CLONING AND EXPRESSION OF EPITHELIAL SODIUM CHANNELS IN SACCHAROMYCES CEREVISIAE

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ABSTRACT

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Epithelial Sodium Channels (ENaC) are integral membrane proteins which regulate sodium re-absorption within cells. Current knowledge about these channels is limited and includes physiological functional studies involving the investigation of cellular responses upon activation and inhibition of these channels. The subunit composition of this particular channel is unknown, and researchers currently debate on whether functional ENaC is a tetramer, with 2-α, 1-β, and 1-γ ENaC, or larger multimeric complex composed of equal numbers of each subunit. Knowledge about the number and

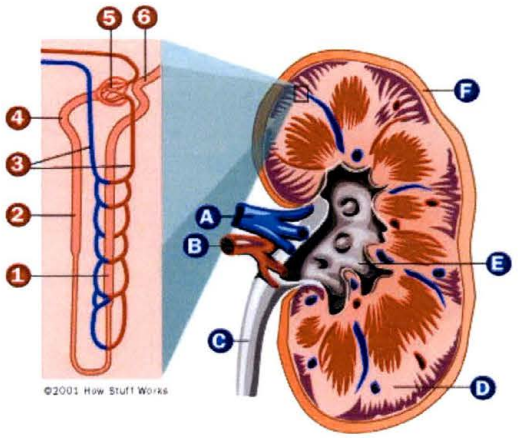
association of subunits as well as a crystal structure would give researchers the ability to study interactions between the protein's subunits and make predictions in how alterations in structure might affect the protein's function. My research will aid in determining ENaC's structure by increasing the amount of expressed protein available for future studies. By cloning ENaC into *Saccharomyces cerevisiae*, we will be able to overcome the limitations of low protein expression seen with traditional mammalian expression systems currently used. Thus, researchers will have the necessary information to carry out studies identifying structural features and further explaining the physiological role in animals.

For this study, mammalian expression vectors containing each ENaC gene were used as template in PCR to amplify each of the ENaC genes. The three ENaC genes were cloned into separate yeast vectors and transformed into competent *S. cerevisiae* cells for expression. The ENaC subunits were expressed individually and in combination in yeast cells as seen by western blot analysis. Expression conditions were optimized using time, sugar source, and various yeast strains. In all strains induction times of ~4 hours result in maximum expression when raffinose was used to replace glucose, expression of alpha ENaC in the yeast strain S1InsE4A *S. cerevisiae* resulted in a significant increase in protein yield. In this study, we have demonstrated that ENaC expression was achieved at the microgram level, which will provide adequate quantities that can ultimately be used to achieve mass spectroscopy analysis and gain adequate knowledge into the stoichiometry of the ENaC subunits.

CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

As we eat and drink our body breaks down food and produces waste. The waste produced by digestion and the normal breakdown of tissue is processed in our kidneys; this processing consists of the removal of toxins and extra water that are excreted as urine and the re-absorption of ions from the urine. Ions which are absorbed by channels in the kidney re-enter blood circulation. The majority of sodium re-absorption is done by channels located in the proximal tubule of the nephron, the functional unit of the kidney (Figure 1). The main components of the nephron are the loop of Henle, which consists of the ascending limb and the descending limb, the proximal tubule, the Bowman's capsule, and the distal tubule. The majority of ion re-absorption is achieved in the proximal tubule; however, sodium concentration is finely adjusted in the distal tubule by amiloride-sensitive channels known as epithelial Na^+ channels (ENaC).



A. Renal vein
B. Renal artery
C. Ureter
D. Medula
E. Pelvis
F. Cortex
1. Ascending limb of loop of Henle
2. Descending limb of loop of Henle
3. Peritubular capillaries
4. Proximal tubule

Figure 1- Schematic diagram of the kidney and its functional unit, the nephron

Epthelial cells which form the distal tubule and collecting duct in the nephron are typically thought of as rectangular cells. The apical membrane is that which creates the interior lining of the tubule and the basolateral membrane is the exterior of the cells side of the cells that do not directly contact urine. ENaC is located in the apical membrane of epithelial cells in the distal tubule and collecting duct of the nephron. ENaC complexes are composed of integral membrane proteins that transport sodium from the lumen to the interior portion of the epithelial cells and are classified as channel-mediated passive transporters. This classification is used because ENaC transports sodium through a channel formed by the protein's subunits rather than using a mechanical motion to shuttle the ions across the membrane. Passive transport is used in the classification because the sodium ions move from an area of high concentration to an area of low concentration and the channel requires no energy input for the ion diffusion. To maintain the concentration gradient necessary for ENaC to function properly, a sodium/potassium ATPase pump is located on the basolateral membrane of the epithelial cells. This pump uses adenosine triphosphate (ATP) to move sodium ions against their concentration gradient and remove them from the interior portion of the cell. As sodium

ions are transported out of the epithelial cells by the ATPase pump, they are re-absorbed by the peritubular capillaries, which move them into renal veins where the ions re-enter blood circulation.

ENaC is composed of three distinct homologous subunits, alpha, beta, and gamma. ENaC's subunits contain two hydrophobic membrane spanning domains and a large extracellular loop in their tertiary structure. It is this structural arrangement which classifies the ENaC protein subunits in the ENaC/ Degenerin family (1). The association and number of protein subunits that create the quaternary structure of ENaC is currently unknown; however, it has been shown that while the alpha subunit of ENaC forms a functional channel on its own, sodium re-absorption is increased when all three subunits are expressed together to form the membrane channel (2;3). There are two proposed subunit models of ENaC which are supported in the literature; the first is a four subunit model which is composed of two alpha subunits, one beta subunit, and one gamma subunit (4;5). The second supported model is an eight or nine subunit model composed of four alpha, two beta, and two gamma subunits or three alpha, three beta, and three gamma subunits, respectively (6;7). The eight and nine subunit models of ENaC are not distinguished and are used together in the supporting literature. A cartoon representation of the four subunit model of ENaC is shown below (Figure 2).

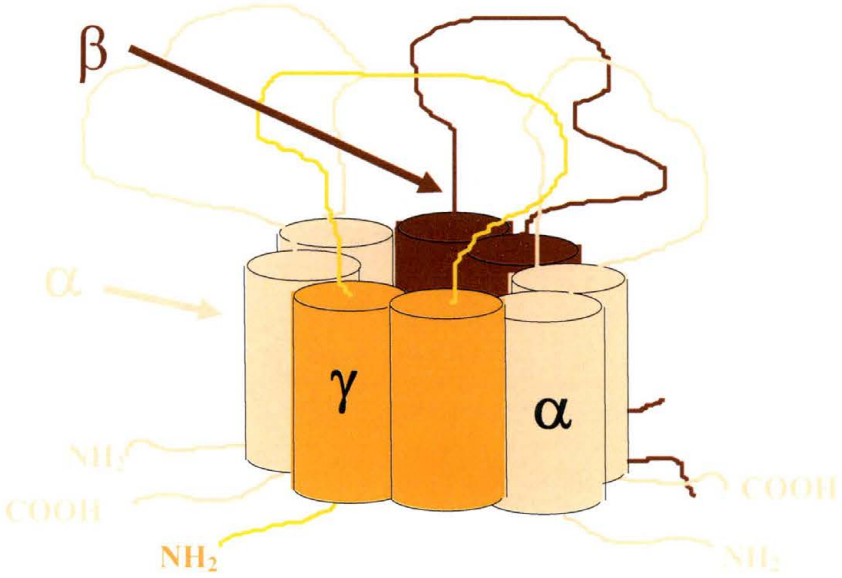


Figure 2- Graphic representation of the proposed subunit model of ENaC: Each subunit is composed of two alpha helices shown as cylindrical shapes and an extracellular loop.

In an effort to determine the quaternary structure of ENaC, a biophysical assay using mutants of each of the three protein subunits that make up a functional ENaC channel was developed (5). These mutant subunits along with wild type controls were expressed in *Xenopus* oocytes and patch-clamped to measure the electrical current generated as sodium passed through the channels. The mutant subunit channels were then exposed to amiloride, a chemical which is known to block ENaC channels. Channels containing mutant subunits were less sensitive to amiloride blockage than wild type subunits; therefore, by injecting varying ratios of mutant and wild-type subunits into the cells Kleyman's lab was able to determine the ratios of each subunit. This study was repeated using the sulfhydryl reagent, MTSEA, which only blocks ENaC when the mutant subunits are present (5). Based on the differences displayed by the mutant channels, the stoichiometry was deduced to be composed of two alpha subunits, one beta subunit, and one gamma subunit (5).

In an alternative experiment, the three subunits were expressed in *Xenopus* oocytes and sequentially labeled using iodinated monoclonal antibodies to label the extracellular loop of each of the ENaC subunits (4). The measurements revealed that the radioactive signal produced was two times more intense for alpha subunits as beta or gamma. In addition, they set up an experiment using channel blockers and mutant subunits. Mutation of serine 583 to cysteine on the alpha subunit caused zinc to block the channel. Increasing concentrations of zinc caused a decrease in the current measured across the membrane by patch-clamping. When alpha wild type and alpha mutants were co-expressed in equal concentrations, the measured current across the channel was similar to the current readings observed when only wild type alpha was expressed therefore indicating that there is more than one alpha subunit present. When the ratio of mutant alpha to wild type alpha was approximately 0.75 the current measurements resembled those observed by only mutated alpha expression. These data are consistent with a two alpha subunit model. However, when beta and gamma mutants were analyzed with amiloride as the channel blocker they were consistent with a single beta or gamma subunit model rather than two subunits. This electrophysiological data in conjunction with the FLAG studies provides evidence of a four subunit model of ENaC (4).

Studies involving fluorescence resonance energy transfer (FRET) concluded that the stoichiometry of ENaC was consistent with either an eight or nine subunit model (8). To investigate the subunit associations, the three ENaC proteins were produced fused to fluorescent proteins. For example, alpha subunits were tagged with cyano fluorescent protein (CFP) on yellow fluorescent protein (YFP), where CFP was the energy donor, and YFP was the energy recipient. These tagged subunits were then expressed along

with the remaining two wild type subunits and allowed to form channels in the membrane. Separate trials were designed for both independent studies of beta and gamma subunits. By observing energy transfer from the CFP to the YFP in each trial, it was determined the stoichiometry must contain more than one beta and gamma subunit per channel. This supports either an eight or nine subunit model (8).

