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**Expressing a Mammalian Signaling Protein in E. coli**

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Chapter 1

Cloning Rhodopsin: An Introduction

1.1 What are GPCRs?

Examining the role and function of G-protein coupled receptors (GPCRs) within the human body is critical. GPCRs are transmembrane proteins (Trabanino et al., 2004) and encompass an estimated 950 genes within the human genome (Fredriksson and Schioth, 2005; Mirzadegan et al., 2003; Takeda et al., 2002). Their importance rests upon their ability to transduce extracellular signals, detect and mediate hormonal and sensory stimuli (Heideman and Bourne, 1990), and participate in electron transfer and ion transport (Trabanino et al., 2004).

1.2 Mechanism of action

GPCRs consist of 7 transmembrane helices; hence, inclusion within the 7-transmembrane receptor family (Peirce et al., 2002; Leftkowitz, 2004). They possess an extracellular N-terminus, 3 extracellular loops, 3 intracellular loops, and an intracellular C-terminus (Salom et al., 2005). The activation of a GPCR occurs from the binding of an extracellular ligand (receptor-specific) to its transmembrane domain. Immediately after ligand binding, the GPCR undergoes a conformational change that allows for the binding of a G-protein
to the intracellular domain of the GPCR, causing the activation of the G-protein and the internal relaying of the extracellular signal. However, various subclasses of GPCRs are highly similar in structure and are affected not only by the agonists and antagonists within their individual subclass, but by the agonists and antagonists of other subclasses as well. This most often results in unwanted side effects. Drug design targeting only one GPCR is frequently encountered with difficulty and frequently results in adverse side effects (Trabanino et al., 2004). Yet, of the presently available therapeutic drugs, approximately 40% of them target GPCRs (Muller, 2000; Sautel et al., 2000).

Properly functioning GPCRs are critical components of normal body function and sound health. Minor mutations in GPCR structures potentially result in diseases such as diabetes and hypertension (mutated in the Glucagon receptor), dwarfism (deficient Growth hormone releasing hormone), obesity (malfunctioning ß-3-Adrenoceptor), and retinitis pigmentosa (mutated Rhodopsin) (Wilson, et al., 1998). Other manifested conditions resulting from deficient GPCRs include: allergies, anxiety, asthma, congestive heart failure, migraines, Parkinson’s, and ulcers (Wilson and Bergsma, 2000).

Fully operative GPCRs are therefore essential in proper conduction of many biological processes and malfunctioning GPCRs are potentially detrimental. Pharmaceutical companies are diligent in their attempts to design therapeutic agents and improve existing ones to mimic the natural agonists and antagonists that are specific in their target of GPCRs. These companies would be more successful in their efforts if the identity and structures of more
GPCRs were known. However, much of what is currently known about GPCR structure was based on X-ray crystallographic measurements of rhodopsin. This was the only GPCR with a known structure until the recent determination of the crystal structure of the human \( \beta_2 \) adrenergic G-protein coupled receptor (Rasmussen et al., 2007).

1.3 Why study rhodopsin?

Rhodopsin is the prototype for GPCRs and therefore widely studied (Menon et al., 2001; Okada et al., 2001; Shichide and Yamashita, 2003). The extensive study conducted on rhodopsin is based on its ability to be highly expressed in eyes, availability and access for electrophysiological manipulations, rapid, quantifiable conversion of its chromophore\(^1\) ligand between inverse states, and its vital role in vision (Ebrey and Koutalos, 2001; Hargrave, 2001; Okada et al., 2001; Okada and Palczewski, 2001; Filipek et al., 2003(b)). The crystallized structure of rhodopsin is invaluable to current information collected on GPCRs. Earliest crystallized structures detailed the helical arrangements, interhelical interactions, cytoplasmic helix 8, chromophore binding site, and extracellular ligand binding interactions in other types of GPCRs (Palczewski et al., 2000). The advent of high resolution diffracted rhodopsin crystals provided further in-depth analysis on the cytoplasmic loops and water molecule interactions of the chromophore (Li et al., 2004; Okada et al., 2004).

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\(^1\) A chromophore is a pigmented molecule that exhibits color due to its absorption of specific wavelengths of light. The chromophore in rhodopsin consists of the covalent interactions of the protein opsin and retinal.
1.4 What is rhodopsin?

Rhodopsin, an extremely light-sensitive membrane protein, is classifiable as photosensitive (Salom et al., 2005). Rhodopsin is composed of a retinal-protein complex, where retinal is covalently bonded to opsin (Wolken, 1995). Formation of the covalent bond occurs when an aldehyde group within retinal bonds to a lysine residue (ε-amino acid) in opsin, creating a protonated Schiff base. As rhodopsin absorbs a photon of light, 11-cis retinal is photoisomerized to the all-trans form, generating the active form.

Figure-1.1 Structures of 11-cis and all-trans retinal.

