM data obtained from the counter was converted to DPM, which was then used to determine the pH_i by utilizing the following formula (Wadell and Butler, 1959):

$$pH_i = pKa + \log ([DMO]_{inside}/[DMO]_{outside} (10^{pHo-pKa+1})-1)$$

pH_i	= intracellular pH (unknown)
pKa	= pKa of DMO = 6.32
$[DMO]_{inside}$	= concentration of DMO inside the oocyte
	= DPM/μl (water volume of a stage VI oocyte = 0.45 μl)
$[DMO]_{outside}$	= concentration of DMO outside the oocyte (medium)
	=DPM/μl
pH_o	= outside pH = 7.6

There was one basic experimental design that was followed throughout all experiments that dealt with regulation of pH_i. One group of oocytes was left untreated and served as the negative control group; one group was treated with progesterone and served as the positive control group; one group of oocytes was microinjected with either the desired protein kinase or mRNA encoding the desired protein kinase and served as the experimental group. In addition to examining the pH changes in the oocytes, the oocytes were also scored for GVBD. All of the experiments were replicated at least three times, utilizing oocytes from three different animals on three different days.

Protein Kinases and Transcription Vectors

The MBP-c-mos protein fusion protein was obtained from Dr. George F. VandeWoude. This construct consists of bacterially expressed active c-mos kinase bound to Maltose Binding Protein (MBP), which is eluted from a Maltose-Sepharose column with maltose to obtain the purified protein. The protein kinase was in a buffer consisting of 100 mM NaCl and 20 mM Hepes at a pH of 7.4. The protein concentration of the

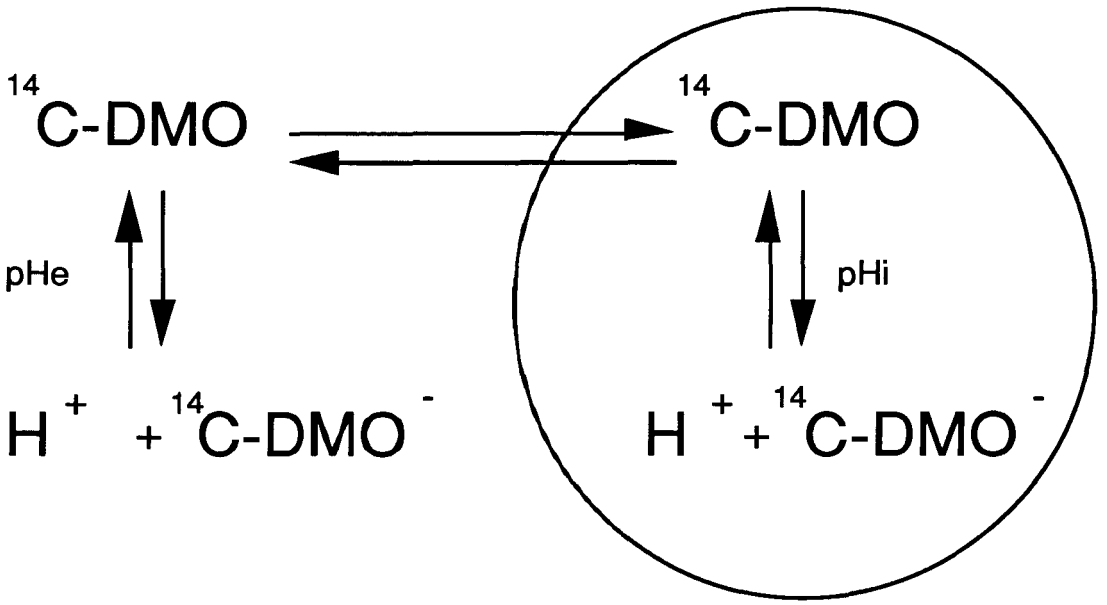

Figure 2. DMO equilibration across the oocyte plasma membrane. Oocytes were incubated in ^{14}C-DMO (0.25 µCi/ml) for 2.5 hours at 20°C to allow for equilibration. The equilibrium of DMO across the cell membrane was established according to the extracellular pH (pH_e) and intracellular pH (pH_i) environments.

stock was 0.95 mg/ml, which was diluted with buffer as needed to concentrations of 1.25 ng/40 nl to 40.0 ng/40 nl for microinjection into the oocytes.

MAP kinase kinase was obtained from Dr. Steven Pelech as a bacterial expressed glutathione synthetase transferase (GST) -fusion protein that was eluted from GST-Sepharose beads with glutathione. Three different mutant proteins were received. K97A is a kinase-inactive protein by virtue of a lysine to alanine amino acid substitution that is necessary for ATP binding. S218A/S222A is a regulatory double-mutant which is an inactive kinase in terms of autophosphorylation and upstream activation. S218E/S222E is a regulatory double-mutant which is constitutively active and requires no upstream activation. The preparations were dialysed against the following buffer: 200 mM MOPS, 50 mM EGTA, 20 mM EDTA, 1 mM $NaCO_3$, 10 mM NaF, 240 mM ß-glycerophosphate, and 50 µM ß-methyl aspartic acid. The stock concentration of all three protein kinase preparations was 3 µg per 20 µl.

Active S6 kinase was obtained from Dr. Raymond Erikson as a purified protein kinase preparation. The concentration of S6 kinase was 1.22 mg/ml in a buffer consisting of 100 mM NaCl, 20 mM Hepes, pH 7.4, and 1.0 mg/ml BSA. The preparation was assayed using *Xenopus* 40s ribosome subunits as substrate.

Transcription vectors with inserts coding three mutants of raf-1 kinase were obtained from Dr. Deborah K. Morrison. The three transcription vectors coded for: wild-type raf-1 protein (wt-raf-1), which is the full length protein (69 kD) in its unphosphorylated form; constitutively active raf-1 protein (ΔN'-raf-1), which is just the catalytic region of the protein (40 kD) with the regulatory region removed; kinase deficient raf-1 protein (KD-raf-1), which is the wt-raf-1 protein (69 kD) with a substitution of alanine for serine at position 621 rendering it incapable of kinase activity. The expected sizes of the three mRNAs was as follows: wt-raf-1 = 2.2 kilobases, ΔN'-raf-1 = 1.3 kilobases, and KD-raf-1 = 2.2 kilobases.

Transcription of Plasmid Vectors

1. Linearization of Plasmids. Each of the plasmids had to be linearized before it could be transcribed into mRNA. All of the plasmids were cut with the restriction enzyme EcoR I since there was only one EcoR I restriction site in the plasmid, located downstream of the insert. The following protocol was utilized for all of the plasmids. Into a RNase-free eppendorf tube was added 0.5 µl plasmid solution (approximately 2.0 µg DNA), 2.0 µl 10x EcoR I Buffer (NEBuffer System, BioLabs, New England), 1.0 µl EcoR I (20 Units/µl) (BioLabs, New England), and 16.5 µl RNase-free H_2O. The solutions were placed into a 37° C waterbath for one hour, after which time a 2 µl aliquot was removed from each reaction tube and run on a gel to verify proper digestion of the plasmids. The remainder of the reaction mixture was precipitated with 1.8 µl 5 M ammonium acetate and 36 µl 100% ethanol at -20° C. To the 2 µl aliquots were added 3 µl 6x loading buffer (0.25% Bromophenol blue, 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 30% glycerol, dH_2O to 50 ml) and 13 µl RNase-free H_2O. All samples were heated for ten minutes at 65° C and run on an 0.8% agarose gel at 100 volts for 45 minutes. The gel was then stained for 15 minutes with Ethidium Bromide (EtBr, 0.5 µg/ml), rinsed with RNase-free H_2O, and viewed under an ultraviolet light source comparing the bands of linearized DNA to known lambda DNA markers (GIBCO/BRL) run in adjoining lanes.

2. Transcription of the linearized DNA. The linearized plasmids were centrifuged at 12,500 x g for 20 minutes, the supernatant was removed, and the pellets were desiccated and resuspended in TE Buffer (10 mM Tris-HCl, 1.0 mM EDTA, pH 8.0). The linearized plasmids were transcribed using an *in vitro* SP6 polymerase transcription kit (mMESSAGE mMACHINE Kit, Ambion, Inc.) following the protocol supplied with the kit, with the termination step being the following protocol for mRNA purification rather than the procedures suggested in the kit. To purify the mRNA that was transcribed,

the RNeasy Total RNA Kit following the RNeasy Clean-up Protocol (Qiagen, Inc.) was used. The final product was suspended in RNase-free H_2O. Two separate methods were used to determine if the transcription reaction had worked successfully. First, the concentration of the mRNA transcribed was determined. Two µl sample were added to 1 ml RNase-free H_2O and the absorbance (A_{260}/A_{280}) readings were taken with a spectrophotometer. Second, aliquots of the samples were run on an agarose gel to determine if the proper size mRNA was transcribed. All samples were first denatured in the following solution: 4.7 µl mRNA (2 µg), 2.0 µl 5x MOPS, 3.3 µl formaldehyde, 10.0 µl formamide, and 1.5 µl EtBr (stock = 10 mg/ml) for 15 minutes at 55° C, after which 5 µl of 6x loading buffer (same buffer as was used in DNA agarose gels) was added. The samples were then run on a 1.0% agarose gel containing 1x MOPS and 0.67 M formaldehyde at 70 volts for 45 minutes. The gel was then thoroughly rinsed with RNase-free H_2O (approximately 30 minutes, changing it periodically) and viewed on an ultraviolet light source. The sizes of the transcripts were compared to RNA markers (Promega) run in adjoining lanes.

Microinjection of Protein Kinases and mRNA

Throughout my study, I used either protein kinases or mRNA encoding protein kinases that would not readily pass across the plasma membrane of the oocyte and therefore had to be microinjected directly into the oocyte cytoplasm. Microinjection needles were made from 10 mm x 100 mm glass capillary tubes using a De-Fonbrune microforge, and were designed to deliver 1.25 ng to 40.0 ng per 40 nl per oocyte. This delivery was made into a single site within each oocyte's cytoplasm. A Brinkman micromanipulator was used to guide the injection needles.

Electrophoretic Analysis of Raf-1 Protein Products

To examine the raf-1 kinase proteins synthesized in mRNA-injected oocytes, 10 stage VI oocytes were left untreated, treated with progesterone (1 μM, Sigma), or microinjected with mRNA for the three forms of raf-1 kinase and allowed to incubate for 6 hours at 20° C. The oocytes were then labelled in ^{35}S-methionine at 50 μCi/ml for 1 hour at 20° C. After the 1 hour of labelling, 6 oocytes from each treatment group were homogenized in 1.0 ml protein extraction homogenization buffer (100 mM NaCl, 20 mM Tris-HCl, 10 mM ß-mercaptoethanol, 0.5 mM phenylmethylsulfonyl fluoride) and centrifuged for 10 minutes at 12,500 x g. After centrifugation, 0.5 ml supernatant was drawn off and added to 1.0 ml 100% ethanol and stored at -20° C for a minimum of 8 hours to precipitate proteins.

The precipitates were then centrifuged for 20 minutes at 12,500 x g, the supernatant was removed, and the protein pellets were dried for 1 hour under vaccum. The pellets were resuspended in 50 μl SDS sample buffer (Tris, pH 6.8, glycerol, bromphenol blue, ß-mercaptoethanol, sodium dodecyl sulfate, and dH_2O), boiled, and then loaded onto a 10% Laemmli gel (1970). After running at 25 milliamps for 3.5 hours, the gel was stained with Coomassie Blue to confirm that equal amounts of protein were present in each lane. The gel was then subjected to flurography with 2,5-Diphenyloxazole (PPO) and glacial acetic acid, dried for 2 hours at 80° C, and put up with XAR-5 film (Kodak) and stored at -85° C for 3 days. After film development, the newly synthesized raf-1 kinase protein bands were compared to ^{14}C-marker proteins (Amersham) that were run in separate lanes.

Dose Response Curves for Protein Kinases or mRNA

Preliminary experiments consisted of microinjecting the stock concentration of the protein or mRNA into stage VI oocytes and determining if a statistically significant

increase in pH_i had occurred compared to control and progesterone-treated oocytes. All preliminary experiments were replicated at least three times for each protein or mRNA. If a significant increase in pH_i was observed, the following experiments were carried out.

Groups of 20 stage VI oocytes were microinjected with different doses of the protein kinase or mRNA ranging from 1.25 ng to 40.0 ng per oocyte and were incubated in OR-2 media containing ^{14}C-DMO at 20° C for the times indicated in the text. After this incubation period, the oocyte pH_i was determined for the various doses and compared to the pH_i in control and progesterone-treated oocytes. The lowest dose of protein or mRNA which gave a pH_i increase that was statistically similar to the pH_i of progesterone-treated oocytes was used in all subsequent experiments .