Freeze fracture microscopy was also used to determine the stoichiometry of the ENaC subunits (6). Human ENaC subunits were expressed in *Xenopus* oocytes and the current generated by sodium flowing through the channels was measured using whole cell patch-clamping techniques. This allowed them to compare their observed current to that seen by other labs and check for channel functionality. They found that the channels were functional and consistent with the currents observed by other labs when all three subunits were present. They then tested amiloride sensitivity to ensure that the channels could be blocked as a second indicator of the presence of ENaC. Once investigators confirmed the presence of ENaC in the channels, standard protocols for membrane fixation and freeze fracture microscopy were performed. The images were examined and the diameter of the channels was measured. These data were then compared to other known and confirmed membrane-spanning channels with similar ENaC/Deg family alpha helices. When compared the group found that the diameter was consistent with channels having seventeen or more membrane spanning alpha helices. ENaC subunits each consist of two membrane spanning alpha helices and therefore the channel would have at least eight subunits (6). Other studies have also expressed and examined the stoichiometry of the ENaC subunits and concluded an eight or nine subunit model (9).

The studies previously discussed which support a four subunit model conducted research in *Xenopus* oocytes; it is possible that in this simplistic system channels are able to form and be functional as a four subunit model. When examining Firsov's studies it is possible that ENaC exists as an eight-subunit model and the signals, given as ratios of signals, are being produced from four alpha subunits, two beta and two gamma. This research concludes that there are twice as many alpha subunits as beta and gamma, not that there are exactly four subunits in the system (4). Similarly, the data from Staruschenko cannot discredit the proposed four subunit model, because it is unknown whether or not functional channels within the membrane are close enough to have inter-channel energy transfer as opposed to only having intra-channel energy transfer (8). Studies such as these cannot conclusively deduce the channel stoichiometry of ENaC and therefore further investigation is needed.

ENaC plays a role in two monogenetic disorders, Liddle's Syndrome and Pseudohypoaldosteronism Type I (PHA I). Liddle's syndrome is a disorder in which a mutation in the beta or gamma subunit of ENaC that alters the Nedd4 binding domain in the carboxy-terminus, increasing the amount of sodium that is re-absorbed in epithelial cells (10;11). Nedd4 is a ubiquitinating protein which labels ENaC channels for degredation and removal from the membrane. Patients with Liddles Syndrome have an increase in sodium re-absorption which leads to hypertension or high blood pressure. PHA I has the opposite effect; patients who have PHA I show low sodium re-absorption and often have hypotension or low blood pressure. This genetic mutation is typically caused by deletions or frameshift mutations that impair the effect of aldosterone. Aldosterone typically increases ENaC activity in the cells and therefore increases sodium

re-absorption; PHA I characteristically does not respond to aldosterone hormone therapy (10).

Aldosterone is a hormone secreted naturally in the body which increases sodium re-absorption in most patients as well as in *in vitro* study systems. Aldosterone operates in two distinct ways; the first is through genomic regulation and the second is non-genomic regulation. In the genomic pathway, aldosterone binds to a particular receptor site known as mineralocorticoid receptor (MR) which interacts with the ENaC promoter region of DNA. Once bound, this complex promotes transcription and translation of the alpha ENaC subunit as well as of particular Nedd4 inhibitory proteins. Nedd4 has a WW binding domain which recognizes and binds to the PPXY binding domain on the carboxy-termini of the beta or gamma subunit of ENaC (12). This binding targets the channel for degradation and removal from the cellular membrane by a protease. This removal decreases sodium re-absorption due to a decreased number of channels present to transport sodium ions. However, when aldosterone is secreted, Nedd4 is inhibited by an inhibitory protein known as serum- and glucocorticoid-induced protein kinase (sgk), and is unable to interact with the beta subunit of ENaC. The channels remain in the cell membranes and sodium re-absorption increases as a consequence of this inhibition. When a patient has PHA I, responses to aldosterone are not detected. This is thought to be caused by a mutation which deletes the MR receptor site and prevents aldosterone activity (10;13). Research also suggests that aldosterone regulates ENaC through non-genomic mechanisms which increase sodium re-absorption. This response appears to be species dependent, and the mammalian regulation pathway is not fully understood; however, it is believed that in frog A6 kidney cells aldosterone interacts with a methylase

which activates a G-protein known as k-Ras. The methylation of k-Ras causes a cascade which allows results in the inability of Nedd 4 to interact with the beta subunit of ENaC increasing sodium re-absorption (14).

ENaC is also regulated by mitogen activated protein kinase (MAPK) phosphorylation. The beta and gamma subunits of ENaC contain a threonine residue at amino acid 628 (15). This residue has a free hydroxyl group which is phosphorylated by MAPK; this action enhances Nedd4 binding and follows the removal and degradation cascade (16). Other proposed ENaC regulation mechanisms affect gating of the channel. This type of modulation is less understood, and without a known structure is difficult to further examine. Until the stoichiometry and geometry of the subunits is known, the mechanism by which ENaC is regulated is difficult to determine. Studies often use a diuretic, amiloride, to block the channel and prevent sodium re-absorption. This action leads many researchers to believe that there is a regulation mechanism that does not involve ENaC expression and/or physical presence in the membrane. However, there is no definite evidence to support this theory.

Because ENaC is a membrane protein, research techniques are difficult, and these techniques must be modified to study the native protein. Membrane proteins are expressed in low levels due to the physical limitation in space of a cell's membrane. ENaC is expressed and inserted in the membrane when sodium concentrations are low in blood circulation and degraded when the salt concentration is raised to physiologically acceptable limits. Membrane proteins also contain large hydrophobic regions which interact with the hydrocarbon tails of membrane phospholipids. These regions often cause insoluble ENaC aggregates to form spontaneously in solution leading to

precipitation of the proteins. The hydrophobic regions of the subunits can also cause re-folding of the protein and loss of the native structure in order to reduce contact of these regions with the aqueous environment. The ideal method to study ENaC in the proposed native human form is by expressing the channel in a mammalian system; however, mammals have very slow cellular generation times, and therefore take longer to generate high concentrations of membrane protein. When membrane protein expression is achieved and purification of the channels is conducted the relative amounts of protein recovered are extremely low; therefore, efficiency in a mammalian system is low and impractical. In a mammalian cell, membrane proteins are glycosylated before they are inserted into the membrane. Glycosylation is not necessary in prokaryotic cells for membrane insertion of proteins and the mechanism by which proteins are inserted is modified from the eukaryotic pathway; therefore it is unknown if proteins expressed in prokaryotes would be in their native form after expression and membrane insertion. Each of these creates specific problems which have led to the difficulties in determining the native protein associations of the ENaC subunits.

In order to address these issues, an expression system that will maintain the mammalian post-translational modifications, but allow for cellular replication times similar to those of prokaryotes is necessary. *Saccharomyces cerevisiae,* or baker's yeast, is an ideal system that meets these requirements. According to Canessa *et al.,* expressing alpha ENaC in yeast cell membranes creates a functional channel which exhibits sodium currents that mimic the equivalent mammalian channel (17). To further investigate this claim, major goals of the current project were to create DNA expressing constructs for all three protein subunits which associate to form the ENaC channel. These constructs were

created by amplifying each of the three genes for ENaC using polymerase chain reaction (PCR). These PCR products were then cloned into three distinct yeast expression vectors. Each of the vectors contains a unique selection marker so that control over protein expression can occur. They also contain either a Myc or V5 epitope for isolation and purification. Once the new gene constructs were confirmed by automated sequencing, the plasmids were transformed into *S. cerevisiae* for protein expression. Protein expression was confirmed using western blotting and the expressed proteins were purified using Immobilized metal affinity chromatography (IMAC), as well as cation exchange chromatography. The focus of this project was to increase protein concentration from the current nanomole scale into the micromole scale by creating the gene constructs described above followed by protein expression and isolation in yeast. Following this project, studies can be performed such as mass spectroscopy and X-ray crystallography to determine the protein associations in ENaC. Once the yeast membrane expressed protein structure is confirmed, patch clamping experiments will be necessary to demonstrate that yeast channels and mammalian channels have analogous sodium transport capabilities.

CHAPTER II

MATERIALS AND METHODS

Plasmid Isolation- *E. coli* cells containing plasmids were grown in 5 mL LB ampicillin cultures at 37 °C for approximately 16 hours. All plasmids were isolated from Top10 *E. coli* cells using a QIAprep Spin Miniprep kit (Qiagen) and following the protocol provided with the kit. This consisted of re-suspension of a cell pellet from an overnight culture, lysis of the cells, neutralizing the solution, removing the cell debris by centrifugation, binding the plasmid to a spin column, washing the plasmid, and eluting the plasmid with water. All of the ENaC plasmids and two of the yeast expression vectors were isolated using this method and were analyzed using horizontal gel electrophoresis.

Plasmid Concentration Determination- Each plasmid was allowed to thaw on ice for 5 minutes along with the 100 ng/mL Calf Thymus DNA standard. Two milliliters of Assay Buffer A (0.1 μg/mL Hoechst 33258 from Sigma in 1X TNE buffer (100 mM Tris, 10 mM EDTA, 2 M NaCl, pH 7.4)) was added into the quartz cuvette and the fluorometer was blanked using the solution. The fluorometer was calibrated for 5 ng/mL to 500 ng/mL by adding 2 μL of the DNA standard to the Assay Buffer A in the cuvette. Once calibrated, the solution was removed and the cuvette was rinsed with water. Two milliliters of Assay Buffer A was again added to the cuvette and the fluorometer was

blanked. For each sample, 2 μL of the plasmid prep was introduced into the Assay Buffer A and the concentration was record.

Horizontal Gel Electrophoresis- A 1 % w/v agarose gel was made using 1X Tris-acetate (TAE) buffer (20 mM acetic acid, 40 mM tris base, 2 mM EDTA pH 7.4). The gel was electrophoresed at 100 V for one hour in an IBI QS-710 Quick Screen gel rig (Shelton Scientific). The gel was stained using ethidium bromide (1 mg/mL) in the 1X TAE buffer as the gel was running. Once complete, the gel was analyzed using a Kodak Digital Sciences Image Station 440 and Kodak 1D imaging software.