Further, rhodopsin and retinal are universally found in all animal retinas. Even unicellular organisms, such as algae, halophilic bacteria, and protozoa utilize rhodopsins which function as light detectors for phototactic movement rather than visual excitation as in animals (Wolken, 1995).
In its inactive form rhodopsin exhibits red to purple hues and absorbs light at 500 nm. Contrarily, when it is activated by light, it bleaches to a white color and absorbs light at 370 nm (Wolken, 1995). Photobleaching, fortunately, is avoidable by conducting rhodopsin experiments in the dark (Salom et al., 2005).

In previous procedures, rhodopsin has been extracted from fresh bovine retinas (Salom et al., 2005; Tachibanaki, et al., 1998). By expressing bovine rhodopsin in Escherichia coli (E. coli) (or other unicellular organisms) through cloning, extraction of the protein is cheaper, less complex, and advantageous.

1.5 Importance of cloning

The fields of biology, medicine, and biotechnology have been revolutionized through the employment of recombinant DNA technology with gene cloning as its central component. As a vital tool in biology, gene cloning conveniently isolates a desired gene, further allowing its assessment. Gene cloning has also provided a way for mammalian proteins and genes to be inserted and produced in bacterial cells. Cloned genes are frequently manipulated and made to exist and function in an organism in which it is not naturally found (Brown, 1990). Nevertheless, gene cloning has significantly impacted biological research and biotechnology, and the information obtained from the structure and function of a cloned gene is very valuable (Brown, 1990).
1.6 What is gene cloning?

The process of gene cloning can best be conveyed in a series of basic steps. In order for the cloning process to begin, two essential components are needed, purified DNA from the cloning vehicle and purified DNA from the prospective gene to be cloned.

1. The prospective gene to be cloned is inserted into a vector, or a circular piece of DNA. The cloned gene plus the vector is a product called recombinant DNA.

2. The vector’s primary function is to transport the gene to be cloned into a host cell, which is usually a bacterium. So in essence, the vector acts as a vehicle. In the experiment, \textit{E.coli} cells are used.

3. When in the host cell, the vector multiplies and produces identical copies of itself along with the inserted gene.

4. As the host cell divides, copies of the vector containing the cloned gene are passed on to its progeny.

5. The host cell continuously divides until it forms a colony – a collection of cells, identical to the host, that all contain the recombinant DNA molecule.

The single most important aspect of the gene cloning process is the method by which a recombinant DNA molecule is transported and integrated into the host cell. In order for a vector to perform its function as a vehicle of cloning, it must possess two key features: it must be readily and easily able to enter the host cell, and it must be self-replicating or able to produce multiple
copies of itself. Fortunately, two types of DNA, plasmids and bacteriophages, are natural cloning vehicles that exist to facilitate the cloning process. Plasmids are small, circular extrachromosomal pieces of DNA that can replicate independently of the host cell’s chromosome. Bacteriophages, on the other hand, are viral chromosomes that invade and infect bacteria. (Brown, 1990).

The Cloning Process

Figure 1.2 summarizes the gene cloning process. Figure is adopted from (Brown, 1990).
1.7 Problems with cloning

Cloning a foreign gene into *E.coli*, specifically a mammalian protein, is not without complications. Difficulties often ensue with large-scale expression of protein from these cloned genes. Problems found on the transcription and translation levels, whether in between or after both stages, are at the cellular level.

1. One common problem arising during the transcription phase is the presence of a sequence within the foreign gene that performs as a termination sequence within *E. coli*. The aforementioned sequence is innocuous within the native host cell; nevertheless, it causes an untimely loss of gene expression in *E.coli*.

2. Between transcription and translation one major problem is the presence of introns in the non-native gene. The removal of introns must occur before the non-native gene can be cloned into *E.coli* because the bacteria are incapable of excising the introns.

3. Problems surfacing at the translation phase are complex and surround the use of codons. Even though the same genetic code is employed by almost all living creatures, some organisms exhibit a preference for select codons. Transfer RNAs (tRNAs) preference for the use of certain codons in *E.coli* may cause an over abundance of that specific tRNA and relatively little amounts of various types of tRNAs for decoding the infrequent codons. If the cloned gene has a relatively large amount of unfavorable codons (in relation to *E.coli*) then the
translation phase in *E.coli* may be thwarted by low levels of the infrequent tRNAs.

4. Immediately after translation, the cloned gene product may be subjected to rapid degradation. The use of a mutant *E.coli* strain (which lacks many proteases) smartly avoids the degradation of the cloned gene product. An additional problem that may surface after the completion of translation involves processing. In most organisms, sugar groups are added to proteins (specifically those from animals) after translation, and addition of these functional groups aids normal protein functioning. *E.coli*, however, will not add the sugar groups to the recombinant proteins, which results in the formation of a non-functional protein.

5. At the cellular level, a cloned mammalian gene may be harmful to the host cell. Even when not toxic to the bacterial cells, fast expression of the recombinant protein is thought to cause delayed growth and cellular instability, especially when compared to an *E.coli* cell lacking the recombinant gene (Brown, 1990).