Protein Synthesis Requirement for Antiporter Activation

It has been shown that protein synthesis is necessary for GVBD to occur in response to progesterone treatment (Wasserman and Masui, 1975). It has also been shown that a new protein(s) has to be synthesized in order to activate the Na^+/H^+ antiporter in response to progesterone stimulation (Rezai, 1994). It is known that c-mos kinase is newly synthesized upon hormonal stimulation (Sagata *et al.*, 1989), but is c-mos kinase the only newly synthesized protein needed to activate the Na^+/H^+ antiporter?

Groups of 20 stage VI oocytes were preincubated in OR-2 medium containing ^{14}C-DMO with or without cycloheximide (10 μg/ml, Sigma), a specific inhibitor of protein synthesis for 12 to 14 hours. This concentration of cycloheximide has been shown to inhibit protein synthesis by 98% (Wasserman *et al.*, 1986). The oocytes were then either left untreated, treated with progesterone (1 μM, Sigma), or microinjected with a particular protein kinase and allowed to incubate at 20° C for 8 to 12 hours. After this incubation period, the oocyte pH_i was determined for all treatment groups. The protein-microinjected groups were compared to the control and progesterone-treated groups in the

absence or presence of cycloheximide.

The Effect of cAMP on Antiporter Activation

It has been shown that progesterone has an inhibitory effect on adenylate cyclase in *Xenopus* oocytes causing a decrease in oocyte cAMP levels (Maller and Krebs, 1977; Schorderet-Slatkine *et al.*, 1982; Matten *et al.*, 1994). It has also been shown that if intracellular cAMP concentrations remain elevated, progesterone-induced GVBD will not occur (Schorderet-Slatkine *et al.*, 1982). Recent studies by Rezai (1994) have indicated that elevated levels of cAMP are also antagonistic toward progesterone-induced activation of the Na^+/H^+ antiporter. My goal was to determine at what point within the protein kinase cascade elevated levels of intracellular cAMP inhibit activation of the Na^+/H^+ antiporter. Previous studies have shown that there are at least two different experimental methods of altering cAMP levels in *Xenopus* oocytes: forskolin, an activator of adenylate cyclase; and 8-Bromo-adenosine-3':5'-monophosphate, cyclic (8-Br-cAMP), a cell-permeable cAMP analogue.

1. Forskolin. Groups of 20 stage VI oocytes were preincubated in OR-2 medium containing ^{14}C-DMO with or without forskolin (100 μM, Calbiochem) for 8 to 14 hours at 20° C. This concentration of forskolin as well as the incubation period were selected based on previous studies (Rezai, 1994). The oocytes were then either left untreated, treated with progesterone (1 μM, Sigma), or microinjected with a particular protein kinase or mRNA and allowed to incubate at 20° C for the times indicated in the text. After this incubation period, the oocyte pH_i was determined for all treatment groups.

2. 8-Br-cAMP. Groups of 20 stage VI oocytes were preincubated in OR-2 medium containing ^{14}C-DMO with or without 8-Br-cAMP (1.0 mM, Boehringer Mannheim) for 8 to 14 hours at 20° C. This concentration of 8-Br-cAMP was selected based on other studies (Nagasaka *et al.*, 1994). The oocytes were then either left

untreated, treated with progesterone (1 μM, Sigma), or microinjected with a particular protein kinase or mRNA and allowed to incubate at 20° C for the times indicated in the text. After this incubation period, the oocyte pH_i was determined for all treatment groups.

Electrophoretic Analysis of S6 Phosphorylation in MAP Kinase Kinase or S6 Kinase Injected Oocytes

To verify that active MAP kinase kinase and S6 kinase were being microinjected into the oocytes, the phosphorylation of the S6 protein on the 40s ribosomal subunit was monitored since the cascade follows from MAP kinase kinase to MAP kinase to S6 kinase to the phosphorylation of the S6 protein. To examine ribosomal S6 protein phosphorylation, 10 stage VI oocytes were left untreated, treated with progesterone (1 μM, Sigma), or microinjected with MAP kinase kinase (which was concentrated 3-fold with centricon 30 microconcentrators, Micon) or S6 kinase, labelled in ^{32}P-orthophosphate at 50 μCi/ ml for 8 to 12 hours at 20° C. After this incubation, oocytes were homogenized, protein was extracted and precipitated as mentioned previously. The precipitated proteins were resuspended and run on a gel, checked for equal loading of proteins, and dried as described above without flurography. The dried gel, XAR-5 film (Kodak), and an intensification screen were then stored at -85° C for 3 days. After film development, the phosphorylated ribosomal S6 protein bands were compared in the various treatment groups.

Statistical Analysis

In each experiment, three groups of five oocytes each (15 oocytes total) were used to measure the intracellular pH of the oocyte in triplicate. All experiments were replicated using oocytes from a minimum of three different animals on three different days (n = 3). All intracellular pH values are the mean pH_i value $\pm$ the standard error of the mean. The

data were analyzed by two-way analysis of variance (ANOVA), and when a significant F-value was encountered, the different treatments were compared using the Tukey's multiple comparisons test. The data were analyzed using MYSTAT and SYSTAT statistical programs.

CHAPTER III

RESULTS

The order of the following experiments reflects the order in which the various protein kinases were received from other laboratories.

Part I: c-mos kinase

The first protein kinase to be investigated in this study was c-mos kinase. A previous study from our laboratory has implicated c-mos kinase in activation of the Na+/H+ antiporter in *Xenopus* oocytes during meiotic maturation (Rezai, 1994). Preliminary results using high doses of kinase microinjected into oocytes indicated that either c-mos kinase, or some protein kinase downstream of c-mos kinase played a role in the activation of the Na+/H+ antiporter. The focus of my initial experiments was to confirm the role of c-mos kinase in regulation of oocyte pH_i during progesterone-induced meiotic maturation.

A. c-mos kinase Dose Response Curve:

Injecting 20 ng c-mos protein per oocyte induced an increase in oocyte pH_i that was statistically similar to the increase in pH_i caused by progesterone (Rezai, 1994). To determine the lowest concentration of c-mos protein needed to cause an increase in oocyte pH_i that was similar to the increase in pH_i caused by progesterone, oocytes were injected with different concentrations of c-mos protein. As shown in Figure 3, oocytes were microinjected with concentrations of c-mos kinase ranging from 1.25 ng to 40.0 ng per oocyte. Increasing concentrations of c-mos kinase induced an increase in pH_i,

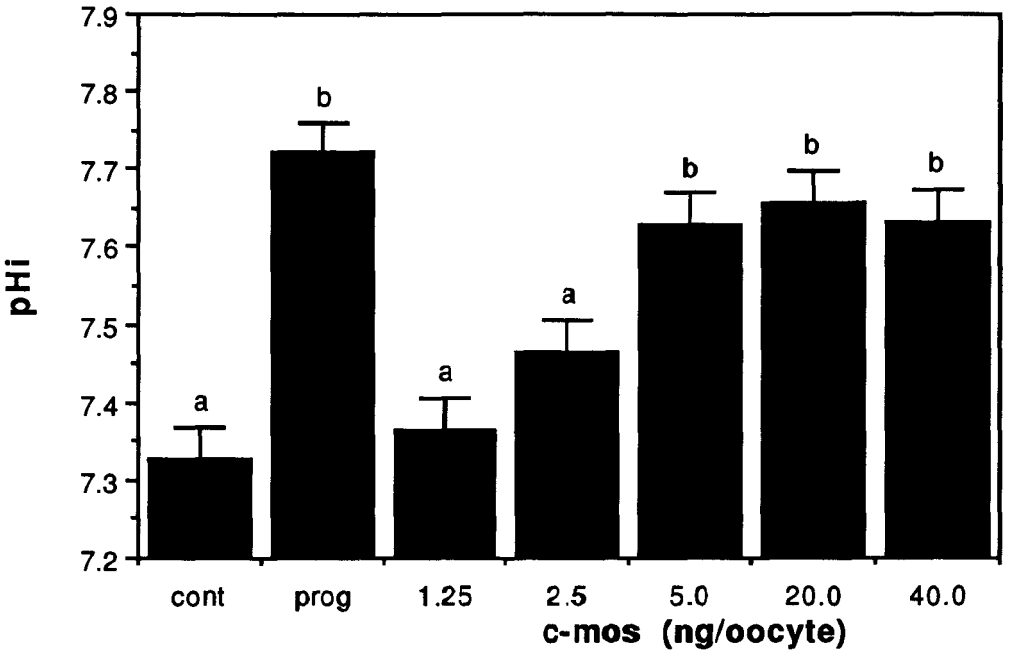

Figure 3. c-mos kinase dose response curve. Oocytes were either left untreated (cont), treated with progesterone (1 μM), or microinjected with concentrations of c-mos kinase ranging from 1.25 to 40.0 ng of protein per oocyte. At concentrations of 5.0 ng per oocyte and higher, the increase in pH_i was statistically similar to the increase in pH_i induced by progesterone. All subsequent experiments used 5.0 ng of c-mos protein per oocyte. Columns with different letters above them are significantly different from each other using Tukey's multiple test comparison. The data are expressed as means $\pm$ the standard error of the mean, where n=3.

which appeared to level off at concentrations greater than 5.0 ng protein per oocyte. 5.0 ng c-mos protein was the lowest concentration that caused an increase in oocyte pH_i statistically similar to the increase in oocyte pH_i induced by progesterone. All subsequent experiments used 5.0 ng c-mos protein per oocyte.

B. Protein Synthesis Requirement for Na^+/H^+ Antiporter Activation:

It has been shown that oocyte protein synthesis is required for activation of the Na^+/H^+ antiporter in response to progesterone stimulation (Rezai, 1994). It is known that c-mos kinase is newly synthesized upon hormonal stimulation (Sagata *et al.*, 1989). To determine if c-mos kinase is the only newly synthesized protein required for the activation the Na^+/H^+ antiporter during oocyte meiotic maturation, the following experiment was performed. Oocytes were treated either with 10 μg/ml cycloheximide (a specific inhibitor of protein synthesis, at a concentration which has been shown to inhibit protein synthesis by 98%, Wasserman *et al.*, 1986) and ^{14}C-DMO or just with ^{14}C-DMO for 12 to 14 hours. As shown in Figure 4, oocytes from each of the two groups were either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with c-mos protein (5 ng/oocyte). The results showed that the pH_i in steroid-stimulated oocytes treated with cycloheximide remained at levels that were statistically similar to the pH_i of both control oocytes with or without cycloheximide. The results showed that the pH_i of oocytes microinjected with c-mos protein, even in the presence of cycloheximide, was statistically similar to the pH_i of progesterone-stimulated oocytes. Thus, c-mos kinase appears to be the only newly synthesized protein needed for the activation of the Na^+/H^+ antiporter during oocyte meiotic maturation.

C. cAMP Block of Antiporter Activation:

Progesterone has been shown to have an inhibitory effect on adenylate cyclase in *Xenopus* oocytes causing a decrease in oocyte cAMP levels (Sadler and Maller, 1981). Not only has it been demonstrated that elevated cAMP concentrations within the oocyte

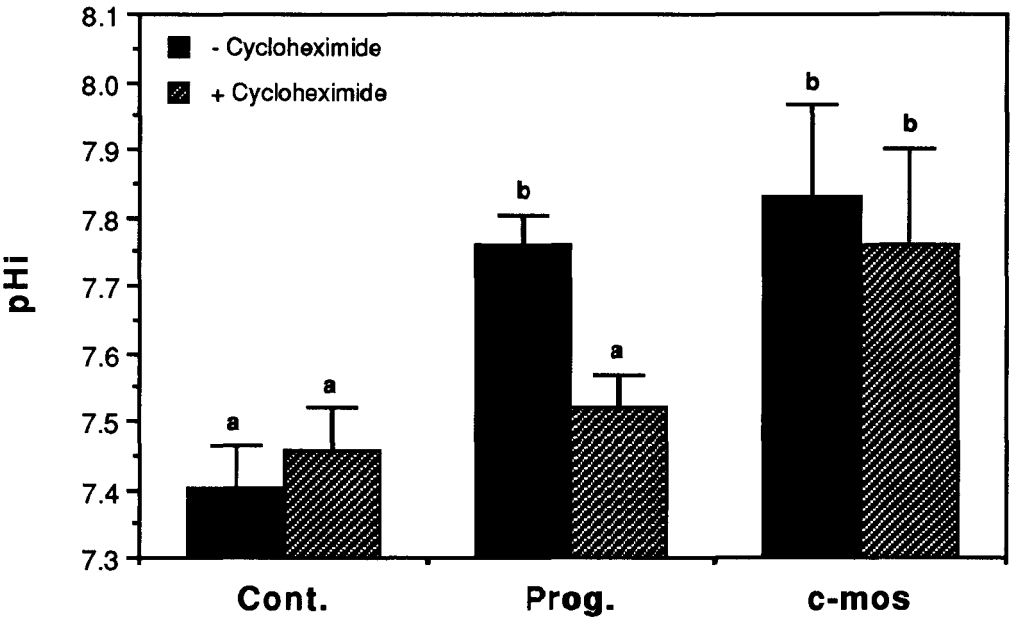

Figure 4. c-mos kinase is the only newly synthesized protein needed for activation of the Na+/H+ antiporter. Oocytes were incubated in ^{14}C-DMO for 12 to 14 hours in the presence or absence of cycloheximide (10 μg/ml). The oocytes from both groups were the either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with c-mos kinase (5.0 ng/oocyte). The pH_i of oocytes microinjected with c-mos kinase, both treated and not treated with cycloheximide, was statistically similar to the pH_i of progesterone-treated oocytes that had not been treated with cycloheximide. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are expressed as means ± the standard error of the mean, where n=3.

inhibit GVBD (Schorderet-Slatkine *et al.*, 1982), but a recent study from our laboratory has indicated that elevated levels of cAMP are also antagonistic toward progesterone-induced activation of the Na^+/H^+ antiporter during oocyte meiotic maturation (Rezai, 1994). To determine if elevated levels of intracellular cAMP are antagonistic toward c-mos kinase-induced activation of the Na^+/H^+ antiporter, the following two methods of altering cAMP levels in *Xenopus* oocytes where used.