PCR- Approximately 100 ng of DNA template was added to each PCR reaction. The PCR reactions included 1 μM custom primers (IDT Integrated DNA Technologies, Inc), 0.4 mM dNTPs (Stratagene), 1X ThermoPol buffer (New England Biolabs), and 0.1 units VENT DNA polymerase (New England Biolabs). The reactions were carried out in an Applied Biosystems 2720 Thermo Cycler under the following run conditions: 94 °C for 2 minutes; twenty-five cycles of 94 °C for 30 seconds, 45 °C for 30 seconds, 72 °C for 2 minutes; 72 °C for 10 minutes; 4 °C until samples were removed. Once the PCR reactions were complete, the products were analyzed and purified using horizontal gel electrophoresis and then gel extracted.

Alternatively, the alpha ENaC gene was amplified using PCR in standard Taq buffer from New England Biolabs and *Taq* DNA Polymerase, which produces adenine overhangs. A traditional PCR touch down method was implemented with a starting annealing temperature of 65 °C dropping one half of a degree per cycle to 50 °C for twenty-five cycles.

ENaC PCR primers had the following sequences:

Alpha forward 5'-ATGCTGGACCACACCAGAGCCCCTGAGC -3'

Alpha reverse 5'-GAGTGCCATGGCCGGAGCACAGGC-3'

Beta forward 5'-CGAAGGATCCATGGCATCAATGCAGAAGCTGATCTCAGAGG-3'

Beta reverse 5'-GCAAGCGGCCGCCTAGATGGCCTCCACCTCACTG-3'

Gamma forward 5'-CGAAACTAGTATGGCATCAATGCAGAAGCTGATCTCAGAGG-3'

Gamma reverse 5'-GCAAGCGGCCGCTTAGAACTCATTGGTCAACTG-3'

Gel Extraction- The bands were excised and cleaned using the Wizard PCR Preps DNA Purification System (Promega). Agarose gel slices were mixed with the PCR binding resin and incubated at 65 °C until the agarose completely melted and were then placed into a column provided in the kit. The column was washed with 85 % v/v isopropyl alcohol and the DNA was eluted with water.

Digestion- PCR products along with the cloning vectors were prepped for ligation using restriction enzyme digestion. The restriction digest protocol called for 450 ng of DNA, 2.5 μL of restriction enzyme (Table 1), and the corresponding New England Biolabs buffer (1X final concentration). The digestions were carried out for three hours at 37 °C and stopped using 1X EndoR Stop, (10 mM EDTA, 5 % v/v glycerol, 0.1 % w/v SDS, 0.01 % w/v bromophenol blue, pH 8.0) or when appropriate were stopped by incubating the reaction at 65 °C for 15 minutes.

Table 1: List of Restriction Enzymes used to Digest Samples

Plasmid Name	Enzyme 1	Enzyme 2	New England Biolabs Buffer
Beta ENaC PCR	BamHI	NotI	2- (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.9)
Gamma ENaC PCR	SpeI	NotI	2
pRS313-Gal1 (18)	BamHI	NotI	2
pTCG (19)	SpeI	NotI	2
Alpha ENaC- pYES2.1-TOPO	XbaI	N/A	2
Beta ENaC- pRS313-Gal1	ApaI	N/A	4- (50 mM potassium acetate, 20 mM Tris-HCl, 10 mM magnesium acetate, 1 mM DTT, pH 7.9)
Gamma ENaC- pTCG	ApaI	N/A	4

Phosphatase reaction- After inactivating the restriction enzymes at 65 °C, digestion samples were cooled to room temperature and the DNA vectors pRS313-Gal1 and pTCG were phosphatased with Calf Intestinal Phosphatase (New England Biolabs). The phosphatase reaction was carried out at 37 °C for 45 minutes and stopped by adding EndoR Stop.

Ligation- The Alpha ENaC gene was ligated into an Invitrogen TOPO expression vector, pYES2.1-TOPO. To achieve ligation, 50 ng of PCR product with adenine overhangs was mixed with 10 ng of TOPO vector and 1 μL of the supplied salt solution (1.2 M NaCl, 0.06 M $MgCl_2$). The reaction was carried out at room temperature for 30 minutes gently mixing every five minutes and placing the reaction on ice after the incubation period expired.

Fragments containing the beta and gamma genes were ligated into yeast vectors by T4 DNA Ligase (400 units). A series of ligation reactions with various ratios of vector to insert were performed to determine the optimum ligation conditions for each

pair. Two controls were used; one contained only digested phosphatased vector and the other was purified digested PCR product only. The three reactions used ratios of insert to vector of 3:1, 1:1, and 1:3. For the 3:1 ratio, 6 μL of PCR product (100 ng) was mixed with 2 μL of digested vector (100 ng). The 1:3 ratio was set up exactly reverse to the 3:1. The 1:1 ratio was carried out by adding 6 μL of both insert and vector (each 100 ng) into the reaction. All of the reactions included 1X T4 DNA ligase buffer (New England Biolabs) and T4 DNA ligase (1X) and were incubated at room temperature for 30 minutes.

***E. coli* Transformation-** Plasmids were amplified and purified from competent Top10 *E. coli* cells. This procedure involved the addition of approximately 0.1-1 μg of plasmid into 100 μL of competent cells. After ice incubation for 20 minutes, the cells were subjected to heat shock for one minute at 42 °C followed by an additional two minute ice incubation. One milliliter of LB broth was added to each of the reactions and they were incubated in a 37 °C Max Q 4000 E-class shaker (Barnstead Lab-Line) for 1 hour. Once the incubation period expired, 500 μL of the transformation reaction was plated on LB ampicillin plates (100 μg/mL ampicillin). The plates were placed into a 37 °C incubator for 24 hours and observed for colony formation.

Sequencing- The constructed plasmids were sent to Davis Sequencing for sequencing. To prepare the samples for sequencing each plasmid was isolated using the Qiagen kit and protocol above and sent at a concentration of 400-500 ng/mL in water. Primers (3 μM) were designed against the ligated plasmid sequence and used in the reactions (see sequences below).

Alpha ENaC- pYES2.1-TOPO:

GAL1 Forward primer- 5'-AATATACCTCTATACTTTAACGTC-3'

V5 C-term Reverse primer- 5'-ACCGAGGAGAGGGTTAGGGAT-3'

Alpha Internal primer- 5'-ACAACTCTTCCTACACTCGC-3'

Beta ENaC- pRS313-Gal1:

GAL1 Forward primer- 5'-CCGCACTGCTCCGAACAATA-3'

T7 Reverse primer- 5'-GCTAGTTATTGCTCAGCGG-3'

Beta Internal primer- 5'-ATGGAACTGTGTGTACCTTGCG-3'

Gamma ENaC- pTCG:

GAL1 Forward primer- 5'-CCGCACTGCTCCGAACAATA-3'

T3 Reverse primer- 5'-ATTAACCCTCACTAAAGGGA-3'

Gamma Internal primer- 5'-TGTCATGCACGTTCATGAGTCG-3'

Yeast Transformation- The three plasmids, Alpha ENaC- pYES2.1-TOPO, Beta ENaC- pRS313-Gal1, and Gamma ENaC- pTCG, were transformed into *Saccharomyces cerevisiae* cells using the following protocol. Each plasmid was transformed into BY4742 *S. cerevisiae* unless otherwise stated (all strains were transformed using this protocol). A 15 mL culture of YPD broth (1 % w/v yeast extract, 2 % w/v peptone, 2 % w/v dextrose) was inoculated with *S. cerevisiae* cells until broth became cloudy; the solution was shaken for 16 hours at 30 °C in an Envirn Shaker (Lab-Line). Three samples of 1.5 mL each of the cell culture were spun at 18,000 × g for thirty seconds in the microfuge. The supernatant was removed and 6 µL of sonicated salmon sperm DNA (10 mg/mL from Stratagene) and 10 µL of the appropriate plasmid were added to the cells. After vortexing briefly, 500 µL of PEG/LiAc and 56 µL of DMSO

were added into the reactions. The samples were gently mixed and placed into the 30 °C shaker for 10 minutes. The tubes were immediately transferred into a 42 °C water bath for 15 minutes and spun for 30 seconds at 18,000 × g in the microfuge to pellet the cells. The supernatant was removed and the cells were re-suspended in 200 µL of water. After re-suspension, the cells were plated on selective minimal media (2 % w/v glucose; 0.67 % w/v yeast nitrogen base; 0.01 % w/v of adenine, arginine, cysteine, leucine, lysine, threonine, tryptophan, and uracil; 0.005 % w/v aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine; 2 % w/v agar; and 0.5 % w/v ammonium sulfate) depending on their specific selection markers. Alpha ENaC-pYES2.1-TOPO was plated on glucose minus uracil media because it has a *URA3* gene and galactose promoter, Beta ENaC-pRS313-Gal1 was plated on glucose minus histidine media due to its *HIS3* gene, and Gamma ENaC-pTCG was plated on glucose minus tryptophan media since there is a *TRP1* gene present on the plasmid. All were grown in a 30 °C incubator for three to four days.

Table 2: Strains of *S. cerevisiae* used in the Expression of ENaC

Strain	**Genotype**
BY4742	MATα *his*3Δ*1 leu2*Δ *lys2*Δ *ura*3Δ (20)
S1InsE4A	MATα *ura3-52 leu2-3,112 trp1-289 his*7-2 *ade*5-1 *lys*2::*Ins*E-*4*A (21)
$VL6_{\alpha}$	MATα *ura3-52 trp1-*Δ*63 lys2 his3 met14 ade*1 (22)
BWG1-7a	MAT**a** *ura3-52 leu2-3,112 his4-519 ade1-100* (23)

Expression- Each of the ENaC subunits was expressed separately in selective minimal broth (2 % w/v glucose; 0.67 % w/v yeast nitrogen base; 0.01 % w/v of adenine,

arginine, cysteine, leucine, lysine, threonine, tryptophan, and uracil; 0.005 % w/v aspartic acid, histidine, isoleucine, methionine, phenylalanine, proline, serine, tyrosine, and valine; and 0.5 % w/v ammonium sulfate) without one amino acid according to the selective markers described above. Colonies were selected from the transformation plates and were placed into 15 mL of selective media and grown in the 30 °C shaker for approximately 16 hours. OD_{600} readings of each culture were taken and the following formula was used to determine how many milliliters of the cell culture would be used for expression:

$$\text{Number of mL needed for expression} = \frac{(0.4\ OD_{600})\ (\text{Number of mL expressed})}{(OD_{600}\ \text{of culture})}$$