1.8 Summary

Cloning a mammalian gene into bacterial cells is a huge undertaking as uncertainties lie in the ability of the cloned gene to be expressed. Despite problems associated with mammalian gene cloning, an optimistic outlook

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2 Proteases are enzymes that solely function to degrade proteins into their constituent peptides.
should still be employed because not all recombinant genes (when expressed in *E. coli*) fail to produce insufficient amounts of recombinant proteins. This is reason enough to believe the cloning of bovine rhodopsin into *E. coli* will work.

In the current experiment, the bovine rhodopsin protein, a mammalian gene, has been cloned into a non-native *E. coli* strain UT5600. The main goal is to assess the capability of the bovine rhodopsin gene, a GPCR, to be expressed as a functional protein in bacterial cells.
Chapter 2
Methodology

Before the cloning process can ensue, two essential components are needed, the cloning vehicle and purified DNA from the prospective gene to be cloned. The pBAD TOPO vector was used as a cloning vehicle in the experiment, and the prospective gene to be cloned was the Bovine rhodopsin gene.

2.1 Cloning Vector

The pBAD TOPO TA Expression kit purchased from Invitrogen was used to conduct the cloning process. The pBAD TOPO plasmid contains special properties making it a suitable, efficient cloning vehicle. These two properties are: 1). A topoisomerase that is already bound to the pBAD TOPO vector and 2). A solitary 3’-thymidine (T) overhang on both ends of the vector. (Thymidine or deoxythymidine is one of the 4 bases found in DNA that pairs with deoxyadenosine (A).)

The 3’-(T) overhang’s principal purpose is to bind to the PCR product (the prospective gene to be cloned) which contains (A) overhangs, thus constituting the self-ligating properties of the pBAD TOPO vector. Consequently, the use of a ligase to bind the PCR product to the pBAD TOPO
vector is unnecessary. A Taq polymerase (which will be subsequently discussed and also supplied by the pBAD TOPO TA Expression kit) is responsible for adding a single (A) to either ends of the PCR product.

Functions of the Vaccinia Topoisomerase 1

Bound to the pBAD TOPO vector is a topoisomerase 1\(^3\) derived from the Vaccinia virus. The Vaccinia topoisomerase 1 is 314 amino acids in length (Shuman and Moss, 1987), and its primary function is to bind to duplex DNA and cleave the phosphodiester backbone of one strand (Shuman, 1994). The extreme sequence specificity of Vaccinia topoisomerase 1 allows it to recognize and cleave DNA at the 5’ CCCTT in the scissile strand (Shuman and Prescott, 1990; Shuman and Prescott, 1991a. 1991b). Also, it is an especially useful tool because it operates as a site-specific restriction endonuclease, which selectively cleaves DNA at highly specific sites; however, it also religates DNA strands with great proficiency. Restriction endonucleases have been traditionally used to cleave DNA at specific sites to produce linear DNA that would then be religated at their ends with DNA ligase. With the advent of Vaccinia topoisomerase 1, what was once a two-step method for inserting a non-native gene into a vector is now one single step. Furthermore, this single-step method is used in vitro to label DNA ends and to clone DNA molecules (Shuman, S., 1994). Moreover, Invitrogen has utilized the specificity of and the discriminatory power in end ligation of Vaccinia topoisomerase 1 and incorporated it into the pBAD TOPO\(^\circ\) vector.

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\(^3\) A topoisomerase is an enzyme that functions to alter and manipulate DNA structure whether by purposefully introducing supercoils or contrastingly, relieving supercoils in the conformation of DNA (Brown, 1990).
2.2 Properties of PCR

An exciting moment in scientific history occurred during 1985-1986, when researchers at Cetus Corporation, USA, established a revolutionary in vitro technique for amplifying DNA fragments. This process is called the polymerase chain reaction, but is more commonly referred to as PCR (Chawla, 2000).

PCR is especially useful because it quickly and easily amplifies a minute amount of DNA. Amplification of a DNA sequence in PCR occurs in 3 main steps.

1. The double stranded DNA template is heated to 94 degrees Celsius to achieve denaturation.

2. Synthetic DNA primers anneal to the target sequences on either side of the DNA region to be amplified. This process is carried out at 35-65 degrees Celsius.

3. DNA synthesis of the targeted region occurs from the 3’ hydroxyl end of each DNA primer by Taq polymerase. This step occurs in repetitive cycles, and the primer extension products of antecedent cycles serve as a new template for subsequent cycles. Hence, the number of targeted DNA copies may double each cycle, hence, the term amplification.

It should be noted that a minute amount of Taq polymerase, approximately 1 unit (but no more than 2 units) is need to carry out PCR, as an excess may cause the amplification of non-target sequences (Chawla, 2000).
Comprehending the central components of PCR is vital for further understanding its complexity. PCR is best conveyed when broken into its central components. Three significant components of PCR include:

1. Known nucleotide sequences, at both ends of the selected DNA region to be amplified, allow for DNA primers (both forward and reverse) to anneal to the targeted DNA molecule.