1. Forskolin Activation of Adenylate Cyclase:

The first method used was incubating the oocytes in forskolin, an activator of adenylate cyclase, to elevate the levels of intracellular cAMP. Oocytes were treated either with forskolin (100 μM) at a concentration shown to have an inhibitory effect on the progesterone-induced increase in pH_i (Rezai, 1994) in ^{14}C-DMO, or just with ^{14}C-DMO for 12 to 14 hours. Oocytes from each of the two groups were left untreated (Cont.), treated with progesterone (1 μM), or microinjected with c-mos protein (5 ng/oocyte). As shown in Figure 5, forskolin inhibited the increase in pH_i in progesterone-stimulated oocytes to a level that was statistically similar to control pH_i levels. Forskolin did not appear to be antagonistic to c-mos kinase's ability to activate the Na^+/H^+ antiporter. The pH_is in c-mos kinase-injected oocytes with or without forskolin pretreatment were statistically similar.

2. 8-Br-cAMP Elevation of Intracellular cAMP Levels:

To verify the forskolin results, a second method of elevating levels of intracellular cAMP was utilized. 8-Br-cAMP (8-Bromo-adenosine-3':5'-monophosphate, cyclic) is a cAMP derivative capable of passing through the oocyte's membrane into the cytoplasm, thus causing an elevation in intracellular cAMP levels. Oocytes were treated with 8-Br-cAMP (1.0 mM) in ^{14}C-DMO or just with ^{14}C-DMO for 12 to 14 hours. Oocytes from each of the two groups were either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with c-mos protein (5 ng/oocyte). As shownin Figure 6,

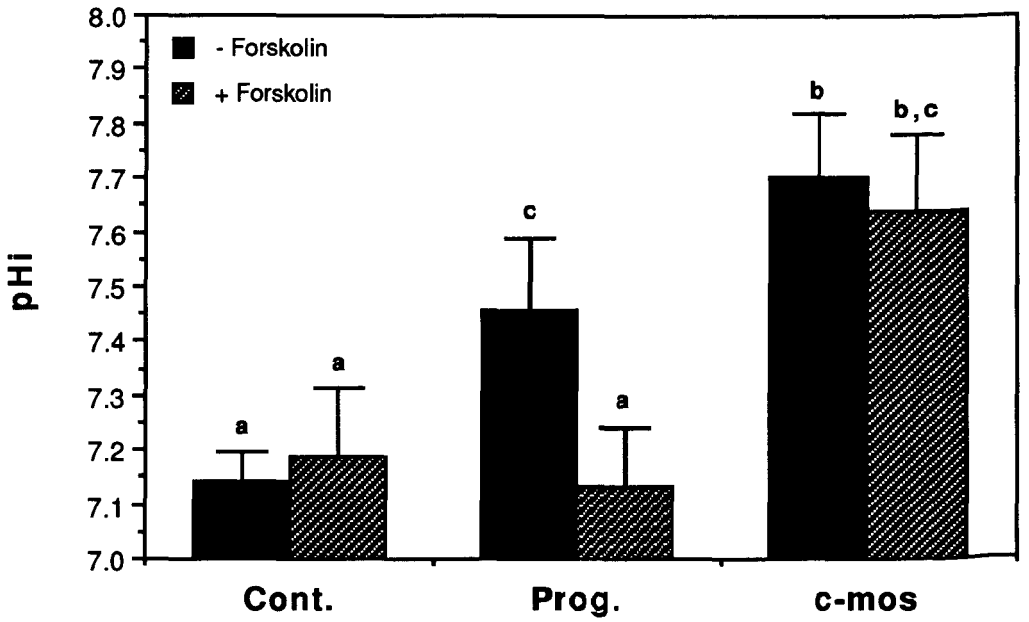

Figure 5. Forskolin does not appear to be antagonistic to c-mos kinase's ability to activate the Na+/H+ antiporter. Oocytes were incubated in ^{14}C-DMO for 12 to 14 hours in the presence or absence of forskolin (100 μM). The oocytes from both groups were then either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with c-mos kinase (5.0 ng/oocyte). The pH_i of the oocytes microinjected with c-mos did not differ significantly regardless if treated with forskolin or not. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are expressed as means ± the standard error of the mean, where n=4.

8-Br-cAMP was antagonistic to progesterone's ability to cause an increase in pH_i, maintaining pH_i at levels statistically similar to the control pH_i values. The results with c-mos kinase and 8-Br-cAMP confirmed the results of the forskolin experiment. Oocytes injected with c-mos kinase and treated with 8-Br-cAMP maintained levels of elevated pH_i that were statistically similar to the group of oocytes that was just stimulated with progesterone. The results from both the forskolin experiments and the 8-Br-cAMP experiments showed that elevated levels of intracellular cAMP were antagonistic toward a progesterone-induced increase in pH_i but have no effect on the ability of c-mos kinase to cause an increase in pH_i. This indicates that the cAMP block is downstream of the progesterone receptor, but upstream of c-mos kinase in the protein kinase cascade.

Part II: S6 kinase

S6 kinase was the next protein kinase investigated. S6 kinase, when activated, is responsible for phosphorylating the 40S ribosomal protein S6. S6 kinase is one of the terminal kinases, located directly downstream of MAP kinase. It has further been shown that the substrate of S6 kinase, 40S ribosomal protein S6, becomes phosphorylated during oocyte meiotic maturation. I therefore wanted to investigate if S6 kinase, in addition to phosphorylating the ribosomal protein, was involved in regulation of the Na^+/H^+ antiporter during oocyte meiotic maturation.

A. S6 kinase Effect on pH_i:

To determine if S6 kinase is involved in activating the Na^+/H^+ antiporter, groups of oocytes were preincubated in ^{14}C-DMO for 12 to 14 hours, and then were either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with active S6 kinase (50 ng/oocyte). As shown in Figure 7, oocytes injected with active S6 kinase did not exhibit an increase in pH_i. Oocytes injected with S6 kinase maintained pH_i levels that were statistically similar to those in untreated oocytes.

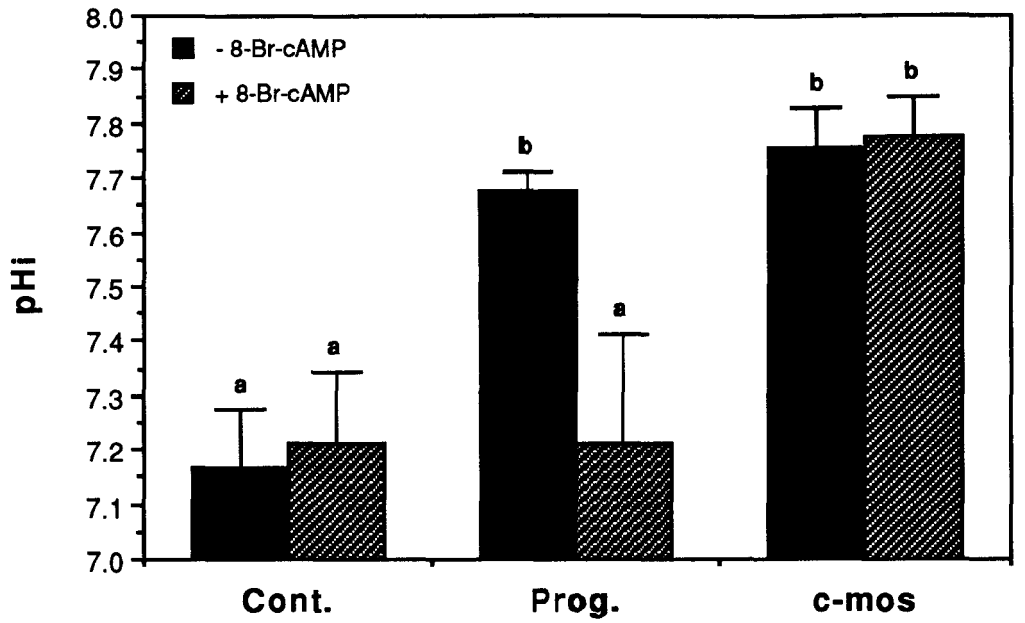

Figure 6. Elevating intracellular levels of cAMP with 8-Br-cAMP does not appear to be antagonistic to c-mos kinase's ability to activate the Na^+/H^+ antiporter. Oocytes were incubated in ^{14}C-DMO for 12 to 14 hours in the presence or absence of 8-Br-cAMP (1.0 mM). The oocytes from both groups were then either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with c-mos kinase (5.0 ng/oocyte). The pH_i of oocytes microinjected with c-mos kinase, both treated and not treated with 8-Br-cAMP, were statistically similar to one another, as well as to the pH_i of progesterone-treated oocytes not treated with 8-Br-cAMP. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are expressed as means ± the standard error of the mean, where n=3.

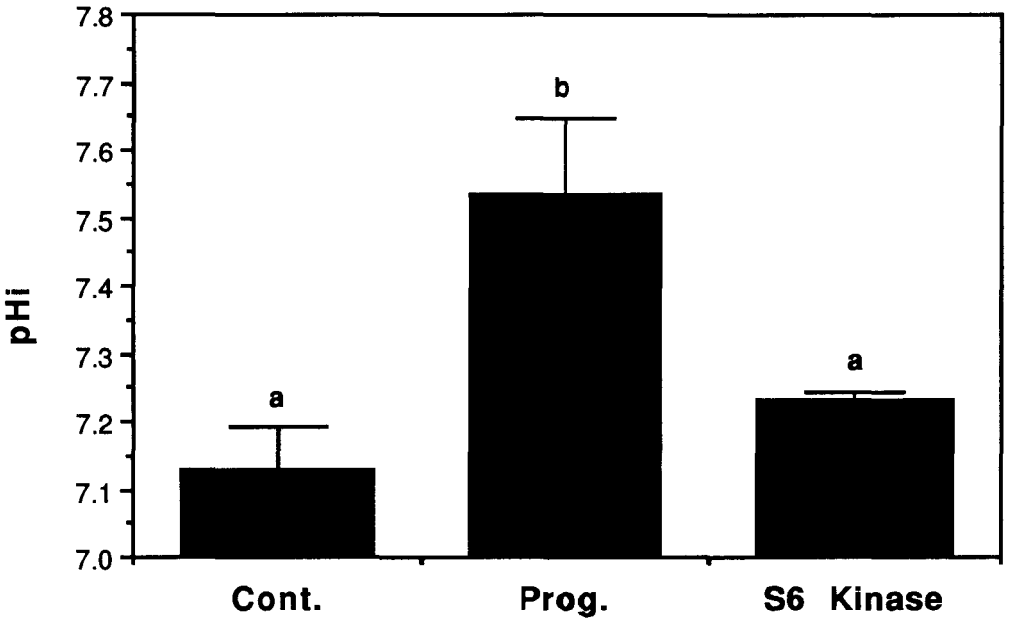

Figure 7. S6 kinase does not activate the Na^+/H^+ antiporter. Oocytes were incubated in ^{14}C-DMO for 12 to 14 hours and were then either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with S6 kinase (50 ng/oocyte). The pH_i of the oocytes microinjected with S6 kinase was statistically different from the pH_i of the progesterone-treated oocytes, but statistically similar to the pH_i of oocytes that were left untreated (Cont.). Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are presented as means ± the standard error of the mean, where n=4.