The calculated number of milliliters was centrifuged at 1500 × g for 5 minutes at 4 °C in a Beckman Coulter Allegra 25R Centrifuge. The supernatant was removed and the cells were resuspended in 1 mL of galactose selective minimal broth. The suspension was transferred into 50 mL of galactose selective minimal media (composition same as above with 2 % w/v galactose rather than glucose) and placed in the 30 °C shaker. One milliliter aliquots were removed from the media every four hours over a twenty-four hour period; OD_{600} readings were taken at each time point. The 1 mL aliquots were centrifuged at 1500 × g for 5 minutes at 4 °C, the supernatant was removed, and the cell pellets were stored at -20 °C. The culture remaining after the last time point aliquot was removed was centrifuged and also stored at -20 °C. The aliquots were thawed on ice and resuspended in 500 µL of lysis buffer (50 mM Na_3PO_4, 1 mM EDTA, 1 mM PMSF, and 5 % Glycerol). The mixture was vortexed briefly and centrifuged at 1500 × g for 5 minutes to re-pellet the cells. The supernatant was once again removed and the cells were re-suspended in enough lysis buffer to yield an OD_{600} reading of 50. An equal

volume of acid washed beads was added to the mixture and the cells were vortexed for 30 seconds. After vortexing, the cells were placed back on ice for 30 seconds; this process was repeated 4 times. The cells were then centrifuged at 18,000 × g for 10 minutes and the supernatant was removed. SDS – PAGE 1X sample buffer (12 mM Tris-HCl, pH 6.8, 4.8 % v/v glycerol, 0.4 % v/v SDS, 2.9 mM β-mercaptoethanol, 0.2 % v/v bromophenol blue) was added to the supernatant and the samples were heated at 100 °C for 5 minutes.

SDS-PAGE Vertical Gel Electrophoresis- Samples were loaded into a stacking gel consiting of a 4 % polyacrylamide gel (30 % v/v bis-acrylamide, 0.5 M tris-HCl, pH 6.8, 10 % v/v SDS, 0.1 % v/v TEMED, and 10 % w/v ammonium persulfate) and a 7.5 % polyacrylamide gel (30 % v/v bis-acrylamide, 1.5 M Tris-HCl pH 8.8, 10 % v/v SDS, 0.05 % v/v TEMED, and 10 % w/v ammonium persulfate) and ran at 150 V for 1.25 hours in 1X Running buffer (25 mM tris base, 0.19 M glycine, and 0.1 % v/v SDS, pH 8).

Western Blot- The proteins in the gel were then transferred to nitrocellulose using a standard western blot protocol. A piece of nitrocellulose membrane (Bio-Rad Trans-Blot Transfer Medium) was placed on top of the gel and sandwiched in between two pieces of filter paper. The blot was placed into a vertical gel rig inside a transfer module. The rig was filled with 1X Transfer buffer (25 mM tris base, 1.9 M glycine, and 20 % v/v methanol) and was run for 45 minutes at 100 V. After completion of the transfer, the blot was placed in blocking solution containing 1 % w/v dry milk in 1X TBST (1.5 mM tris-HCl, 50 mM NaCl, 0.1 % v/v Tween 20, pH 7.4). The solution was agitated for 15 minutes at room temperature and the blocking solution was removed. Primary antibody (anti-Myc monoclonal antibody for beta and gamma ENaC and anti-V5 monoclonal antibody for alpha ENaC) in blocking solution was applied to the blot

overnight at 4 °C. The next morning the solution was removed from the blot, and it was washed three times in 1X TBST for 5 minutes each. Secondary antibody (anti-Mouse) was added to the blot in blocking solution, agitated for 45 minutes, and the blot was washed two times in 1X TBST and once in 1X TBS (1.5 mM Tris-HCl, 50 mM NaCl, pH 7.4). One milliliter each of a two part Western Lighting kit (Perkin Elmer LAS, Inc.) solution was applied to the blot for 1 minute. The excess solution was removed and the blot was placed in Saran wrap and placed into a film cassette. The blot was exposed to X-ray film for 1 to 10 minutes and was placed into a film developer.

CHAPTER III

RESEARCH RESULTS AND DISCUSSION

In order to obtain structural and stoichiometric data on ENaC, the genes need to be cloned into a new host system. This would allow larger quantities of expressed protein to be produced, easily isolated, and purified. These conditions must be met to allow definitive determination of the stoichiometry of the three ENaC subunits as well as assessment of the extent of glycosylation and subunit association using mass spectrometry. To achieve the goal of determining subunit stoichiometry, the three ENaC genes were cloned and expressed in *Saccharomyces cerevisiae.*

ENaC Gene Amplification Using PCR: In order to clone the ENaC subunits, alpha, beta, and gamma, each gene was amplified using PCR. Primers for PCR were designed against the N-terminal Myc region of the pCMV-Myc Beta and Gamma ENaC sequences as well as the C-terminus, encompassing the stop codon, for amplification of each of the ENaC genes. Primers were also designed for alpha ENaC slightly downstream from the N-terminal Myc region and in front of, but not including, the stop codon, at the end of the pCMV-Myc-Alpha sequence. Specific restriction enzymes were engineered on each end of the beta and gamma primers, which were used to clone the genes in the correct orientation. PCR was performed using two different polymerases: *Taq* DNA polymerase was used for alpha and VENT DNA polymerase was used for beta and gamma.

These two polymerases differ in both their proofreading capabilities and terminal ends of the PCR products they produce. Since alpha ENaC was cloned into the commercially purchased TOPO vector with thymine overhangs, the *Taq* DNA polymerase was used to achieve adenine overhangs on each end of the alpha ENaC PCR product at the cost of possible errors. While VENT DNA polymerase has proofreading ability, unlike *Taq*, the PCR products resulting from VENT have blunt ends. Beta and gamma ENaC PCR products were digested to create sticky ends which could be cloned into corresponding digested vectors. Since the ends of the PCR products were insignificant to the cloning process, the proofreading Vent polymerase was used to minimize the number of mutations. After 25 cycles of PCR were performed, the lengths of the PCR products were examined using horizontal gel electrophoresis in a 1 % w/v agarose gel stained with ethidium bromide (Figure 3). PCR product migration patterns were compared to the predicted migration patterns based on sequence size analysis. The expected PCR products for alpha, beta, and gamma were 2106 bp, 2011 bp, and 2060 bp, respectively. All PCR products were observed in the agarose gel at approximately 2000 bp. These PCR products were gel extracted and purified using the Promega PCR clean-up kit. Beta and gamma ENaC were digested using BamHI/NotI and SpeI/NotI, respectively (Table 2) and gel purified a second time (data not shown). Alpha ENaC was not digested because the pYES2.1 TOPO vector ligates PCR products containing adenine overhangs directly.

Table 3: Gel Electrophoresis Results after PCR Amplification

Plasmid Name	Expected Size (bp)	Actual Size (bp)
Beta ENaC PCR	2011	~2000
Gamma ENaC PCR	2060	~2000
Alpha ENaC PCR	2106	~2000

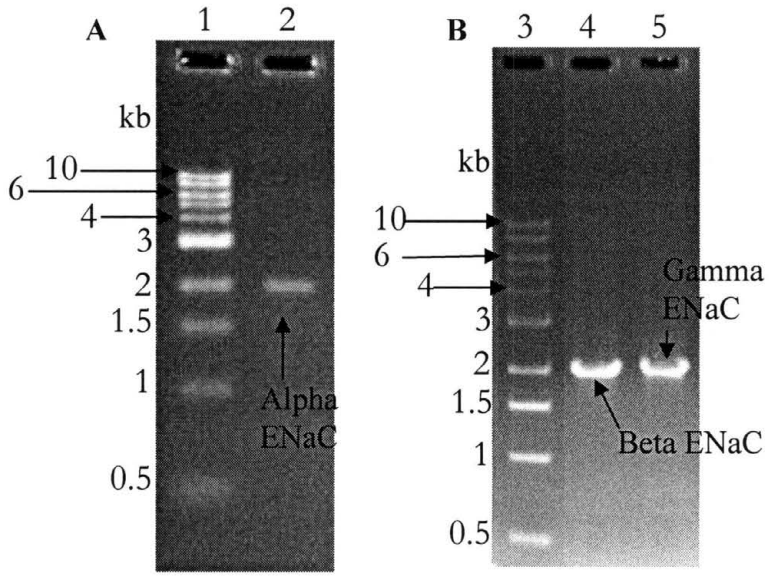


Figure 3: PCR amplification of α, β, and γ ENaC genes. Figure 3-A, lane 1- 1kb ladder and lane 2- Alpha-ENaC PCR product. Figure 3-B, lane 3- 1 kb ladder, lane 4- Beta-ENaC PCR product, and lane 5- Gamma-ENaC PCR product. Gel was stained with ethidium bromide.

Alpha ENaC Cloning: The Alpha ENaC gene was incubated for thirty minutes at room temperature with the pYES2.1 TOPO cloning vector according to the TOPO cloning manual protocol. This ligation reaction was then transformed into competent Top 10 *E. coli* cells. The cells were grown overnight on LB Amp plates for selection. Individual colonies were selected and placed into LB Amp broth and were incubated overnight at 37 °C with constant shaking to promote cell proliferation and gene

amplification. The plasmids were purified from the overnight culture using a Qiagen Plasmid Purification kit. Alpha ENaC-pYES2.1-TOPO clones were digested to check for correctly oriented ligated products using AgeI. Alpha ENaC- pYES2.1-TOPO was digested resulting in DNA fragments expected near 2780 bp and 5313 bp. After gel analysis, bands were observed at approximately 3000 bp and 5800 bp confirming that correct ligation had occurred and Alpha ENaC was inserted in the correct orientation (Figure 5). Confirmed clones were re-transformed and isolated; plasmid concentrations were 600 ng/µL, and samples were sent for automated sequencing. From the three potentially successful clones, clone number 2 (Lane- 2 of Figure 4) was selected for protein expression because it didn't have mutations in the coding region of the Alpha ENaC gene.