2. The sole function of DNA primers is to demarcate the region of DNA that will be enzymatically amplified. DNA primers are also known as oligonucleotides, which are short, synthesized sequences of DNA or RNA that are typically 25 nucleotides or fewer in length.

3. Taq polymerase, or DNA polymerase 1 from Thermus aquaticus⁴, is necessary for the amplification of the selected DNA region. Taq polymerase, a thermostable enzyme discovered by Kary Mullis, ably endures denaturation in environments up to 94 degrees Celsius. The Klenow fragment, obtained from DNA polymerase 1 in E. coli, was initially used to execute PCR. While functional and structural properties of Taq polymerase resembled those of DNA polymerase 1 of E. coli (Joyce and Grindley, 1983), DNA polymerase 1 was ill-suited for the denaturation cycle in PCR.

Other important components included in PCR are the amplification buffer, which typically contains KCl, Tris-chloride, and MgCl₂, and deoxyribonucleoside triphosphates (DNTPs) at a concentration of 200 µM (Chawla, 2000).

⁴Thermus aquaticus is a bacterium native to hot springs.
Additional Functions of *Taq* Polymerase

*Taq* polymerase functions to replace nucleosides in DNA repair and also remove RNA primers during the processing of Okazaki fragments (Kernberg and Baker, 1992). In nucleotide editing reactions, two prominent domains allow *Taq* polymerase to exhibit dual functions. The N-terminal domain has 5’-nuclease activity which cleaves RNA primers or defective DNA strands, and the C-terminal domain has DNA polymerase activity which synthesizes new DNA strands (Ho, D. et al., 2004). One limitation to the function of Taq polymerase is its lack of 3’ to 5’ exonuclease activity. It is thereby unable to proofread and remove mismatched bases making it slightly less efficient than other naturally existing polymerases. Fortunately, other thermostable enzymes with proofreading capabilities have been isolated (Chawla, 2000). In summary, the nuclease and polymerase activity of Taq polymerase has been utilized by Invitrogen to allow the non-template transfer of a solitary (A) to each 3’ end of the PCR product.

2.3 PCR Product

Complementary DNA (cDNA) is a single-stranded DNA transcript synthesized from an mRNA template, and its synthesis is catalyzed by reverse transcriptase. Once the cDNA transcript is produced, the second strand of cDNA is made from DNA polymerase 1. Formation of the double-stranded cDNA may then be fused into a vector and cloned (Brown, 1990).
Genomic DNA of cells containing cloned cDNA of bovine rhodopsin was used as a PCR template in my experiment. The PCR product, cDNA of bovine rhodopsin, was contributed by Jeremy Nathans, et al. (1989). In addition, Nathans et al. sub-cloned the cDNA encoding bovine rhodopsin and the protein was expressed in human cells. When treated in vitro with 11-cis retinal, these cells containing the cloned cDNA of bovine rhodopsin generated a red pigment with an absorbance identical to the original bovine rhodopsin (Nathans, J. et al., 1989).

I used PCR to amplify the cDNA of bovine rhodopsin and to add new restriction enzyme sites using the template provided by Nathans. The PCR product was then treated with Taq polymerase provided by the Invitrogen pBAD TOPO® cloning system, in order to add (A) overhangs required to ligate the PCR product to the 3’ (T) overhangs within the pBAD TOPO vector.

As previously mentioned, DNA primers are needed to mark or isolate the region of DNA that will be enzymatically amplified. The following DNA primers, as shown in Figure-2.1, were designed and purchased for the PCR product. The forward primer begins with an in-frame stop codon (TAG) and is subsequently followed by the ribosome binding site (AGGA) which functions as a translation re-initiation sequence. The ATG start codon of the bovine rhodopsin cDNA follows the ribosome binding site 7 nucleotides later.
This construction of the rhodopsin polypeptide sequence permits expression from the arabinose (araBAD) promoter without the enterokinase leader sequence. Devoid of the protective space provided by the 6 nucleotides (between the ribosome binding site (AGGA) and the start codon (ATG)), the bacterial cells would be incapable of restarting translation. Additionally, restriction endonucleases, BamHI and NdeI, were introduced into the forward primer. HindIII, also a restriction endonuclease, was introduced in the reverse primer as well. To summarize, the purpose of the introduced restriction enzyme sites was to provide sites where N- or C-terminal modifications of the rhodopsin sequence could be inserted.

2.4 TOPO® Cloning Reaction

With the presence of the cloning vehicle (pBAD TOPO® vector) and PCR product from the prospective gene to be cloned (cDNA encoding bovine rhodopsin), the cloning process was executed.
1. In sequential order: 1 microliter (µl) of the PCR product containing the cDNA of the bovine rhodopsin protein, 1 µl of salt solution (concentration of 1.2 M NaCl and 0.06 M MgCl$_2$), 3 µl of sterile water, and 1 µl of the pBAD TOPO® vector (for a final volume of 6 µl) were added to a sterile 1.5 milliliter (ml) test tube. The reagents were mixed gently and allowed to rest undisturbed at room temperature (21-23 °C) for a total incubation time of 5 minutes. The (A) overhangs within the PCR product easily allowed its ligation to the (T) overhangs in the pBAD TOPO® vector, constituting the complete insertion of the PCR product into the plasmid.