B. Electrophoretic Analysis of S6 Phosphorylation:

The above result indicated that S6 kinase does not play a role in regulating pH_i. To verify that the injected S6 kinase was active, phosphorylation of S6 protein in the injected oocyte ribosomal subunits was monitored. Oocytes were either left untreated (Cont.), stimulated with progesterone (1 μM), or microinjected with S6 kinase and labelled in OR-2 medium with ^{32}P-orthophosphate (100 μCi/ml) for 8 hours. Oocytes were removed from their respective treatments and proteins were extracted and electrophoresed using a SDS-polyacrylamide gel (SDS-PAGE). Figure 8 shows an autoradiograph comparing the phosphorylation of S6 protein between the three treatments. S6 protein has a molecular weight of 32,000. Based on this experiment, S6 protein (32,000 Da) was phosphorylated in the progesterone-stimulated oocytes as well as in the S6 kinase-microinjected oocytes, but not in the untreated oocytes. This indicates that the S6 kinase that was used in previous experiments was active and that the negative effect of S6 kinase on oocyte pH_i was valid.

Part III: raf-1 kinase

I was unable to obtain raf-1 kinase protein, but rather, received transcription vectors with inserts coding for three forms of raf-1 kinase. The three transcription vectors coded for: 1) wild-type raf-1 protein (wt-raf-1), the full length protein in its unphosphorylated form; 2) constitutively active raf-1 protein (ΔN'-raf-1), just the catalytic region of the protein with the regulatory region removed; 3) kinase deficient raf-1 protein (KD-raf-1), the wt-raf-1 protein with an alanine for serine substitution at position 621 rendering it incapable of kinase activity (Fabian *et al.*, 1993).

A. Transcription of Plasmid Vectors and Translation of mRNA for raf-1:

1. Linearization of raf-1 Plasmids:

Each of the three plasmids was first linearized before it could be transcribed into

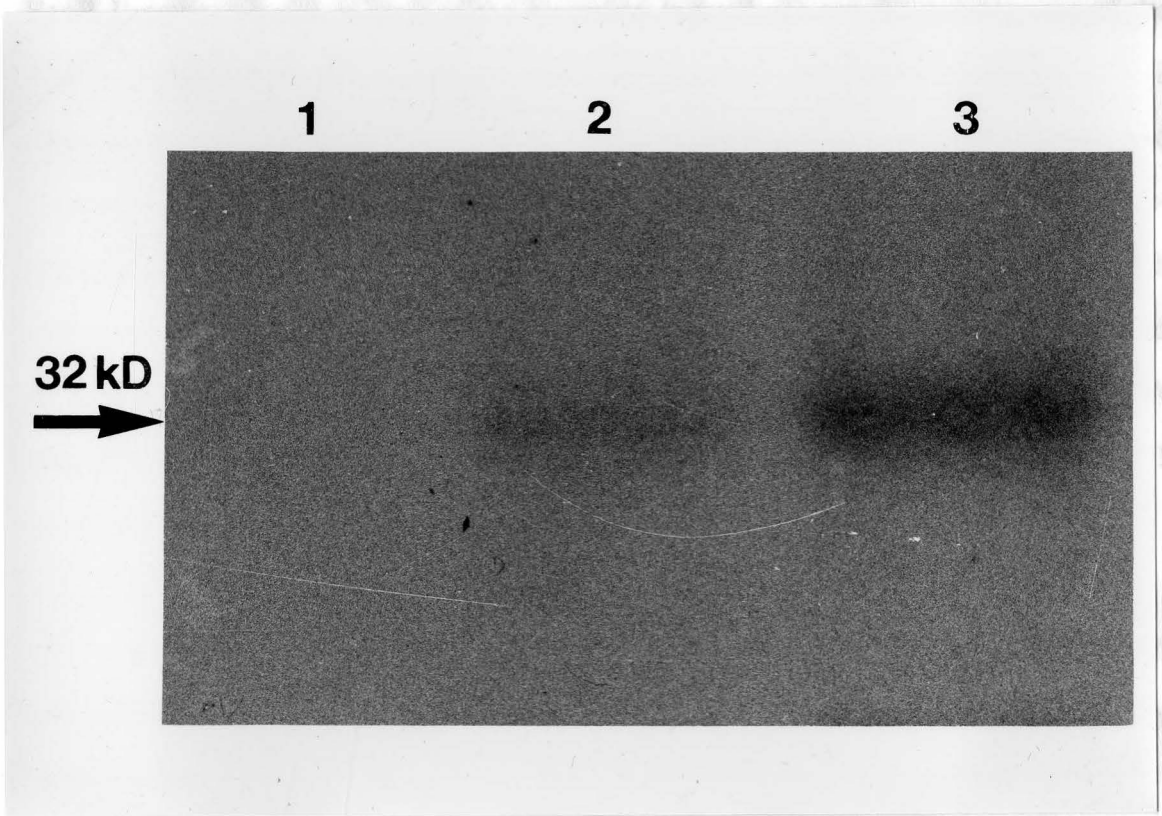

Figure 8. S6 kinase phosphorylates the S6 protein when injected into oocytes. Oocytes were left either untreated (lane 1), treated with progesterone (1 μM) (lane 2), or microinjected with S6 kinase (lane 3) and labelled in OR-2 medium with ^{32}P-orthophosphate (100 μCi/ml) for 8 hours. Protein was extracted from each of the treatment groups and run on a 10% SDS-polyacrylamide gel. Phosphorylation of the S6 protein (32,000 Da) was observed in both the progesterone-treated oocytes, as well as in the S6 kinase microinjected oocytes, but not in the untreated oocytes as compared with ^{14}C-methylated protein markers (lane not shown).

mRNA. The plasmids were cut with the restriction enzyme EcoR I. There was only one EcoR I restriction site in each of the plasmids, located not too far downstream of the inserts. After the digest, a sample of each DNA product was electrophoresed on a 0.8% agarose gel to verify that the plasmids were cut only once, and that the linearized fragments were of the proper length. Figure 9 is a photograph of an ethidium bromide-stained agarose gel which has alternating lanes of uncut and cut plasmids. Lanes at both ends of the gel are lambda DNA markers. As seen in figure 9, the uncut plasmid existed as both a supercoiled and a relaxed form, while the digest produced a single DNA fragment of 4.8, 4.8, and 4.3 kilobases in length for wt-raf-1, KD-raf-1 and ΔN'-raf-1 respectively.

2. Transcription of the Linearized raf-1 DNA:

The linearized DNA for all three plasmids was transcribed using an *in vitro* SP6 polymerase transcription kit (Ambion). In order to purify the transcribed mRNA, RNeasy centrifuge columns were used and the final product was suspended in RNase-free H_2O. To ensure that the transcription product mRNA was of the proper length, 2.0 μg of each mRNA sample was run on a 1.0% agarose denaturing gel. Figure 10 shows a photograph of an ethidium bromide-stained gel with two lanes of each mRNA sample flanked by known RNA markers. As seen in figure 10, the sizes of the transcribed mRNA were 2.2 kilobases for the wt-raf-1 and KD-raf-1 mRNAs, and 1.7 kilobases for the ΔN'-raf-1 mRNA coding just for the catalytic region of the protein. These transcript sizes agree with published values.

3. Electrophoretic Analysis of raf-1 mRNA Translation Products:

The transcribed mRNA coding for the three forms of raf-1 kinase were microinjected into different groups of oocytes. If the oocytes were capable of translating the mRNA, then the possible effects of these protein products on pH_i could be monitored after microinjecting the mRNA samples. To confirm that the microinjected mRNAs were

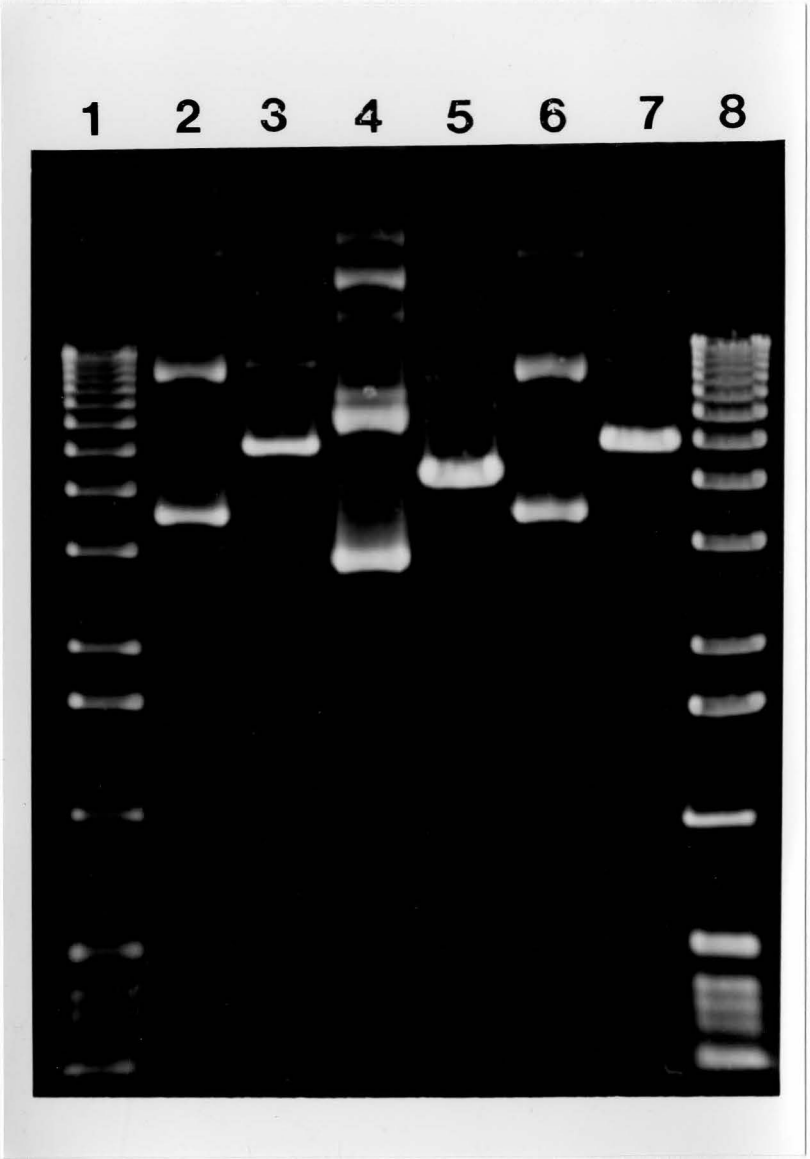

Figure 9. Linearization of raf-1 DNA plasmids. Each of three plasmids were cut with the restriction enzyme EcoR I to linearize them for transcription. Samples from each digest were run on a 0.8% agarose gel and stained with ethidium bromide. Lanes 1 and 8: lambda DNA markers; lane 2: uncut wt-raf-1; lane 3: cut wt-raf-1; lane 4: uncut ΔN'-raf-1; lane 5: cut ΔN'-raf-1; lane 6: uncut KD-raf-1; lane 7: cut KD-raf-1. The two bands appearing in all of the uncut lanes represent both the supercoiled (lower band) and relaxed (upper band) forms of the plasmid.

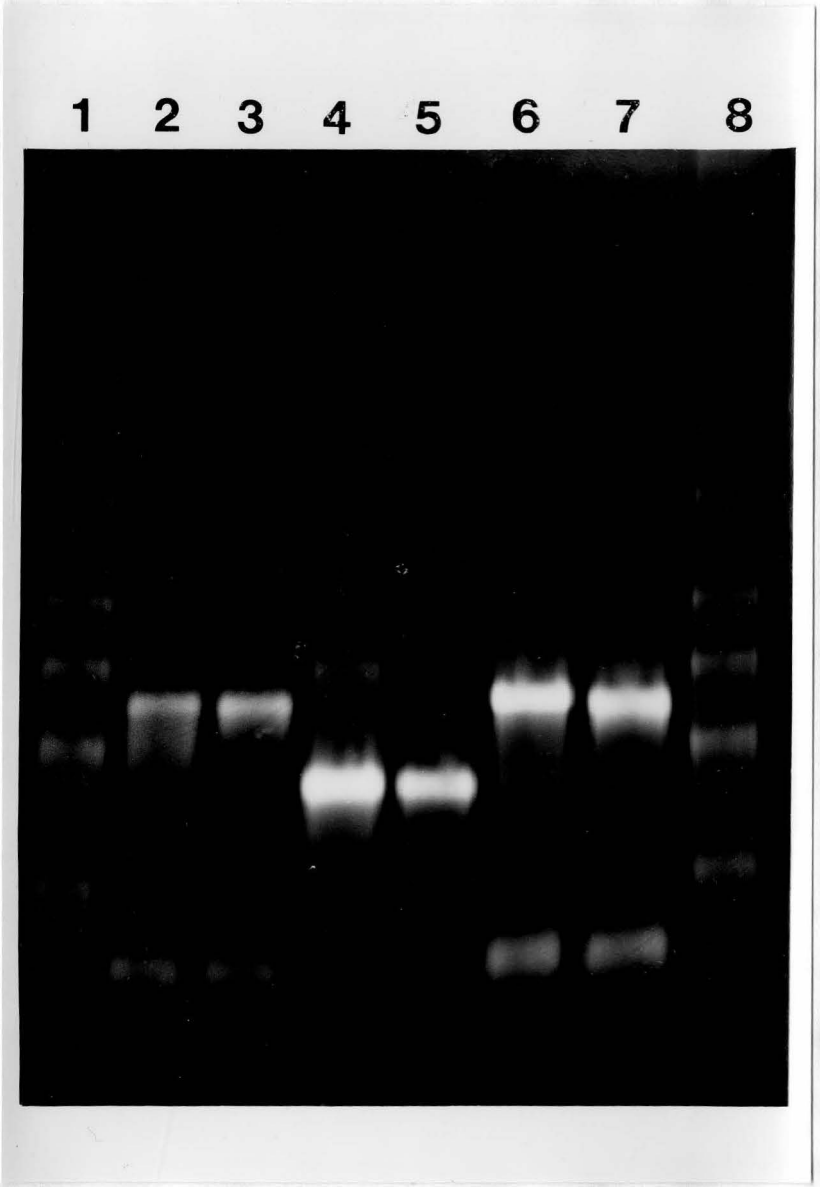

Figure 10. Transcription of the linearized raf-1 DNA. The three linearized plasmids were transcribed using an *in vitro* SP6 transcription kit, and purified using RNeasy centrifuge columns. The transcription products were run on a 1.0% agarose denaturing gel and stained with ethidium bromide. Lanes 1 and 8: RNA markers; lanes 2 and 3: wt-raf-1 mRNA; lanes 4 and 5: ΔN'-raf-1 mRNA; lanes 6 and 7: KD-raf-1 mRNA.