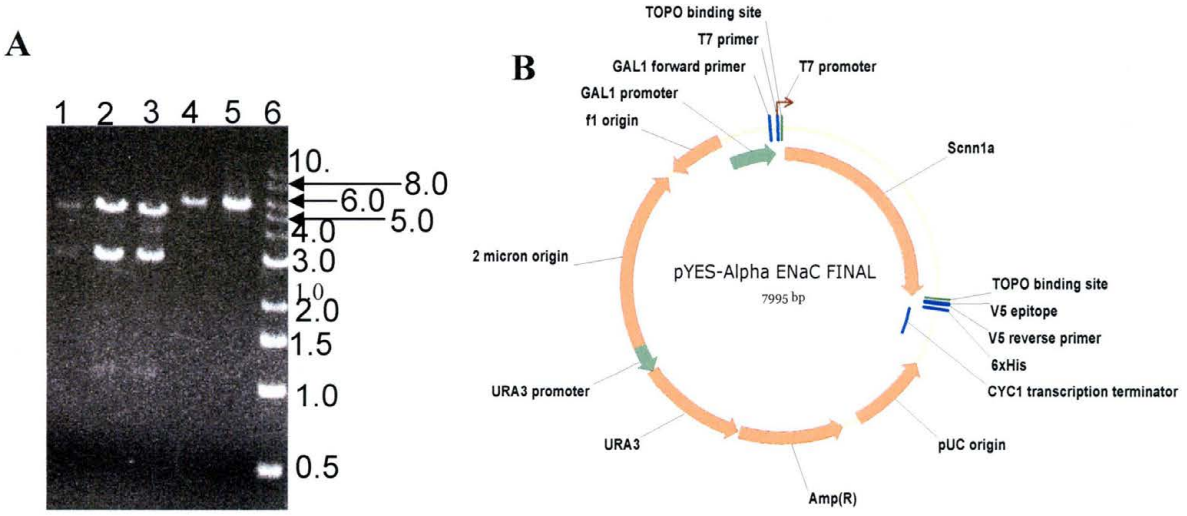


Figure 4- Alpha ENaC-pYES2.1-TOPO Cloning Confirmation: A is a 1 % agarose gel of the Alpha ENaC-pYES2.1-TOPO cloning confirmation. Lanes 1, 2, and 3 are successful ligations, Lanes 4 and 5 are unsuccessful ligations, and Lane 6 is a 1 kb ladder. B, on the right, is a vector map of Alpha ENaC-pYES2.1-TOPO. Agarose gel was stained with ethidium bromide.

pRS313-Gal1 and pTCG Vector Digestion: Cloning vectors for beta and gamma ENaC were digested using restriction enzymes and gel purified before being

cloned. The pRS313-Gal1 plasmid was digested using NotI and BamHI and pTCG was digested using NotI and SpeI. Digestion products of each expression vector were analyzed in an agarose gel stained with ethidium bromide (Table 3). Migration of digested pRS313-Gal1 vector was expected at 5613 bp and was observed in the gel at approximately 5500 bp (data not shown) and the pTCG vector was expected at 7919 bp and was observed at approximately 8000 bp (Figure 5). Size predictions were determined for each digested vector by sequence analysis. After digestion, the 5' phosphate ends of each cut vector were removed with a phosphatase to prevent the vector from ligating back together and each plasmid was gel purified for ligation.

Table 4: Restriction Enzyme Digestion Products for pRS313-Gal1 and pTCG

Plasmid Name	Enzyme 1	Enzyme 2	Expected Size (bp)	Actual Size (bp)
pRS313-Gal1	BamHI	NotI	5613	~5500
pTCG	SpeI	NotI	7919	~8000

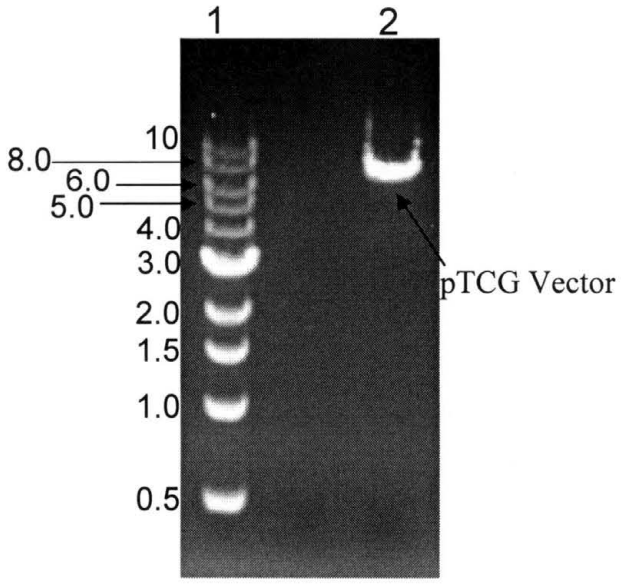


Figure 5- Digested pTCG Gamma ENaC Cloning Vector: A 1 % w/v agarose gel after restriction enzyme digest. Lane 1 is a 1 kb DNA Ladder, and Lane 2 is the digested gamma ENaC vector pTCG cut with NotI and SpeI. Gel stained with ethidium bromide.

Beta ENaC Cloning: The digested pRS313-Gal1 vector and beta ENaC gene were ligated and reactions were transformed into competent Top 10 *E. coli* and selected on LB Amp plates overnight at 37 °C. Individual colonies were selected and grown in LB Amp media overnight in a 37 °C shaker. Plasmid DNA was isolated from overnight cultures using a QIAprep Spin Miniprep kit and analyzed in an agarose gel. In an initial screen, the migration of the ligated constructs was compared against the migration of empty vector (data not shown). Four positive clones, migrating slower (larger in size) than the empty vector, were further screened by digestion with ApaI. The digestion reactions were then analyzed in a 1 % w/v agarose gel (Figure 6). Sequence analysis predicted that correct ligation of beta ENaC into the pRS313-Gal1 vector would produce DNA fragments that migrated at 2020 bp and 5602 bp. The observed migration pattern from the digested Beta ENaC- pRS313-Gal1 clones produced bands at approximately 2050 bp and 6000 bp, which corresponds with the anticipated migration pattern. Successful clones were re-transformed, isolated, and the resulting plasmid DNA concentrations were approximately 600 to 700 ng/μL. The ligated products were then sent for automated sequencing and two of the four potential clones were used for protein expression. Each of the two clones used for expression had a single mutation in the extra-cellular loop of the beta ENaC subunit. The mutation resulted in a lysine to arginine amino acid change. It is suspected that the mutation was in the original sequence used to achieve PCR cloning; since the plasmid used for PCR has been successfully expressed and has demonstrated functionality we are certain the mutation will not affect the expression or function of our yeast clones. The mutation does not affect the functionality of ENaC because of the similarity between the two amino acid's

physical properties and the location of the mutation in the extracellular loop of beta ENaC's tertiary structrure.

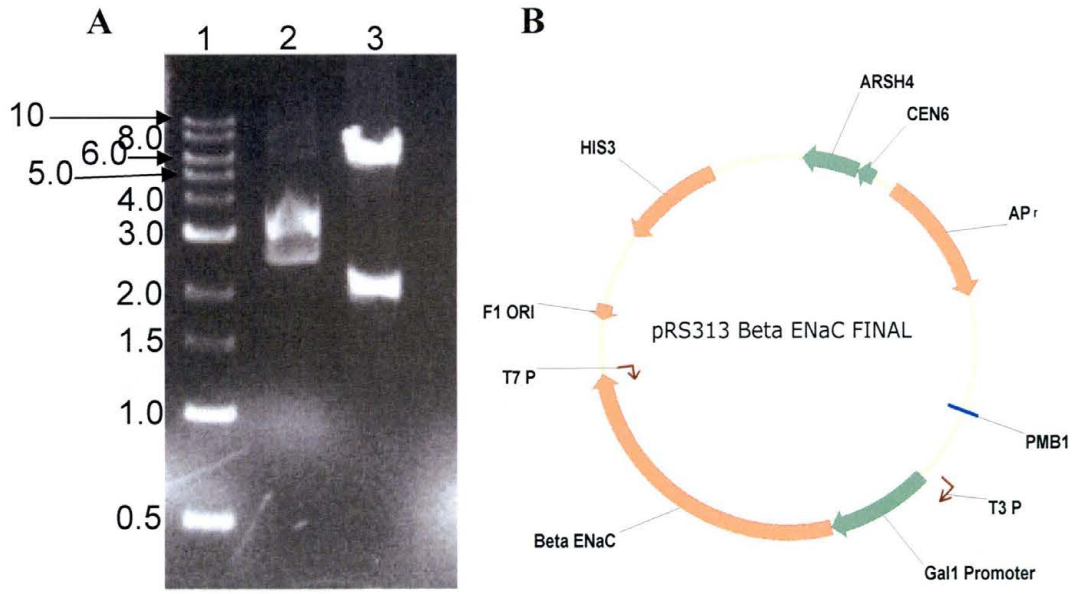


Figure 6- Beta ENaC-pRS313-Gal1 cloning confirmation: A is a 1 % agarose gel containing Beta ENaC-pRS313-Gal1 cloning confirmation. Lane 1 is a 1 kb ladder, Lane 2 is unsuccessful Beta ENaC-pRS313-Gal1 ligation, and Lane 3 is a successful Beta ENaC-pRS313-Gal1 ligation. B, on the right is a pictoral representation of the ligated product sequence. The gel was stained with ethidium bromide.

Gamma ENaC Cloning: Gamma was cloned into the pTCG vector and positive constructs were digested using ApaI. Ligated clones were expected to produce bands at 2978 bp and 7001 bp. After the agarose gel was run and stained with ethidium bromide, fragments were observed at approximately 3000 bp and 7500 bp (Figure 7, Lanes 4 and 6). An unexpected fragment appears at approximately 6 kb in each of these lanes; this is thought to be from un-digested supercoiled ligated product. Also in Lane 6, a second additional band is present at approximately 10 kb which corresponds with single cut linear ligated Gamma ENaC-pTCG. Both clone 4 and clone 6 were sent for automated

sequencing and did not contain mutations; the ligated clone in lane 4 was selected and used for protein expression.