2. The cloning reaction (from the previous step) was placed on crushed ice after 5 minutes. Once chilled, 1 µl of the cloning reaction was added to a microcentrifuge tube containing 50 µl of thawed (on ice), competent cells E.coli cells strain XL-1 blue (Stratagene). Competent cells are especially efficient at DNA re-uptake. It is believed that MgCl$_2$ (or another salt) causes the DNA to precipitate and adhere to the outside of the E.coli cell walls. When the E.coli cells are chilled and immersed in cold salt-solution, they are more proficient in DNA uptake than untreated cells. However, the DNA does not enter the treated cells, but instead, remains attached to the exterior of the cell walls. Only when the chilled cells are shocked or quickly heated to 42°C does the DNA enter the competent cells (Brown, 1990).
3. The tube of competent *E. coli* cells were placed in a hot water bath (stabilized to 42°C) and heat-shocked for 30 seconds. Immediately, the vial of cells were transferred to ice and incubated between 10-15 minutes.

4. 250 µl of room temperature SOC medium (containing 2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, and 20 mM glucose) was added to the competent cells.

5. The vial of cells was incubated at 37°C for 1 hour in a non-shaking incubator.

6. 100 µl of the cells were added and evenly spread on a room temperature LB-ampicillin plates and incubated (in a non-shaking incubator) at 37°C overnight.

2.5 Selection and growth of colonies

A single colony resulted from the TOPO® Cloning reaction. This was inoculated in a capped, sterile 10 ml test tube containing 2 ml of sterile LB media (per liter: 10g tryptone, 5g yeast extract, 10g NaCl, and distilled H₂O, adjusted to a pH of 7.0 using NaOH) and 2 µl of filter-sterilized ampicillin (50 mg/ml). The tube was incubated for 16 hours at 37°C in a non-shaking incubator.

The following day, 1 ml of the cells and 40 µl of ampicillin (50 mg/ml) were added to a 125 ml flask of 40 ml of sterile LB media and grown in a shaker bath overnight at 37°C.
2.6 DNA Purification of the cloned cells

Plasmid DNA from the cloned cells was purified using Wizard® Plus SV Minipreps DNA Purification System.

Harvesting of Cells

1. 1.5 ml (from the 40 ml of cells) was added to a sterile 1.5 ml microcentrifuge tube and centrifuged at 10,000 rpm for 5 minutes.
2. The supernatant was decanted and excess liquid was removed by inverting the tube and gently blotting it on a kimwipe.

Purification

1. 250 µl of Cell Resuspension solution (50 mM Tris-HCl at pH 7.5, 10 mM EDTA, and 100 µg/ml RNase A) was added to the pelleted cells. By gently pipetting the solution, the pellet was thoroughly resuspended.
2. 250 µl of Cell Lysis solution (0.2 M NaOH, 1% SDS) was added to the tube of cells. Cell lysis solution lyses open the cells allowing the DNA (and inside contents) to enter solution. The tube was inverted 4 times and incubated at room temperature until the lysate cleared. (The solution takes on a slimy, viscous consistency).
3. Although not a crucial step, 10 µl of Alkaline Protease solution was added to the tube and inverted 4 times. The sole purpose of the protease solution is to deactivate endonucleases and other degrading enzymes released into the solution during cell lysis. These proteins may degrade the quality of DNA by nicking portions.
4. 350 µl of Neutralization solution (4.09M guanidine hydrochloride, 0.759M potassium acetate, and 2.12M glacial acetic acid at a pH of 4.2) was added to the test tube. Sufficient mixing of the contents was done by inverting the tube 4 times. The acidity of the Neutralization solution restabilizes the pH to neutral, as too basic a pH damages DNA by denaturation.

5. The test tube was centrifuged at 10,000 rpm for 10 minutes at room temperature\(^5\) to separate the soluble products (DNA) from the solid bacterial matter.

Centrifuging the lysate

1. Approximately 850 µl of the cleared lysate was extracted from the white precipitate (carefully avoiding the transfer of the precipitate) and transferred to a spin column (already inserted in the 2 ml collection tube).

2. The spin column and its contents were centrifuged at 10,000 rpm for 1 minute and the flow-through was discarded. At this point, the DNA binds to the spin column, and all the soluble, excess materials (e.g., junk DNA and soluble proteins) are removed in the filtrate.

3. 750 µl of Column Wash solution\(^6\) (previously diluted with 35 ml of 95% ethanol) was added to the spin column and centrifuged at 10,000 rpm for 1 minute. The flow-through was subsequently discarded.

Ethanol precipitates the DNA, which continues to adhere to the spin

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\(^5\) The entire DNA purification process was carried out at room temperature.