translated *in vivo*, three different groups of oocytes were microinjected with a given mRNA, labelled with ^{35}S-methionine, and then homogenized and the protein extracted. The protein extracts were electrophoresed on SDS-PAGE and the final results were viewed via autoradiography techniques. Figure 11 is an autoradiograph of newly synthesized protein made in mRNA-injected oocytes, as well as in a control group of oocytes that were microinjected with RNase-free H_2O. From this autoradiograph it is evident that the oocytes are translating the microinjected mRNA as is observed by the definitive dark bands found in the three treatment lanes and not in the control lane. These bands correspond to the published molecular weights, with wt-raf-1 kinase and KD-raf-1 kinase appearing as proteins of similar size (69 kDa), and ΔN'-raf-1 kinase appearing as a smaller protein (40 kDa) which was expected, as it is just the catalytic region of the protein and not the full-length protein.

B. pH_i Measurement in raf-1 mRNA-injected Oocytes:

1. Oocyte pH_i for mRNA-injected Oocytes:

Oocytes were preincubated in ^{14}C-DMO for 12 to 14 hours. As shown in Figure 12, oocytes were either left untreated, treated with progesterone (1 μM), or microinjected with stock concentrations of the three raf-1 mRNA products. Oocytes microinjected with wt-raf-1 and KD-raf-1 mRNAs coding for inactive proteins did not exhibit an increase in pH_i. Oocytes microinjected with ΔN'-raf-1 mRNA coding for an active kinase did exhibit an increase in pH_i that appeared similar to the increase in pH_i caused by progesterone stimulation. Based on this unreplicated experiment, it appeared that active ΔN'-raf-1 caused an increase in pH_i. Therefore, the role of active raf-1 kinase was investigated further.

2. ΔN'-raf-1 mRNA Dose Response Curve:

Oocytes were injected with different concentrations of ΔN'-raf-1 mRNA, to determine the lowest concentration of mRNA needed to induce an increase in oocyte pH_i

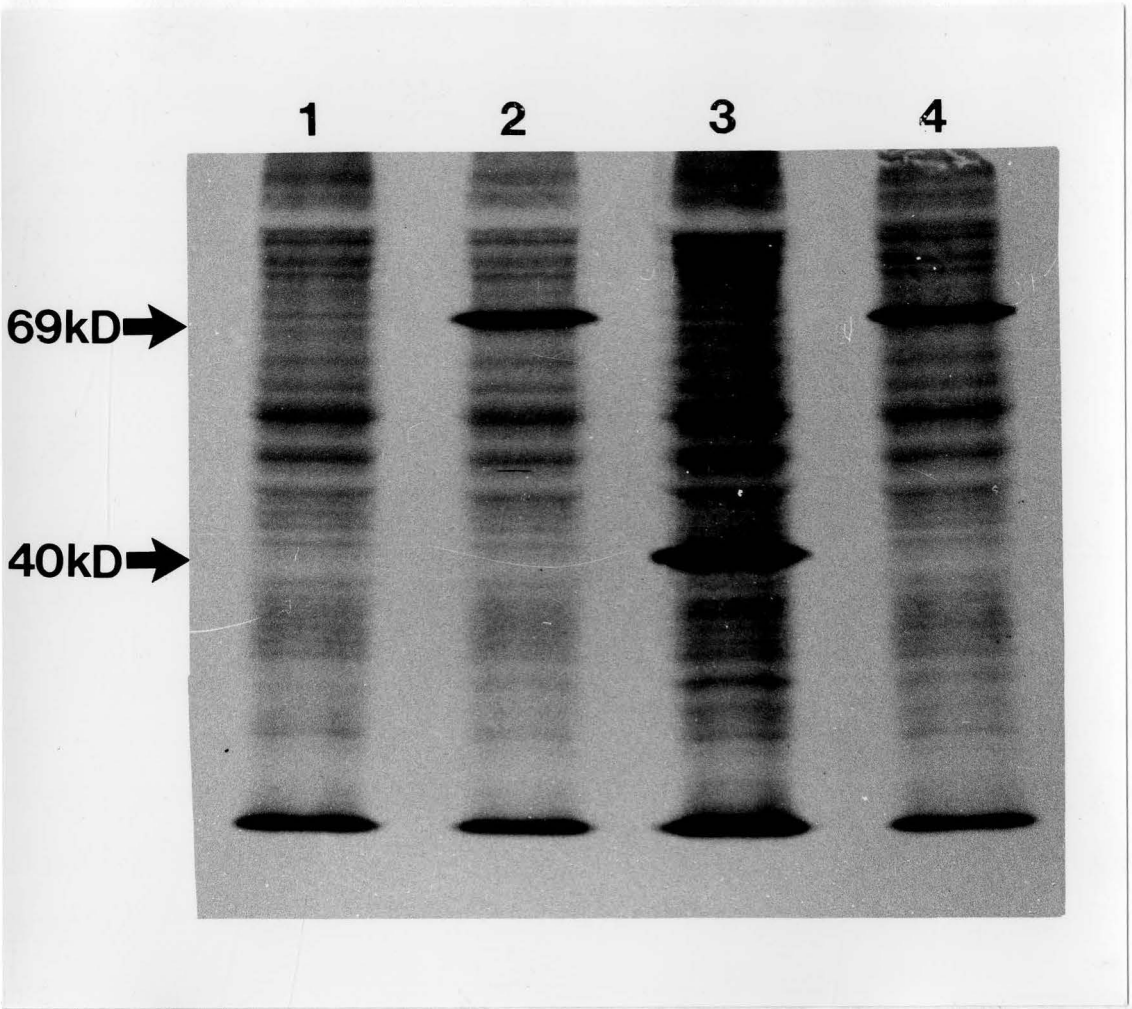

Figure 11. Electrophoretic analysis of raf-1 mRNA translation *in vivo*. Oocytes were microinjected with either H_2O (lane 1) as a control, wt-raf-1 mRNA (lane 2), ΔN'-raf-1 mRNA (lane 3), or KD-raf-1 (lane 4) and labelled in OR-2 media with ^{35}S-methionine. Protein was then extracted and run on a 10% SDS-polyacrylamide gel. The microinjected mRNA was successfully translated by the oocyte (wt-raf-1 and KD-raf-1 = 69 kDa; ΔN'-raf-1 = 40 kDa).

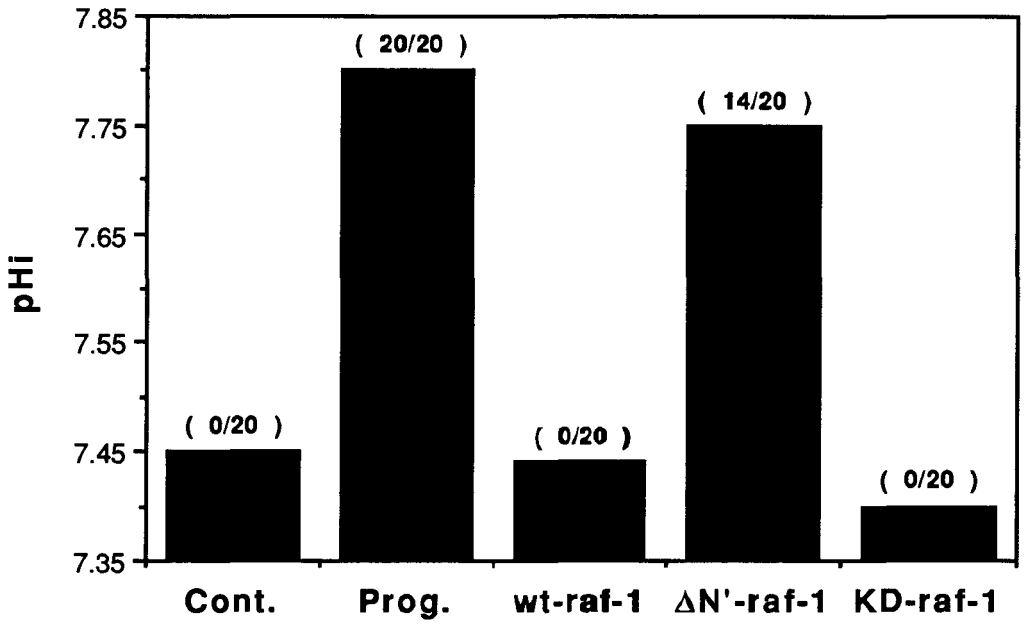

Figure 12. The effects of the three raf-1 mRNAs on oocyte pH_i. Oocytes were either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with one of the three raf-1 mRNAs. Only the ΔN'-raf-1 mRNA caused an increase in oocyte pH_i as well as GVBD (numbers above the bars show number of oocytes that underwent GVBD out of 20 oocytes).

that was similar to the increase in pH_i induced by progesterone. As shown in Figure 13, oocytes were microinjected with concentrations of ΔN'-raf-1 mRNA ranging from 1.25 ng to 10.0 ng per oocyte. The oocytes were then incubated for 12 to 14 hours to allow for translation of the mRNA. The results indicated that increasing concentrations of ΔN'-raf-1 mRNA (and presumably raf-1 protein) induced higher levels of pH_i, which appeared to level off at concentrations greater than 2.5 ng of mRNA per oocyte. Based on these results, 5.0 ng ΔN'-raf-1 mRNA per oocyte was the lowest concentration of mRNA that consistently induced an increase in oocyte pH_i that was statistically similar to the increase in pH_i induced by progesterone. All subsequent experiments were conducted using 5.0 ng ΔN'-raf-1 mRNA per oocyte.

C. The Effect of cAMP on raf-1 Regulation of the Na^+/H^+ Antiporter:

Recent studies have indicated that elevated levels of cAMP are antagonistic toward progesterone-induced activation of the Na^+/H^+ antiporter during oocyte meiotic maturation (Rezai, 1994). To determine if elevated levels of intracellular cAMP are also antagonistic toward raf-1 kinase-induced activation of the Na^+/H^+ antiporter, the following experiments altering cAMP levels in *Xenopus* oocytes were performed.

1. Forskolin Activation of Adenylate Cyclase:

Oocytes were treated with forskolin (100 μM) in ^{14}C-DMO or only with ^{14}C-DMO for 8 hours. As shown in Figure 14, oocytes from each of the two groups were left untreated (Cont.), treated with progesterone (1 μM), or microinjected with ΔN'-raf-1 mRNA (5.0 ng/oocyte) and incubated for 12 to 14 hours. The results show that forskolin significantly inhibited the increase in pH_i in progesterone-stimulated oocytes. Forskolin did not appear to be antagonistic to ΔN'-raf-1's ability to activate the Na^+/H^+ antiporter. The pH_i in ΔN'-raf-1 mRNA injected oocytes pre-treated with forskolin was statistically similar to the pH_i in progesterone-treated oocytes without forskolin.