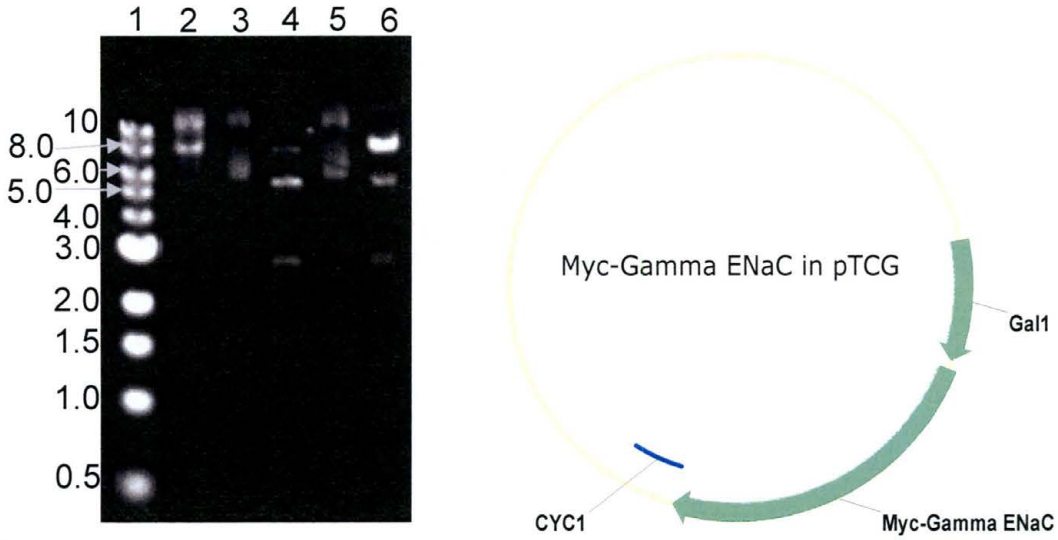


Figure 7- Gamma ENaC-pTCG cloning confirmation: A is a 1 % agarose gel of Gamma ENaC-pTCG cloning confirmation. Lane 1 is a 1 kb ladder, Lane 2, 3, and 5 are unsuccessful ligations of gamma ENaC into pTCG, Lanes 4 and 6 are successful ligations of gamma ENaC into pTCG. The plasmids were digested with ApaI. B, on the right is a pictoral representation of Gamma ENaC-pTCG plasmid. The gel was stained with ethidium bromide.

Expression of Alpha, Beta, and Gamma ENaC: Alpha, beta, and gamma ENaC were expressed in yeast individually to confirm protein production for each of the three subunits. Newly constructed expression vectors, Alpha ENaC-pYES2.1-TOPO, Beta ENaC-pRS313-Gal1, and Gamma ENaC-pTCG were transformed separately into *S. cerevisiae* BY4742 cells. Individual colonies from the transformation were selected and grown overnight in minimal media containing 2 % w/v glucose as a sugar source. OD_{600} readings were taken to determine the cell density after approximately 14 hours of growth; these readings were then used to make new overnight expression cultures in minimal media containing an inducer, 2 % w/v galactose, at an OD_{600} of 0.4. Five milliliter aliquots were collected at set time intervals over 12 or 24 hours. These aliquots were

centrifuged and the media was decanted from the cell pellet. Samples were then frozen at -20 °C until protein extraction could be performed. To extract protein from the cells, a standard lysis buffer with EDTA and a protease inhibitor cocktail were added to the samples. Acid-washed glass beads were added to the cell suspension and vortexed to break open the cells. After removal of the cellular debris, the protein samples were loaded into an SDS-PAGE and transferred onto nitrocellulose. In order to view the protein subunits, the alpha ENaC subunits were probed with anti-V5 primary antibody, while both beta and gamma ENaC were probed with anti-Myc primary antibody. Alpha ENaC was observed around 110 kDa (Figure 8), beta ENaC was observed around 100 kDa (Figure 9-A), and gamma ENaC was observed at approximately 30 kDa (Figure 10). Suspected cleavage products and alternate protein expression products were also observed at 70 kDa, 38 kDa, and 26 kDa in the expression (Figures 9 and 10). Each of the individual protein subunits had an optimal expression time of 12 hours.

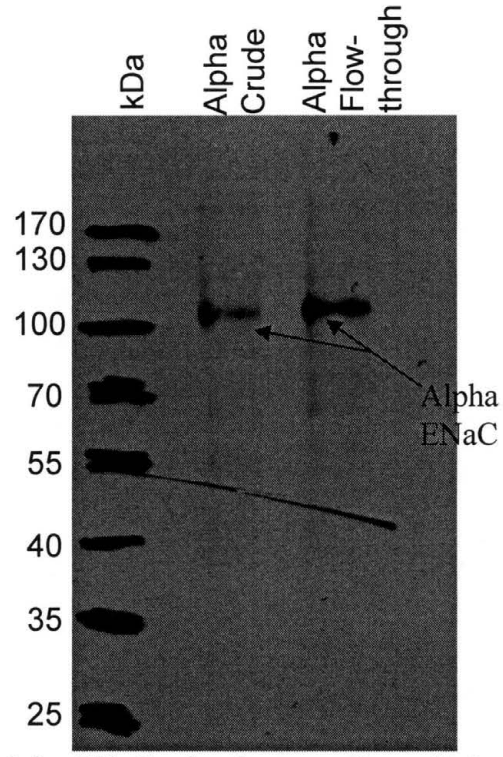


Figure 8- Western blot of alpha ENaC subunit expression: Alpha crude is the expressed product before being run in an IMAC column and alpha Flow-through is the initial collection before elution from the IMAC column. Probed with anti-V5 primary antibody.

Partial purification of His6-tagged alpha ENaC was achieved using a nickel IMAC, metal affinity column. A western blot probed with anti-V5 primary antibody followed by an anti-mouse secondary antibody was used to monitor the expression of alpha ENaC as well as the success of the column purification. His6-tagged alpha ENaC did not bind to the nickel column; this was most likely due to the presence of EDTA in the lysis buffer during column purification. When levels of alpha ENaC expression were compared to a control (LacZ), the relative concentrations of alpha ENaC appeared approximately 10 fold lower (data not shown). This lack of protein expression may be due to the disruption of cell membranes caused by salt intake through homomeric alpha ENaC channels. Alpha subunits have been reported to form functional, homomeric channels, which are distinct from heteromeric channels in their pore size, but allow

sodium transport at a less efficient rate across the membrane (24). Cells which contain mature, functional, alpha subunits may die quickly after expression begins, and only cells which contain few channels or non-functional channels in their membranes are being extracted and thus little ENaC protein would be observed in the western blot.

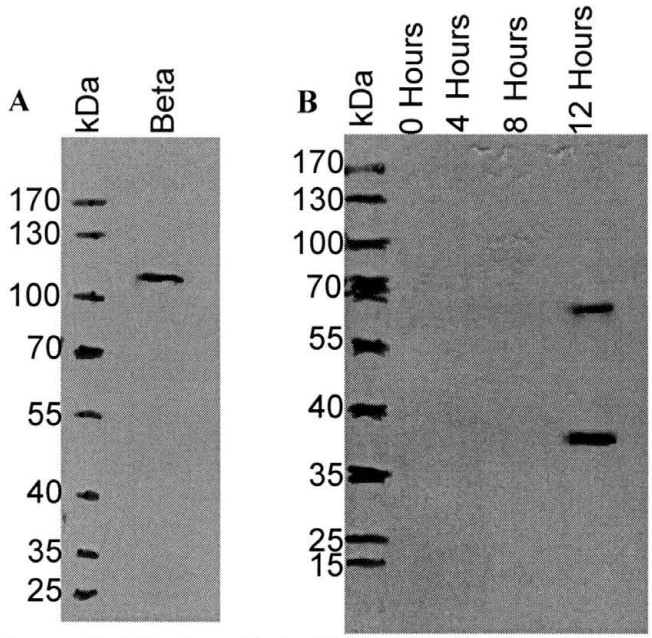


Figure 9- Western blot of beta ENaC subunit expression: A- Beta ENaC after expression over 12 hours. Samples in blot A were extracted using standard lysis buffer with EDTA and an added protease cocktail. B- Time interval collections to determine optimal beta ENaC expression. Expression times included 0 hrs (Lane 2), 4 hrs (Lane 3), 8 hrs (Lane 4) and 12 hours (Lane 5) from glucose overnights. Samples in blot B were extracted using standard lysis buffer without EDTA or a protease cocktail. Both blots were probed with anti-Myc primary antibody and anti-mouse secondary antibody.

Beta ENaC was expressed in BY4742 yeast cells for 24 hours and total protein was extracted from the cells using standard lysis buffer with EDTA and fresh protease inhibitors, and beta ENaC was detected at 12 hours. After expressing and extracting beta ENaC from *S. cerevisiae,* a western blot probed with anti-Myc primary antibody followed by anti-Mouse secondary antibody was developed (Figure 9-A). Following this

observation, a subsequent beta ENaC expression was performed and protein was extracted using standard lysis buffer without EDTA or a protease inhibitor (Figure 9-B). EDTA was eliminated from the lysis buffer so that a nickel column could be used for purification. As observed in Figure 8, when His6-tagged alpha ENaC was extracted with EDTA in the lysis buffer, the protein did not bind well to the nickel column and was therefore eluted in the flow-through instead of the elution fractions. It is suspected that when EDTA and protease inhibitors are removed, cleavage products of beta ENaC are produced. Interestingly, Kleyman and co-workers have reported the activation of ENaC via a proteolytic cleavage (25). The alpha and gamma ENaC subunits are cleaved to activate the channels; although no reports of beta ENaC subunits being cleaved have been made, we have consistently isolated beta ENaC as two separate fragments. We suspect that due to the homology between beta and gamma ENaC, it is possible that like gamma, beta too has proteolytic cleavage capabilities. During the cloning of beta ENaC, the Myc tag was placed on the N-terminal side of the amino acid sequence. It is possible that in the processing of the beta ENaC in yeast cells, two separate cleavage positions are produced. First the subunit would have to be cleaved to yield a 70 kDa N-terminal product, followed by the 38 kDa N-terminal product.

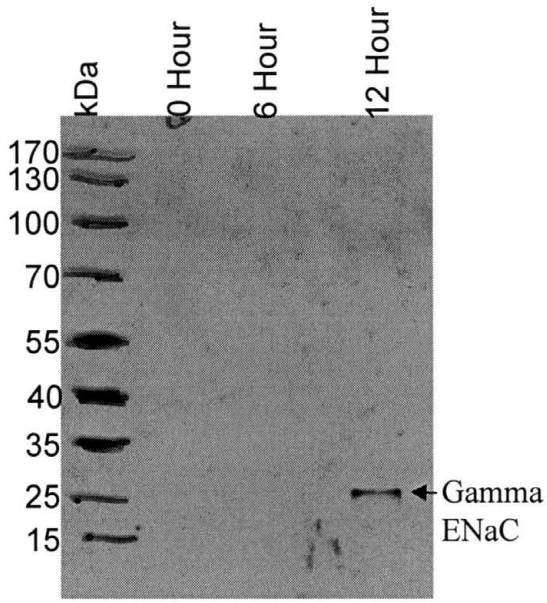


Figure 10- Western blot of gamma ENaC subunit expression: Gamma ENaC was expressed over a 12 hour time period to determine optimal expression and samples were collected at 0 hours (Lane 2), 6 hours (Lane 3) and 12 hours (Lane 4). The blot was probed using anti-Myc primary antibody.