\(^6\) The Column wash solution contains 60% ethanol, 60 mM potassium acetate, 8.3 mM Tris-HCl, 0.04 mM EDTA).
column. Any trace amounts of residual proteins are removed in the flow-through.

4. 250 µl of Column wash solution was added to the spin column and centrifuged for 2 minutes.

5. The spin column was removed, blotted with a Kimwipe to remove excess column wash solution and transferred to a sterile 1.5 ml microcentrifuge tube.

6. 60 µl of Nuclease free water was added to the spin column. The tube was centrifuged for 1 minute at 10,000 rpm for 1 minute and stored in the freezer at -20˚C. Water resolubilizes the DNA, permitting easy passage through the spin column.

2.7 Analysis of the Plasmid DNA

The orientation of the bovine rhodopsin gene within the pBAD TOPO plasmid was analyzed by restriction endonuclease (restriction enzyme) treatment and agarose gel electrophoresis. By performing enzymatic digests on the DNA sample, the generation of DNA fragments of varying sizes directly resulted from the location of the restriction enzyme sites in the DNA sample. An agarose gel aided in the process of separating the DNA fragments and created a visual depiction of the gene’s orientation.

Restriction endonuclease digests

The purified plasmid DNA was treated with restriction endonucleases, NcoI, BamHI, and HindIII. Single digests and double digests were
performed. For the single digests: 5 µl of the DNA sample, 1 µl of 10x Buffer, and 1 µl of the restriction endonuclease (totaling 7 µl) were added to a sterile 0.6 ml microcentrifuge tube. For the double digests: 5 µl of the DNA sample, 2 µl of 10x buffer, and a total of 2 µl of restriction endonucleases (totaling 9 µl) were added to a 0.6 ml microcentrifuge tubes. Three double digests were made in the following combinations: NcoI and HindIII, NcoI and BamHI, and BamHI and HindIII. All samples were incubated in a non-shaking incubator for 1 hour at 37˚C.

Gel preparation

A 0.8% agarose gel was made using 0.4g of agarose\textsuperscript{7} 10 ml of 5x TBE buffer (made from 2.7g Tris base, 1.38g boric acid, and 1 ml EDTA in 50 ml of distilled H\textsubscript{2}O), and 40 ml of distilled H\textsubscript{2}O. The ingredients were added to a 125 ml flask and heated until the agarose dissolved. When the solution roughly cooled to 40˚C, 3 drops of ethidium bromide stock solution (0.5 g/ml) was added. The solution was poured in a gel-casting mold and allowed to solidify. After solidification, the gel was loaded in a horizontal gel box and 200 ml of diluted 5x TBE solution (40 ml of buffer in 160 ml of distilled H\textsubscript{2}O) to which 3 drops of ethidium bromide were added.

Running samples on the Gel

1. 2 µl of gel loading buffer (with tracking dye) were added to each of the 3 single digests and 3 double digests.

\textsuperscript{7} The agarose was made by Fisher BioReagents and has a high melting temperature suitable for separation of large DNA molecules.
2. 5 µl of a DNA ladder\(^8\) (Gene choice molecular weight standards), 9 µl of each of the single digests and 10 µl of each of the double digests were added to the wells of the gel.

3. The gel ran between 30 and 45 minutes in duration at 150 volts.

4. A UV-trans-illuminator/camera Gel Doc (Kodak) provided an image of the gel, making analysis possible.

2.8 Transformation

After determining the orientation of the bovine rhodopsin gene within the pBAD TOPO plasmid, E.coli cells from strain UT5600 were transformed. As an advantageous method, transformation promotes the acquisition and integration of extrachromosomal DNA into a bacterial cell’s chromosome. While many bacterial strains are adept in DNA uptake from their environments, transformation is not entirely efficient primarily because of DNA degradation\(^9\). Easy distinction between cells with successful transformation and those lacking the plasmid DNA involves the expression of specific genes by the plasmid DNA. The presence of an ampicillin resistance gene within the plasmid markedly distinguishes the transformed cells from the non-transformed cells. Antibiotic resistant genes are transferred to the host cell in cloning reactions. Cells that lack the ampicillin-resistant gene are unable to grow on agar plates containing ampicillin (Brown, 1990).

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\(^8\) The DNA ladder determines the size of DNA molecules ranging from 200 to 12,000 base pairs in length. 5 µl of the ladder contains 1% agarose in 1x TAE buffer stained with ethidium bromide.

\(^9\) Uptaken DNA often experiences degradation by the host cell.
Procedure

Transformation of *E.coli* cells from strain UT5600 was completed using 1 µl, 2 µl, and 5 µl of the purified recombinant plasmid DNA (pBAD TOPO + bovine rhodopsin).