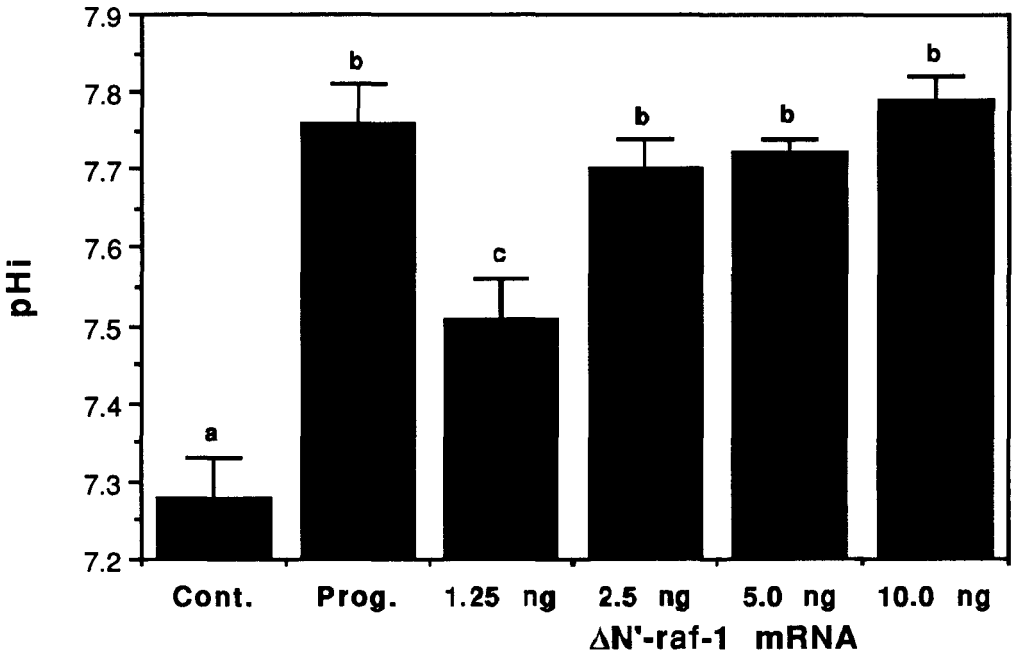

Figure 13. ΔN'-raf-1 mRNA dose response curve. Oocytes were either left untreated (Cont.), treated with progesterone (1 μM), or microinjected with concentrations of ΔN'-raf-1 mRNA ranging from 1.25 to 10.0 ng of mRNA per oocyte. At concentrations of 2.5 ng mRNA per oocyte and higher, the increase in pH_i was statistically similar to the increase in pH_i induced by progesterone. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are expressed as means ± the standard error of the mean, where n=3.

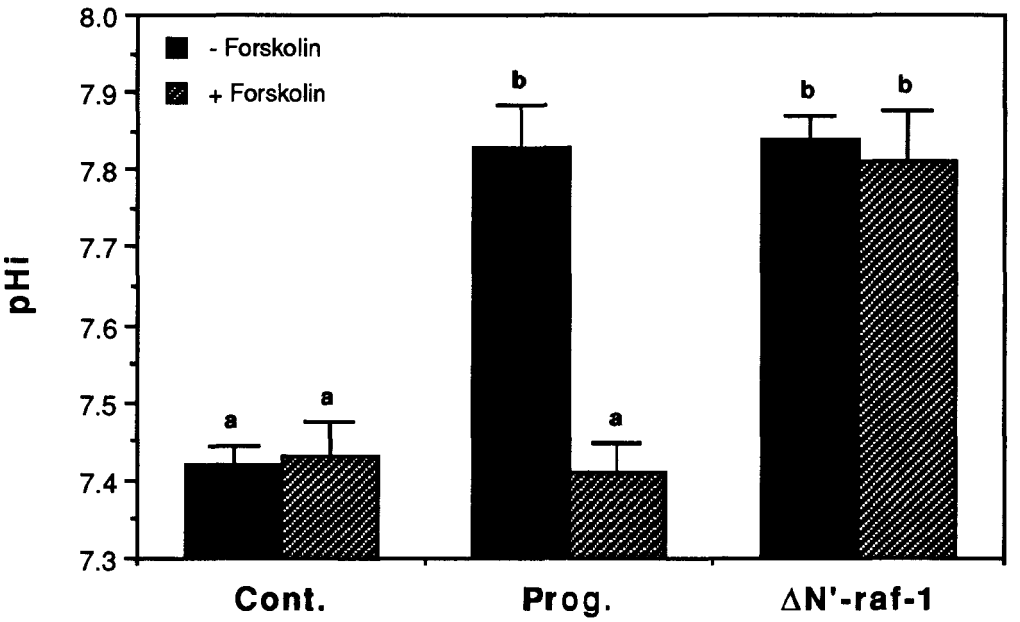

Figure 14. Forskolin does not appear to be antagonistic to ΔN'-raf-1's ability to activate the Na^+/H^+ antiporter. Oocytes were incubated in ^{14}C-DMO for 8 hours in the presence or absence of forskolin (100 μM). The oocytes from both groups were then left untreated (Cont.), treated with progesterone (1 μM), or microinjected with ΔN'-raf-1 mRNA (5.0 ng/oocyte). Forskolin treatment significantly blocked the ability of progesterone to activate the antiporter. The pH_i of the oocytes microinjected with the ΔN'-raf-1 mRNA did not differ significantly from one another whether they were treated with forskolin or not. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are presented as means ± the standard error of the mean, where n=3.

2. 8-Br-cAMP Elevation of Intracellular cAMP Levels:

To verify the forskolin results, a second method of elevating levels of intracellular cAMP was utilized. Oocytes were treated with 8-Br-cAMP (1.0 mM) in ^{14}C-DMO or only in ^{14}C-DMO for 8 hours. As shown in Figure 15, oocytes from each of the two groups were left untreated (Cont.), treated with progesterone (1 μM), or microinjected with ΔN'-raf-1 mRNA (5.0 ng/oocyte) and incubated for 12 to 14 hours. The results indicate that 8-Br-cAMP was antagonistic to progesterone's ability to induce an increase in pH_i, but had no significant effect on ΔN'-raf-1 mRNA injected oocytes. These results confirmed the forskolin experiments. The results from both the forskolin experiments and the 8-Br-cAMP experiments show that elevated levels of intracellular cAMP are antagonistic toward a progesterone-induced increase in pH_i but have no effect on the ability of raf-1 kinase to cause an increase in pH_i. Therefore, it appears that the cAMP block is downstream of the progesterone receptor, but upstream of raf-1 kinase in the protein kinase cascade.

Part IV: MAP kinase kinase

MAP (Mitogen Activated Protein) kinase kinase was the next kinase to be investigated for its possible role in activation of the Na^+/H^+ antiporter. This kinase is a substrate for raf-1 kinase within the protein kinase cascade, as well as the activator of MAP kinase which is located downstream of MAP kinase kinase within the cascade. To determine if MAP kinase kinase was responsible for inducing the increase in the oocyte pH_i, the following experiments were conducted.

A. pH_i Measurement in MAP kinase kinase Injected Oocytes:

Groups of oocytes were preincubated in ^{14}C-DMO for 3 to 4 hours. As shown in Figure 16, oocytes were left untreated, treated with progesterone (1 μM), or microinjected with constitutively active MAP kinase kinase (6.0 ng/oocyte). The oocytes microinjected

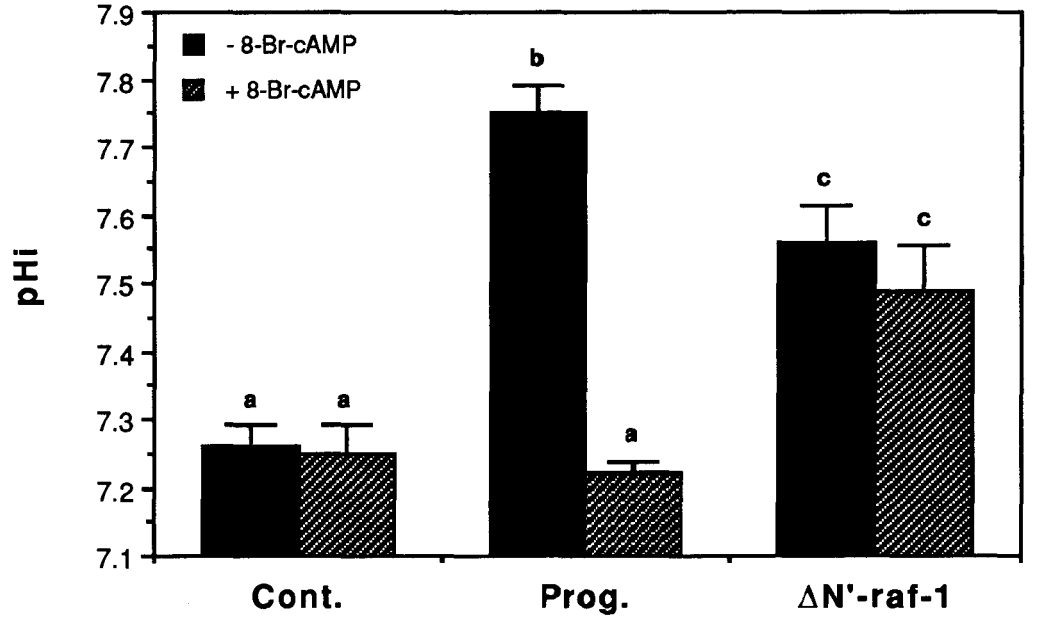

Figure 15. Elevating levels of intracellular cAMP with 8-Br-cAMP does not appear to be antagonistic to the ability of ΔN'-raf-1 to activate the Na+/H+ antiporter. Oocytes were incubated in ^{14}C-DMO for 8 hours in the presence or absence of 8-Br-cAMP (1.0 mM). The oocytes from both groups were left untreated (Cont.), treated with progesterone (1 μM), or microinjected with ΔN'-raf-1 mRNA (5.0 ng/oocyte). 8-Br-cAMP significantly blocked the ability of progesterone to activate the antiporter. The pH_i of the oocytes microinjected with the ΔN'-raf-1 mRNA did not differ from one another significantly whether treated with 8-Br-cAMP or not. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are presented as means ± the standard error of the mean, where n=4.

with constitutively active MAP kinase kinase did not exhibit an increase in pH_i as did those oocytes which were stimulated with progesterone. Oocytes microinjected with constitutively active MAP kinase kinase maintained levels of pH_i that were statistically similar to the pH_i levels found in untreated oocytes (Figure 16).

B. S6 Phosphorylation as a Confirmation of MAP kinase kinase Activity:

The previous results indicated that constitutively active MAP kinase kinase did not cause any significant increase in pH_i. It has been shown that active MAP kinase kinase activates MAP kinase, which in turn activates S6 kinase in the protein kinase cascade. To verify that the MAP kinase kinase microinjected into the oocytes was active, phosphorylation of the S6 protein on the 40S ribosomal subunit was monitored. Oocytes were left untreated (Cont.), stimulated with progesterone (1 μM), or microinjected with constitutively active MAP kinase kinase and labelled in OR-2 medium with ^{32}P-orthophosphate (100 μCi/ml) for 12 to 14 hours. Oocytes were removed from their respective treatments and proteins were extracted and run on a SDS-polyacrylamide gel. Figure 17 shows an autoradiograph comparing the phosphorylation of S6 protein between the three treatments. S6 protein has a molecular weight of 32,000 Da (^{14}C-methylated protein markers: lysozyme (14,300 Da), carbonic anhydrase (30,000 Da), ovalbumin (46,000 Da), bovine serum albumin (69,000 Da), and phosphorylase b (97,400 Da)). S6 was phosphorylated in progesterone-stimulated oocytes, but only weakly phosphorylated in the constitutively active MAP kinase kinase-microinjected oocytes, and not phosphorylated at all in the untreated oocytes. This indicates that the constitutively active MAP kinase kinase that was used was only partially active. Therefore, the lack of effect of MAP kinase kinase on oocyte pH_i seen in Figure 16 must be interpreted with caution.