Gamma ENaC was expressed over a 12 hour period, then protein was extracted from the yeast cells using standard lysis buffer with EDTA and PMSF, but not with additional added protease inhibitor cocktail. Western blot analysis was used to analyze expression and probed with anti-Myc primary antibody and subsequently anti-Mouse secondary antibody. Gamma ENaC was expected to migrate at approximately 100 kDa based on analysis of the amino acid sequence and previous literature reports (25); however, after developing the western blot, gamma ENaC was observed at approximately 28 kDa (Figure 10). Although alternative extraction solutions were made, including the addition of a protease inhibitor cocktail, the gamma subunit consistently migrated at 28 kDa. Like beta ENaC, gamma is suspected to contain a proteolytic cleavage event on the N-terminus to produce the observed band (26).

Co-Expression of alpha, beta, and gamma ENaC: Once the ENaC subunits were individually expressed in *S. cerevisiae,* all three subunits were co-expressed in the same cells over a 24 hour time period to determine optimal protein expression. Because of the apparent truncation of the gamma subunit, alpha and beta ENaC were co-transformed and expressed together in BY4742 yeast cells. Overnights of the co-transformed yeast cells were made using 2 % w/v glucose to repress the galactose promoter and prevent protein expression. The overnights were then diluted to a starting OD_{600} reading of 0.4 in induction media containing 2 % w/v galactose. Five milliliter aliquots were removed at 0, 6, 12, and 24 hours and frozen until all protein expression samples were collected. Each of the samples were extracted using the same method as individual protein extraction in a standard lysis buffer with EDTA, SDS-PAGE followed by western blot to confirm the presence of both subunits (Figure 11). To confirm the presence of both subunits, the blot was first probed with anti-Myc primary antibody followed by anti-mouse secondary antibody to verify the presence of beta ENaC (Figure 11). Next, the blot was stripped, rinsed, and re-probed using anti-V5 primary antibody followed by anti-mouse secondary antibody (data not shown). After developing the blots, it appeared that as with the individual expression of the subunits the co-expression of alpha and beta ENaC had optimum protein expression at 12 hours after being introduced into the induction media. Alpha ENaC again migrated at approximately 110 kDa as seen in individual expression samples (data not shown); however, beta ENaC was observed at approximately 38 kDa. The co-expression of alpha and beta ENaC was repeated and each time the 70 kDa beta ENaC was no longer observed as it had been previously seen in the individual expression of the beta subunit (Figures 9-A and 11). The suspected

proteolytic cleavage event which produces the 70 kDa fragment consistently observed in the beta subunit individual expression may be rapidly cleaved a second time to produce the 38 kDa fragment when alpha ENaC is also present.

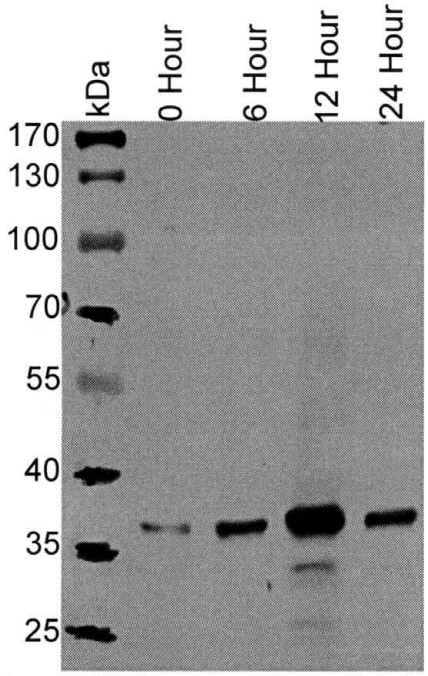


Figure 11- Co-expression of alpha and beta ENaC subunits in *S. cerevisiae:* Western blot probed with anti-Myc to observe the presence of beta ENaC. Lane 1 on the far left is a Fermentas protein standard marker, the subsequent lanes are time intervals indicating when aliquots were removed from the induction media and total cellular protein was extracted from the cells.

After co-expressing alpha and beta ENaC, the gamma ENaC plasmid was transformed in conjunction with the other two subunits and co-expressed in BY4742 yeast cells (Figure 12). Five milliliter aliquots were removed at 0, 4, 6, 12, and 24 hours to observe the progression and optimal time of protein expression. The protein subunits were extracted in standard lysis buffer with EDTA and no protease inhibitor. Both the western blot using anti-V5 primary anti-body to observe the presence of alpha ENaC (Figure 12-A) and the blot using anti-Myc primary anti-body to observe the presence of beta and gamma ENaC (Figure 12-B) are below. Alpha ENaC is again observed above

100 kDa, but beta ENaC is now also observed at 100 kDa. There is a band present at 70 kDa, which is consistent with observations of beta ENaC; however, it disappears after 12 hours. A band at approximately 60 kDa is due to gamma ENaC's presence, and a band at approximately 35 kDa could be a cleavage product of beta ENaC or a product of gamma ENaC. When considering the observations made by Kleyman and colleagues, and considering other supported findings of ENaC, it is possible that since beta ENaC cannot form functional channels on its own it is simply degraded and removed by proteolytic activity within the cell. When alpha and beta are co-expressed without gamma ENaC, alpha forms homolytic channels and beta could still be degraded due to a lack of function in the cell. When all three ENaC subunit are co-expressed together, beta does serve a purpose in the system and is therefore observed at 100 kDa as expected. Small amounts of beta ENaC could be experiencing proteolytic cleavage due to an excess production of the subunit; this cleavage would yield the bands at 70 kDa and perhaps 35 kDa. The same argument could be made for gamma ENaC. When it is expressed alone in the cells, it serves no function and is therefore degraded down to approximately 30 kDa, but when expressed with alpha and beta ENaC it is produced at approximately 60 kDa.

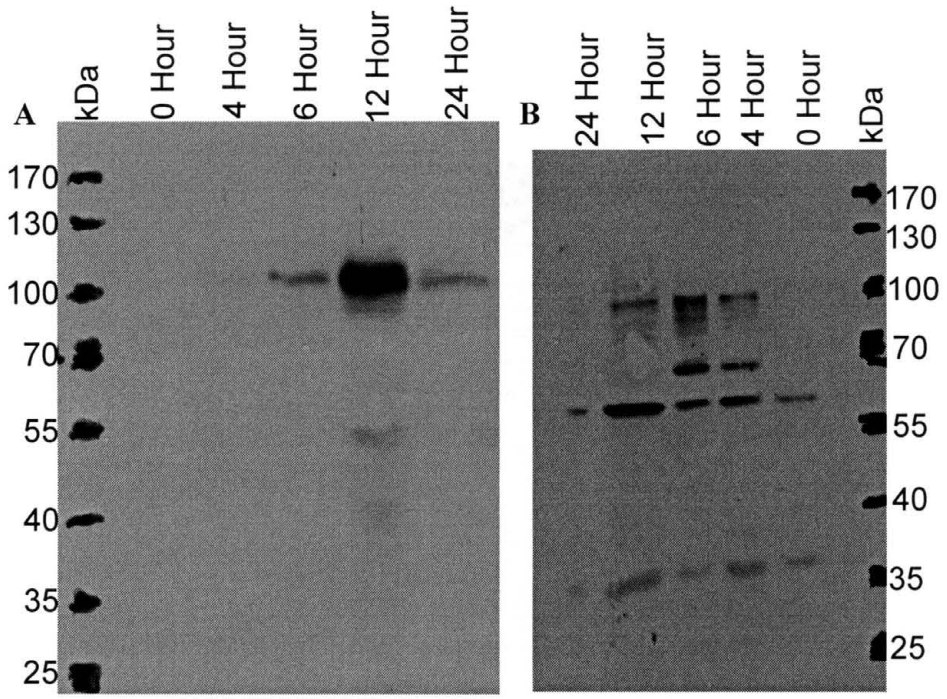


Figure 12- Co-expression of all three ENaC subunits: Blot A on the left is probed with anti-V5 and shows the expression of alpha ENaC to be optimal at 12 hours; blot B on the right is probed with anti-Myc and shows the expression of both beta and gamma ENaC. The 70 kDa band thought to be produced by a proteolytic cleavage of beta ENaC becomes very faint at 12 hours and disappears at 24 hours.

Varying Expression Conditions of Each Subunit for Comparison: After observing beta and gamma ENaC subunits much smaller than was expected from the literature review and seeing results which seemed to vary with extraction and expression conditions; alternative methods of expression were attempted. First, colonies from the *S. cerevisiae* transformations were grown as overnights in minimal media containing 2 % w/v raffinose. These samples were then diluted according to their individual OD_{600} readings and placed in minimal media containing 2 % w/v galactose to start expression. Samples were collected every 2 hours and protein was extracted using standard lysis buffer with EDTA. The samples were analyzed by western blot (Figure 13). The optimal expression times in raffinose were reached sooner, as expected, than what had previously

been observed for each subunit after being grown in glucose overnights. Alpha ENaC is expressed from glucose overnights optimally after 12 hours, but by using raffinose alpha had an observed optimal expression between 4 and 6 hours (Figure 13-A). Beta and gamma expression is also optimal after 12 hours from glucose overnights, but had maximum protein expression after only 4 hours (Figure 13-B and C). When overnights are grown in glucose, a repressor binds the galactose promoter; when raffinose is used instead of glucose, the promoter is suppressed but not bound by a molecule. Therefore, when galactose is introduced into the media there is not a time delay for the bound repressor to be removed and expression begins much quicker (27). In addition to the reduced expression times, less protein degradation was observed for both the beta and gamma subunits. Alpha was observed above 100 kDa as usual, but beta ENaC was seen at 100 kDa and 70 kDa with the previously observed 38 kDa fragment absent. Gamma was also observed much higher than had been previously observed (Figure 10) at approximately 60 kDa rather than 28 or 30 kDa. It is suspected that the rapid production of large quantities of the subunits prevents the dominant degradation products from being seen on the blot. Due to the lack of yeast expression systems for ENaC, there are no data which correlates to the expression from raffinose overnights; however, it is known that since raffinose does not have a bound galactose promoter repressor, expression is initiated much faster and easier in the system.