1. Three, 1.5 ml microcentrifuge tubes containing 50 ml aliquots of the *E.coli* cells (stored -70°C) were thawed in crushed ice.
2. 1 µl, 2 µl, and 5 µl of the purified plasmid DNA were added to the tubes. The contents of the tubes were mixed with gentle stirring of the pipette tip.
3. The tubes were incubated on ice for 5 minutes.
4. Cells were heat shocked at 42°C in a water bath for 45 seconds.
5. Immediately after heat shocking, the tubes were placed on ice.
6. 200 µl of sterile LB media without ampicillin was added to each of the 3 tubes.
7. The tubes were incubated in a non-shaking incubator at 37 °C for 1 hour.
8. 100 µl of cells were taken from each of the 3 tubes and evenly spread onto LB plates containing ampicillin and incubated at 37 °C for 24 hours.

2.9 Protein Expression

Protein expression occurs in two stages, with the first being transcription and the second, translation. A brief examination of transcription
(with particular emphasis on initiation) sufficiently details the ability of the pBAD TOPO plasmid to express the bovine rhodopsin protein.

Transcription\(^\text{10}\) is the process whereby an RNA transcript is synthesized from a DNA template. Two important sub-phases within transcription are initiation and elongation.

1. In the initiation phase (most crucial phase), RNA polymerase must detect and bind to the promoter\(^\text{11}\) in order to initiate transcription. The promoter ultimately controls the expression of a gene. For the bovine rhodopsin protein to be expressed, RNA polymerase must first bind to the pBAD promoter (which will subsequently be discussed).

2. In the elongation phase, RNA polymerase reads the template DNA strand in the 5’ to 3’ direction while simultaneously adding the complementary RNA bases\(^\text{12}\). RNA polymerase produces an RNA transcript complementary to the DNA strand encoding the bovine rhodopsin gene. The RNA transcript is later translated into the bovine rhodopsin protein (Chawla, 2000).

Promoter control of protein expression

The pBAD (araBAD) promoter permits the expression of the bovine rhodopsin protein. Within the pBAD TOPO plasmid, the araC gene lies adjacent to the pBAD promoter (Wilcox et al., 1974) and positively and negatively regulates the pBAD promoter (Carra and Schleif, 1993; Lobell and

\(^{10}\) Transcription, one of two steps (the other being translation) in protein synthesis, is catalyzed by RNA polymerase.

\(^{11}\) A promoter is a short DNA sequence located immediately upstream of the gene to be transcribed.

\(^{12}\) The four RNA bases are uracil (U), guanine (G), cytosine (C), and adenine (A).
Schleif, 1990). The presence of arabinose turns on transcription from the pBAD promoter. Conversely, the opposite is true in the absence of L-arabinose, as the pBAD promoter is suppressed by the araC gene resulting in minimal transcription levels (Lee, 1980; Lee et al., 1987). To summarize, the pBAD promoter, incorporated in the pBAD TOPO plasmid, functions to regulate heterologous gene expression of bovine rhodopsin in the *E. coli* cells (Guzman et al., 1995).

Procedure

1. A colony was selected from a transformation plate\textsuperscript{13} and inoculated into 2 ml of LB media and 2 µl of ampicillin (50 mg/ml) at 37 °C in a non-shaking incubator for 16 hours.

2. The 2 ml overnight culture was added to a sterile 1 L flask containing 350 ml of fresh, enriched LB media (per liter: 10g tryptone, 5g NaCl, 5g yeast extract, 5g NaHPO\(_4\) (anhydrous) and 0.5% glycerol adjusted to pH 7.0) and 350 µl of ampicillin. As the cells grow and consume glycerol in the LB media, they produce wastes that decrease the pH of the solution. Fortunately, the phosphate buffer (HPO\(_4^{2-}\)/H\(_2\)PO\(_4\)) prevents a drastic lowering of pH that would otherwise occur.

3. The flask was shaken at 200 rpm at 37°C and the optical density of the culture was taken every 45 minutes to monitor cell growth.

\textsuperscript{13}The plate was from the transformation reaction conducted with 5 µl of the purified plasmid DNA and E. coli cells strain UT5600.
After 4.5 hours, 3.5 ml of a 20% arabinose solution (made from 2g of L-arabinose in 10 ml of distilled water) and approximately 2-5 µl of retinal stock solution (~50 µM) was added to the cells.

4. The heat of the shaker bath was turned off\textsuperscript{14} and the cells were allowed to grow overnight at room temperature.

5. The color of the cells was noted the following day.

Cell lysis

1. The 350 ml culture of cells was equally divided into 175 ml aliquots and added to two 400 ml centrifuge tubes.

2. The cells were centrifuged at 4,000 rpm for 30 minutes at 4°C.

3. The supernatant was removed and 5 ml of cell lysis solution (in 20 ml: 10 mM HEPES at pH 7.1, 1 mM PMSF protease inhibitor, 3% octyl glucoside, 0.08% lysozyme, and 0.01 mg/ml DNAse) was added to one of the centrifuge tubes (the other was stored at -20°C).

4. On ice, the cells were thoroughly resuspended with continuous gentle stirring and the lysate was transferred to a sterile, capped 50 ml centrifuge tube.