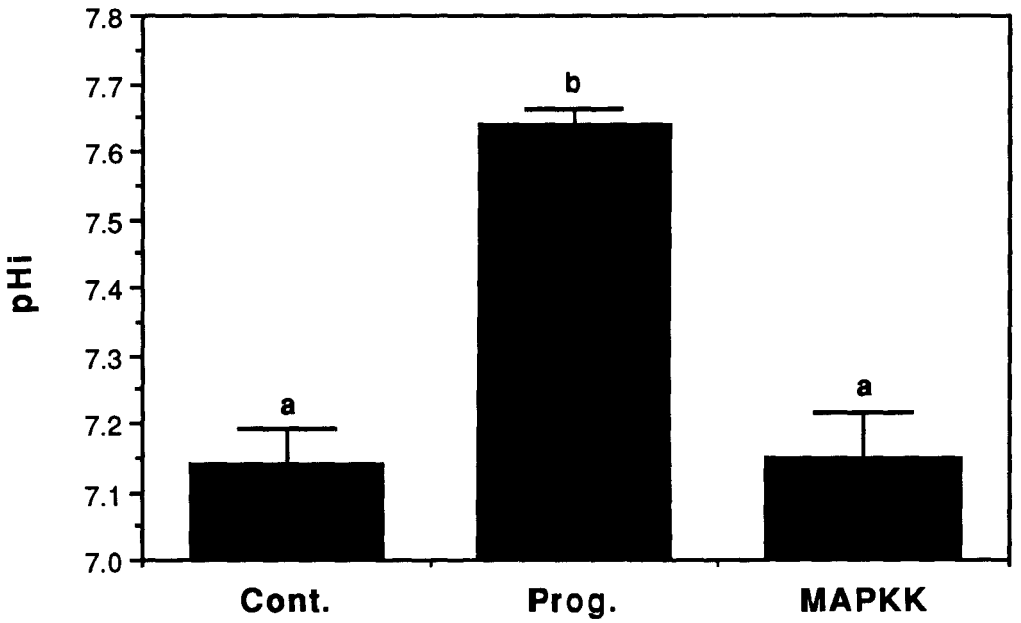

Figure 16. MAP kinase kinase does not appear to activate the Na+/H+ antiporter. Oocytes were incubated in ^{14}C-DMO and were left untreated (Cont.), treated with progesterone (1 μM), or microinjected with MAP kinase kinase (6.0 ng/oocyte). The pH_i of the oocytes microinjected with MAP kinase kinase was statistically different from the pH_i of the progesterone-treated oocytes, but statistically similar to the pH_i of the oocytes that were left untreated. Columns with different letters above them are significantly different from each other using Tukey's multiple comparison test. The data are presented as means ± the standard error of the mean, where n=3.

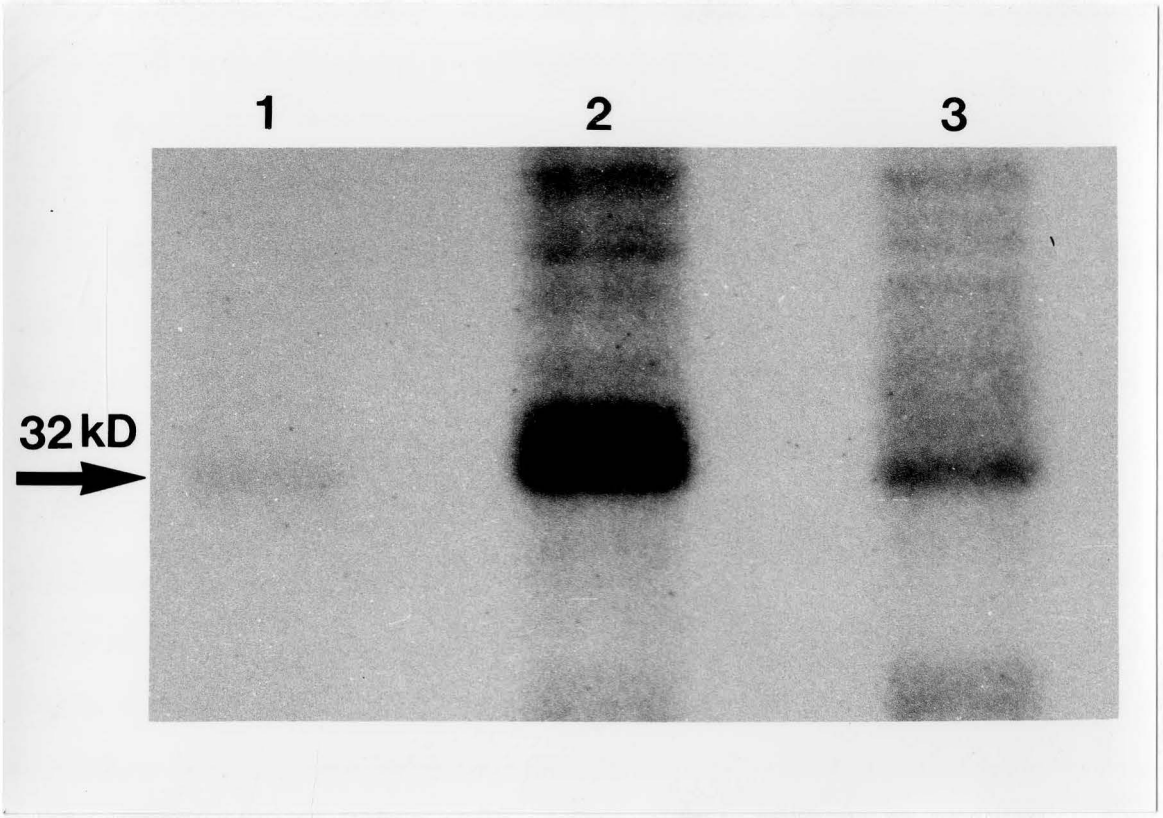

Figure 17. Electrophoretic analysis of S6 protein phosphorylation. Oocytes were left untreated (lane 1), treated with progesterone (1 μM) (lane 2), or microinjected with MAP kinase kinase (6.0 ng/oocyte) (lane 3) and labelled in OR-2 medium with ^{32}P-orthophosphate (100 μCi/ml) for 8 hours. Protein was extracted from each of the treatment groups and run on a 10% SDS-polyacrylamide gel. Phosphorylation of the S6 protein (32,000 kDA) was observed in both the progesterone-treated oocytes and in the MAP kinase kinase-microinjected oocytes, but not in the untreated oocytes as compared with ^{14}C-methylated protein markers (lane not shown).

CHAPTER IV

DISCUSSION

One of the physiological events occurring during oocyte meiotic maturation is an increase in oocyte pH_i. Previous studies have shown that this increase in pH_i appears to be the result of the activation of Na^+/H^+ antiporters present in the plasma membrane of the oocyte. If the extracellular sodium is replaced with choline chloride, or if amiloride (a Na^+/H^+ antiporter inhibitor) is used, the increase in pH_i in response to progesterone stimulation is completely inhibited (Wasserman and Houle, 1984; Stith and Maller, 1985; Towle *et al.*, 1991; Rezai, 1994). The increase in pH_i during *Xenopus laevis* oocyte meiotic maturation has been shown to be very important for preparing the egg for fertilization with the sperm (Houle and Wasserman, 1983). To understand better the role of pH during these cellular processes, an understanding of how the Na^+/H^+ antiporter is regulated during oocyte meiotic maturation needs to be obtained. The Na^+/H^+ antiporter in other cell systems is proposed to be regulated by a phosphorylation event. It has been hypothesized that addition of phosphates induces a conformational change in the structure of the Na^+/H^+ antiporter, the result of which is an increased affinity for cytoplasmic hydrogen ions (Wakabayashi *et al.*, 1992; Borgese *et al.*, 1992). In support of this hypothesis, investigators have demonstrated that as antiporter activity increases, there is a concurrent increase in the phosphorylation of NHE1 (Na^+/H^+ exchanger 1), a transmembrane glycoprotein of 110 kDa, in fibroblasts, epithelial cells, and blood platelets (Livne *et al.*., 1991; Sardet *et al.*, 1990; Wakabayashi *et al.*, 1992; Grinstein *et al.*, 1992; Guizouarn *et al.*, 1993).

In *Xenopus* oocytes, studies have been performed to investigate the possible role of certain protein kinases in regulating the Na^+/H^+ antiporter. Through use of various inhibitors and activators, it was determined that Protein Kinase C, Protein Kinase A, and Calcium-Calmodulin Kinase were not involved in regulation of the Na^+/H^+ antiporter (Rezai, 1994). In addition, MPF H-1 kinase was also found not to play a role in regulating this antiporter (Rezai, 1994). Oocytes stimulated with progesterone undergo meiotic maturation and a series of protein kinases are phosphorylated, and subsequently activated, forming a protein kinase cascade. This cascade consists of the following protein kinases: c-mos kinase, raf-1 kinase, MAP kinase kinase, MAP kinase, and S6 kinase. Preliminary investigations of the possible role of c-mos kinase (the first kinase within the cascade) in activation of the Na^+/H^+ antiporter in *Xenopus* oocytes have been performed in our laboratory. The results of these studies indicated that high doses of microinjected c-mos protein can cause an increase in oocyte pH_i (Rezai, 1994). It could not be concluded however, whether c-mos kinase itself or a protein kinase downstream of c-mos was responsible for the activation of the Na^+/H^+ antiporter.

In the current study, c-mos kinase, as well as the other protein kinases within the cascade, were examined for their potential role in regulating the Na^+/H^+ antiporter during progesterone-induced oocyte meiotic maturation. The first protein kinase that was investigated was c-mos kinase. The proto-oncogene product p39 c-mos kinase has been proposed to be the "initiator" of oocyte maturation. Active c-mos kinase has been shown to induce GVBD when microinjected into stage VI oocytes in the absence of progesterone stimulation (Sagata *et al.*, 1989). It was further demonstrated that oocytes pre-treated with cycloheximide (an inhibitor of protein synthesis) and then microinjected with active c-mos protein underwent GVBD in the absence of progesterone, while oocytes pre-treated with cycloheximide and then stimulated with progesterone did not undergo GVBD (Yew *et al.*, 1992). From these experiments it appears that c-mos kinase is the only newly synthesized

protein needed in the oocyte to trigger GVBD. Preliminary experiments indicated that high doses of c-mos kinase appeared to play a role in regulating the Na^+/H^+ antiporter in *Xenopus* oocytes (Rezai, 1994). Based on the results from the current study, it appears that lower doses of c-mos kinase (down to 5 ng of total protein per oocyte) are also capable of causing an increase in oocyte pH_i similar to the increase induced by progesterone (Figure 3). These results support the earlier conclusion that c-mos kinase does play a role in activation of the Na^+/H^+ antiporter in *Xenopus* oocytes.

We do not know what the endogenous level of active c-mos kinase is in progesterone-stimulated oocytes. The results from experiments performed by other investigators have shown that low doses of injected c-mos kinase have an effect on GVBD as well as MAP kinase activity. 2-5 ng c-mos protein injected per oocyte were capable of inducing an increase in MAP kinase activity and GVBD (Matten *et al.*, 1994). In the current study, I was unable to determine the exact amount of active c-mos protein being injected into each oocyte. Several factors must be taken into consideration when calculating the amount of c-mos kinase that was injected, such as the actual amount of active c-mos kinase versus the total amount of c-mos protein present in the preparation. There is also the problem of leakage of c-mos kinase from the injection wound. Precautions such as injecting deeper into the oocyte cytoplasm and not withdrawing the pipette immediately after delivery were taken to minimize this from occurring. Even with all of these assumptions, I was probably injecting excess active c-mos kinase into the oocytes.

We know that c-mos kinase appears to be the only newly synthesized protein needed to trigger GVBD (Yew *et al.*, 1993). A previous study from our laboratory showed that oocyte protein synthesis is also necessary for the increase in oocyte pH_i in response to progesterone (Rezai, 1994). The results from the current study injecting c-mos kinase into cycloheximide-treated oocytes indicate that c-mos kinase appears to be the

only newly synthesized protein needed to activate the Na^+/H^+ antiporter (Figure 4). These results indicated that c-mos kinase plays a role in the activation of the Na^+/H^+ antiporter. In order to determine if c-mos kinase is activating the antiporter directly or through the activation of a pre-existing protein kinase located downstream in the cascade needed to be examined.

During progesterone-induced oocyte meiotic maturation, adenylate cyclase activity is inhibited, resulting in a decrease in intracellular cAMP and PKA activity (Sadler and Maller, 1981). It has been shown that if intracellular cAMP is artificially maintained at elevated levels, steroid-treated oocytes will neither undergo GVBD (Maller and Krebs, 1977; Schorderet-Slatkine *et al.*, 1982; Matten *et al.*, 1994) nor exhibit an increase in pH_i (Rezai, 1994). Artificially elevated levels of intracellular cAMP did not appear to be antagonistic toward c-mos kinase's ability to activate the Na^+/H^+ antiporter. Oocytes pre-treated with either forskolin (an activator of adenylate cyclase) or 8-Br-cAMP (a cAMP derivative capable of passing through the oocyte's plasma membrane) and then microinjected with c-mos kinase exhibited a normal increase in pH_i. These results indicate that the cAMP/PKA block to activating the antiporter is located in the progesterone-pathway upstream of c-mos kinase.

The effect of cAMP/PKA on c-mos kinase has been studied in Dr. Vande Woude's laboratory. They showed that microinjection of the catalytic subunit of PKA inhibited progesterone-induced translation of c-mos mRNA as well as the activation of downstream MAP kinase. If, however, the catalytic subunit of PKA was coinjected with active c-mos protein, MAP kinase was activated (Matten *et al.*, 1994). These results suggest that the catalytic subunit of PKA acts as a negative regulator during meiotic maturation, blocking translation of c-mos kinase mRNA, and thus blocking the kinase cascade. Microinjection of the catalytic subunit of PKA was not performed in the current study, but intracellular levels of cAMP were artificially elevated. Elevated levels of cAMP should have then

bound the regulatory subunits of endogenous PKA, freeing the catalytic subunits, yielding a result similar to injecting the catalytic subunits themselves. By injecting active c-mos protein, the mRNA translation block by PKA was bypassed. Therefore, the activation of the Na^+/H^+ antiporter indicated that the activator is either c-mos kinase or a protein kinase activated downstream of c-mos.