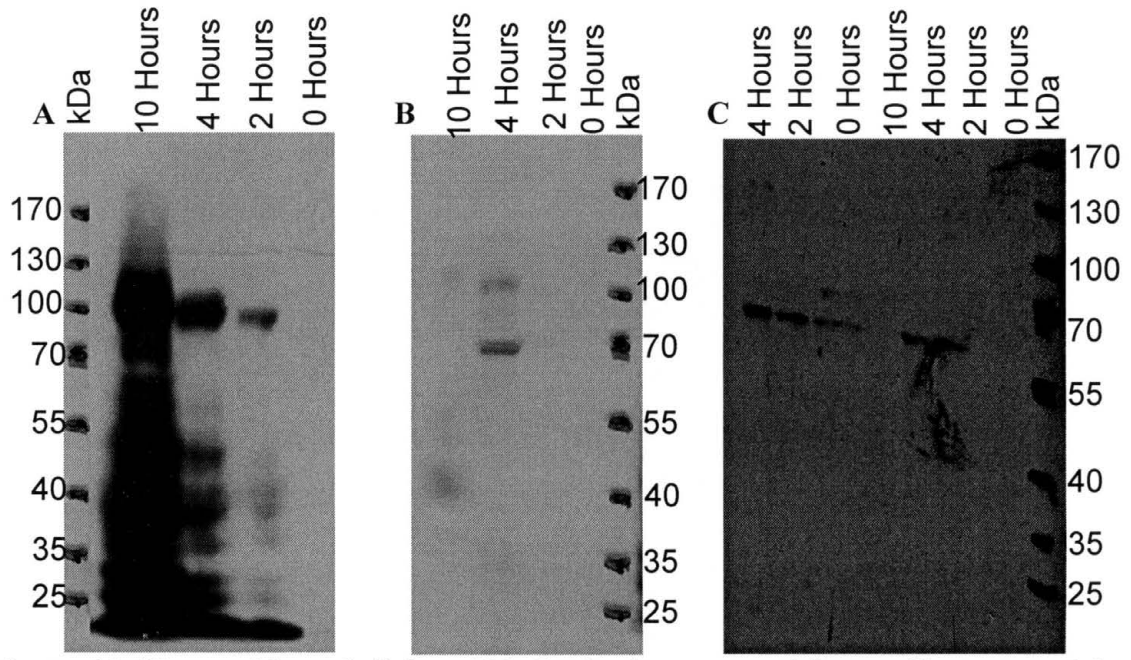


Figure 13- Western blots of all three ENaC subunits expressed from raffinose overnights: Blot A is the expression of the alpha ENaC subunit over a 10 hour time period, probed with Anit-V5 primary antibody, blot B is the beta ENaC subunit, and blot C corresponds to the expression of the gamma ENaC subunit. Both B and C were 10 hour expressions and were probed with anti-Myc primary antibody. Anti-mouse secondary antibody was used for all three of the blots.

Expression of all three ENaC subunits consistently appeared to yield significantly lower yields of protein relative to the LacZ expression control (Figure 14). While all three expression vectors contain the same promoter and replication elements, the alpha subunit is the only one we can directly compare to LacZ. In an effort to optimize alpha ENaC expression, three additional yeast strains, VL6α, BGW1-7a, and S1InsE4A were used to express alpha ENaC using the same expression protocol as the previous strain. After expressing the alpha ENaC subunit in each of the three strains and extracting protein using the standard lysis buffer with EDTA, an SDS-PAGE vertical gel followed by western blot analysis was used to analyze expression. The blot was probed with an anti-V5 primary antibody followed by anti-mouse secondary antibody (Figure 15- A, B,

and C). Of the three new strains being analyzed, S1InsE4A appeared to have the greatest effect on the alpha ENaC yield (Figure 15-A). When compared to previous expression levels of alpha from the original BY4742 strain it also appears that the relative yield of extracted alpha is significantly higher. The difference in the regulation of the Gal promoter between the strains and the processing differences between the strains seems to be, in part, what is responsible for the dramatic differences in expression (28). It is important to point out that these samples were equivalent in every way. They were each normalized to the same starting density as well as probed in the same dishes to ensure that they had equal antibody probes. Therefore, we know that the differences in the level of expression had to occur because of differences among the strains and not by experimental differences.

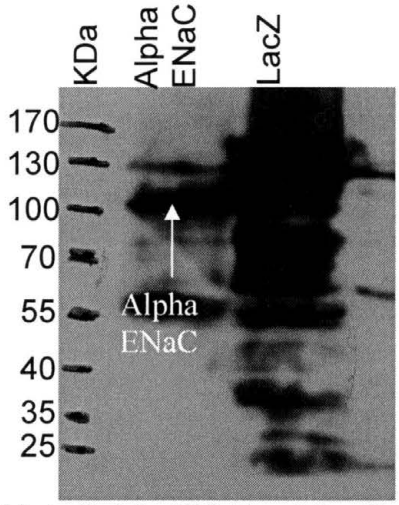


Figure 14- Western blot of alpha ENaC and LacZ expression: The samples were extracted after 12 hours using standard lysis buffer containing EDTA. Blots were probed with anti-V5 primary antibody and anti-mouse secondary antibody.

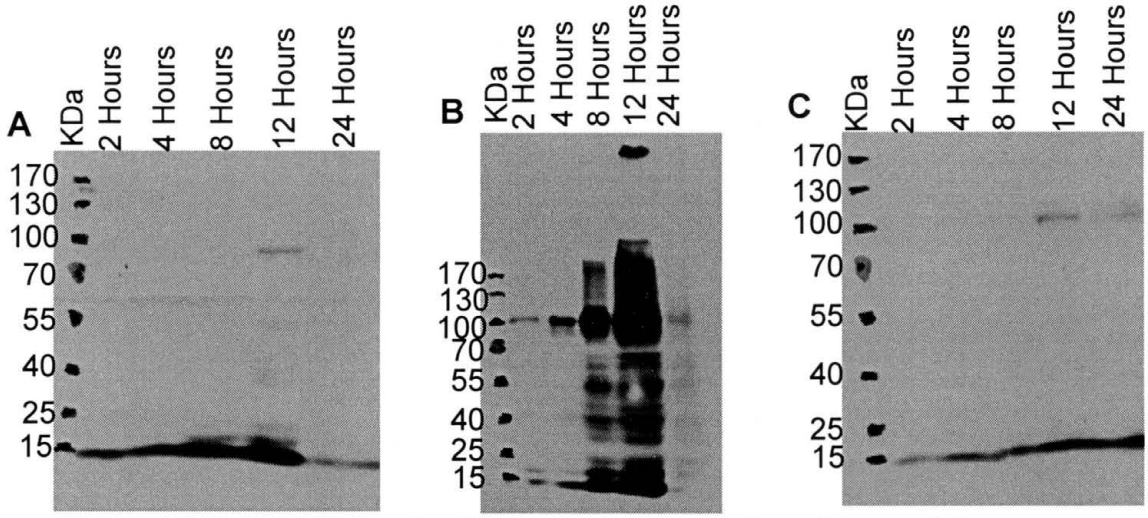


Figure 15- Alpha ENaC Expression in Three Separate Strains of S. cerevisiae: Western blots of alpha ENaC expression in BGW1-7a (A), S1InsE4A (B), and VL6$_\alpha$ (C) over a 12 hour time period. Alpha ENaC was extracted using standard lysis buffer with EDTA and the blots were probed with Ant-V5 primary and anti-mouse secondary antibodies.

A portion of alpha ENaC from S1InsE4A *S. cerevisiae* cells does not enter the gel matrix corresponding to protein remaining in the well of the gel (Figure 15-B). Previous studies in our lab involving ENaC have often reported complexes which do not migrate into the gel but instead stay in the loading well. This could be insoluble protein which was unable to migrate into the gel or alpha ENaC complexes which are too large to enter the gel matrix.

CHAPTER IV

CONCLUSION

Studying ENaC has been difficult for researchers in the past due to the difficulties involved with achieving high yields of purified protein. The limitations of studying ENaC have led to inefficient data about the stoichiometry and assembly of the three homologous subunits alpha, beta, and gamma. To aid in the understanding of ENaC, each of the three genes was cloned into yeast vectors and named Alpha ENaC-pYES2.1-TOPO, Beta ENaC-pRS313-Gal1, and Gamma ENaC-pTCG. These three plasmids were then transformed into *S. cervisiae* cells in an attempt to produce large quantities of each of the three subunits which could be quickly grown and extracted for purification. After extracting protein from the yeast cells it was apparent that the conditions needed to be optimized in the new system. Various sugar sources, expression times, and yeast strains were all tested until the concentration of protein being extracted had reached levels which were close to LacZ (an expression control). When expressing alpha ENaC in yeast we have determined that S1InsE4A *S. cerevisiae* should be used. The ENaC subunits are easily expressed in BY4742 *S. cerevisiae* at an optimal time of 12 hours from glucose overnights, but high levels of protein degradation often occur. When the three subunits are expressed from raffinose overnights, the optimal expression time is 4 hours and protein degradation appears to be significantly less when compared to glucose overnights.

When expressed in yeast, alpha ENaC is observed at approximately 100 kDa, beta ENaC is observed at 100 kDa and as two distinct cleavage products which result in a 70 kDa N-terminal fragment and a 35 kDa N-terminal fragment. Gamma ENaC is observed at 60 kDa, and also as a 28 kDa N-terminal fragment. The proteolytic cleavage by an external protease on the gamma ENaC subunit previously reported in the literature has been reproduced in this study and seems to exist in yeast as well as in other expression systems. The cleavage products can be reduced when the subunits are expressed individually be adding additional protease inhibitors and speeding up expression by growing the cultures in raffinose as opposed to glucose. When the subunits are co-expressed however, the beta ENaC subunit is consistently cleaved and observed as a 35 kDa fragment. This study has shown that it is possible to express ENaC subunits in yeast and under the optimal expression conditions microgram concentrations of protein can be easily and efficiently extracted and purified. Using this expression system, future studies are underway to obtain mass spectrometry data of the subunits which will be used to determine the extent of post-translational modifications and regions of subunit-subunit interactions.

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VITA

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