Ribosomal protein S6 kinase, when activated, is responsible for phosphorylating the 40S ribosomal protein S6 during oocyte meiotic maturation. S6 kinase is also one of the most downstream protein kinases within the cascade (Wasserman and Houle, 1984). Active S6 kinase, however, does not cause activation of the Na^+/H^+ antiporter when microinjected into oocytes. A high dose (50 ng of protein per oocyte) of S6 kinase failed to increase oocyte pH_i when microinjected into stage VI oocytes. The activity of the injected S6 kinase was confirmed by monitoring phosphorylation of ribosome S6 in the injected oocytes. It can therefore be concluded that S6 protein kinase is not responsible for the activation of the Na^+/H^+ antiporter.

Based on the results thus far, the protein kinase that activates the Na^+/H^+ antiporter may be c-mos kinase, or a kinase downstream of c-mos kinase and upstream of S6 kinase within the protein kinase cascade. The kinase which immediately follows c-mos within the cascade has not yet been determined. It has been shown through use of oncogenic as well as dominant negative mutant mRNAs that raf-1 kinase is necessary for progesterone-induced oocyte meiotic maturation and that it functions downstream of c-mos (Muslin *et al.*, 1993). I was unable to obtain raf-1 kinase protein as with c-mos kinase and S6 kinase. I was, however, able to obtain transcription vectors coding for three forms of raf-1 kinase: wild-type (wt), constitutively active (ΔN'), and kinase-deficient (KD). The three vectors were linearized and then transcribed *in vitro*. All three raf-1 mRNAs were efficiently translated into protein when injected into oocytes (Figure 11). mRNAs for wt-raf-1 and KD-raf-1 did not induce an increase in pH_i or GVBD when

injected into oocytes, but, ΔN'-raf-1 mRNA did induce both an increase in pH_i and GVBD in injected oocytes (Figures 12 and 13). Based on these preliminary results, it would appear that a high dose of ΔN'-raf-1 mRNA (10 ng per oocyte) was capable of activating the Na^+/H^+ antiporter in *Xenopus* oocytes. Lower doses of ΔN'-raf-1 mRNA were also capable of inducing an increase in oocyte pHi. As shown in Figure 13, concentrations as low as 1.25 ng ΔN'-raf-1 mRNA per oocyte were able to induce a significant increase in oocyte pH_i, with 2.5 ng mRNA per oocyte and higher inducing an increase in pH_i similar to that caused by progesterone. These concentrations of mRNA were based on absorbance readings taken of each stock sample. Individual dilutions were made as needed to obtain the proper concentration of mRNA for injection. The actual concentration of newly synthesized active raf-1 protein could not be determined due to problems such as leakage of the mRNA out of the injection site, the amount of protein made in the oocyte, and the activity of the translated protein. Active raf-1 kinase, however, does appear to play a role in the activation of the Na^+/H^+ antiporter during oocyte meiotic maturation. I cannot conclude, however, if raf-1 kinase acts directly on the Na^+/H^+ antiporter, or if it is a protein kinase located downstream of raf-1 within the protein kinase cascade.

Results from experiments carried out in other cell systems propose the following model for raf-1 kinase activation. Activated ras protein recruits inactive raf-1 kinase to the plasma membrane of the cell by binding to the amino-terminal region of the raf-1 protein. Once at the membrane, the complex is anchored by cytoskeletal elements and an unknown factor unfolds the amino-terminal domain of raf-1 off of the catalytic domain, exposing the activation sites, serine residues 259 and 621, for phosphorylation. Once raf-1 kinase is phosphorylated and activated it is released from ras (Stokoe *et al.*, 1994; Daum *et al.*, 1994; Williams *et al.*, 1994). Based on this proposed model, active raf-1 kinase is located near the plasma membrane of the cell. This places raf-1 kinase in close proximity to the

Na^+/H^+ antiporters allowing raf-1 kinase the opportunity to phosphorylate the Na^+/H^+ antiporters directly.

Progesterone normally causes a decrease in intracellular levels of cAMP, which, if artificially maintained elevated, inhibit progesterone's ability to activate the antiporter. Elevated levels of intracellular cAMP did not appear to be antagonistic toward c-mos kinase's ability to cause an increase in oocyte pH_i. To determine if elevated levels of intracellular cAMP had any influence on ΔN'-raf-1 kinase's ability, similar experiments were performed. Oocytes pre-treated with forskolin or 8-Br-cAMP and then injected with ΔN'-raf-1 mRNA exhibited a full increase in oocyte pH_i (Figures 14 and 15). These results, along with the results for c-mos kinase, indicate that the cAMP/PKA block is upstream of both raf-1 kinase and c-mos kinase within the progesterone-stimulated pathway.

The only known physiological substrate for raf-1 kinase within the protein kinase cascade is thought to be MAP (Mitogen Activated Protein) kinase kinase. Recently, a group of researchers have shown that cdc25 phosphatase associates with raf-1 kinase in mammalian somatic cells and in frog meiotic oocytes (Galaktionov *et al.*, 1995). The interaction between cdc25 and raf-1 kinase occurred at the carboxy-terminal region of raf-1 kinase, which the investigators believe suggests a strong and specific interaction between the two proteins. Further studies need to be carried out to determine if raf-1 kinase could possibly have multiple substrates.

MAP kinase kinase has been shown to be phosphorylated both *in vitro* as well as *in vivo* by raf-1 kinase (Dent *et al.*, 1992; Huang *et al.*, 1993). Active MAP kinase kinase in turn activates MAP kinase within the protein kinase cascade. The c-mos kinase and raf-1 kinase experiments suggest that the protein kinase responsible for regulating the Na^+/H^+ antiporter is either raf-1 kinase or a kinase downstream of raf-1 kinase. The S6 kinase experiments suggest that the Na^+/H^+ antiporter activating protein kinase is upstream of it,

therefore, MAP kinase kinase was the next protein kinase to be investigated.

Oocytes which were microinjected with constitutively active MAP kinase kinase did not exhibit an increase in pH_i. As seen in Figure 16, the pH_i level of oocytes injected with constitutively active MAP kinase kinase remained at basal pH_i levels (control level) when compared to the pH_i level in progesterone-stimulated oocytes. To accept these negative results, the activity of the microinjected MAP kinase kinase needed to be determined. The activity of injected MAP kinase kinase protein can be monitored by observing phosphorylation of a substrate known to be located downstream of MAP kinase kinase. Ribosomal S6 protein was monitored for phosphorylation in oocytes microinjected with MAP kinase kinase. This level was compared to ribosomal S6 protein phosphorylation in oocytes stimulated with progesterone. The results indicated that the constitutively active MAP kinase kinase was only partially active. This may explain why there was no increase in oocyte pH_i in oocytes injected with this protein. Further studies need to be performed with very active MAP kinase kinase before any conclusion can be made. Since S6 kinase had no effect on pH_i, and MAP kinase kinase had no effect on pH_i, the intermediate kinase, MAP kinase, was not investigated.

Based on the results obtained in this and other studies, the following model of the role of protein kinases in regulation of the Na^+/H^+ antiporter is proposed (Figure 18). Cycloheximide inhibits progesterone's ability to activate the Na^+/H^+ antiporter but not that of injected c-mos protein, indicating that the block is located between the progesterone receptor and c-mos kinase within the cascade. Cycloheximide is most likely blocking translation of endogenous c-mos mRNA. Elevated levels of cAMP/PKA inhibit progesterone's ability to activate the Na^+/H^+ antiporter but not c-mos kinase's or raf-1 kinase's ability to do so. This places the cAMP/PKA block between the progesterone receptor and c-mos kinase. Data from Dr. Vande Woude's laboratory indicates that elevated levels of cAMP/PKA block the cytoplasmic polyadenylation and translation of c-

mos mRNA. Both c-mos kinase and raf-1 kinase can activate the Na^+/H^+ antiporter while S6 kinase does not. A previous study has also ruled out MPF (H1 kinase) (Rezai, 1994). The results from this study indicate that MAP kinase kinase does not activate the Na^+/H^+ antiporter, but the activity of the protein preparation used is questionable, so further experimentation with a more active protein preparation are needed to confirm these findings. Based on the results of this study and the model of raf-1 kinase activation by translocation to the membrane, it can tentatively be concluded that raf-1 kinase may directly phosphorylate and activate the Na^+/H^+ antiporter in *Xenopus* oocytes during meiotic maturation.

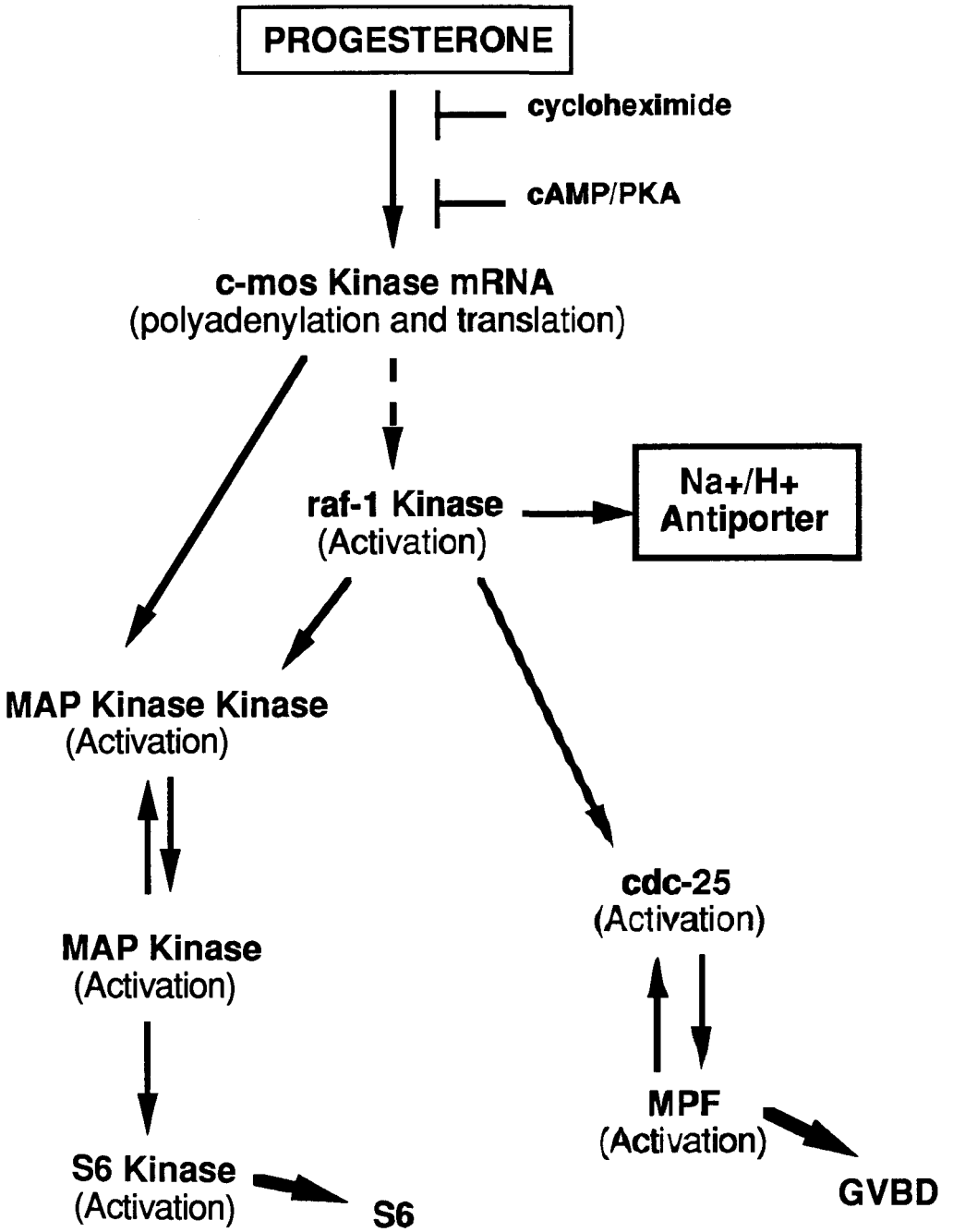

Figure 18. A proposed model for the role of protein kinases in the regulation of the Na^+/H^+ antiporter in *Xenopus laevis* oocytes during progesterone-induced meiotic maturation. Solid arrows indicate direct protein-protein interaction, while dashed arrows indicate indirect action.

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The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the thesis is now given final approval by the committee with reference to content and form.

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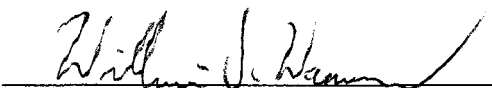
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