5. The tube was incubated at 4°C for approximately 70 hours.

6. The lysate was centrifuged at 4,000 rpm for 20 minutes at 4°C. The supernatant was removed and used for spectral analysis.

\textsuperscript{14} Turning off the heat allows a gradual cooling of cells; otherwise overgrowth and minimal protein expression would occur.
Chapter 3

Results and Conclusions

3.1 Cloning Reaction

With the appearance of a single colony, the probability of selecting a colony possessing the correct orientation\textsuperscript{15} of the bovine rhodopsin gene was only a 50% chance. As luck would have it, it was not necessary to repeat the cloning steps to obtain additional colonies, since the correct orientation was obtained.

![Culture of cells from the only resulting colony of the cloning reaction.](image)

3.2 Restriction Enzyme Digests

A restriction enzyme digest was performed on the purified plasmid DNA containing the bovine rhodopsin gene in order to assess the orientation of the gene. Restriction endonucleases recognize and cleave DNA at specific sequences. NcoI recognizes the DNA sequence, 5’ C/CATGG 3’ and cleaves

\textsuperscript{15} It was possible for the bovine rhodopsin gene to be inserted into the pBAD TOPO plasmid in either the forward or reverse direction. The correct orientation, and thus highly desired, is the forward direction.
between the two cytosines (C). BamHI recognizes the 5’ G/GATCC 3’ sequence and cleaves between the two guanidines (G). HindIII recognizes the sequence, 5’ A/AGCTT 3’ and cleaves between the two adenosines (A).

Depending on the location of the location of the restriction enzyme sites in a DNA sequence, performing a digest on a DNA sample will generate fragments of varying sizes, indicating the direction or size of your DNA product.

The purified plasmid recombinant DNA is approximately 5.2K base pairs. As shown in Fig 3.2, capital letters represent the nucleotides sequence in the pBAD TOPO plasmid and lower case letters represent the bovine rhodopsin gene (id: NM_00104890). Highlighted in green, red, and blue are the restriction enzyme sites of BamHI, NcoI, and HindIII respectively.

NcoI has two sites in the recombinant plasmid DNA. The first is 342 base pairs from the first nucleotide and the second is 498 base pairs from the first nucleotide. Counting from the bottom, the first NcoI site is 4,696 base pairs from the last nucleotide and the second site is 4,582 base pairs from the last. BamHI also has two three in the recombinant plasmid DNA. The first site is 237 base pairs from the first nucleotide, the second is 353 base pairs from the first nucleotide, and the third site begins at 390 base pairs from the first nucleotide. Beginning at the bottom, the first site is approximately 4,957 base pairs from the last nucleotide, the second site is 4,841, and the third is 4,804 base pairs from the last nucleotide. Hind 3 has only one site in the
recombinant plasmid DNA. It is 1,451 nucleotides from the first nucleotide and 3,743 base pairs from the last nucleotide.

Figure-3.2 Sequence of the pBAD TOPO plasmid (obtained from the Invitrogen website) with the inserted bovine rhodopsin gene. The DNA molecule is 5,202 base pairs in length and highlighted in blue, green, red, and blue are the restriction enzyme sites.
Figure 3.3 Restriction enzyme digest performed on the purified plasmid DNA sample.

Single and double digests were performed on the purified plasmid DNA containing the bovine rhodopsin gene was treated with restriction enzymes. It is possible that the position of the cut sites have shifted and this may be to supercoiled DNA.

The first lane from the left is the DNA ladder. The second lane shows purified DNA from the pBAD TOPO with the added bovine rhodopsin gene. The uncut, supercoiled plasmid in lane 2 matches with the 4,000 base pair (bp) fragment, but is actually expected to have a total length of 5,000 bp. The third lane shows the NcoI cut sites with 2 two bands in very close proximity between the 6,000 and 5,000 bp fragments. The fourth lane shows the BamHI
cut sites with the first band appearing between the 8,000 and 7,000 bp fragments and the second appearing between the 6,000 and 5,000 bp fragments. The fifth lane shows HindIII which only has one cut site, giving a clean band around the 5,000 base pair fragment. In lane 6, the NcoI sites (around 4,500 bp on the gel) and the HindIII (around 1,200 bp on gel) are in close proximity to their correct positions and should be far apart from one another. In lane 7, appears to have one band, but the NcoI and BamHI sites are positioned close to one another in the actual DNA sequence. In lane 8, the BamH1 and the HindIII cut sites are in the correct places at 5,000 bp and around 1,400 base pairs respectively. If the bovine rhodopsin gene was inserted in the incorrect direction, a strong 1,400 bp fragment should have been visible in lanes 3, 4, and 7. Moreover, the agarose gel displaying the digested DNA sample indicates the correct insertion of the bovine rhodopsin plasmid.

3.3 DNA Sequence

The purified plasmid DNA (concentration of 129 nanograms per microliter) and the pBAD forward sequencing primer (concentration of 0.1 µg/µl in TE Buffer) was diluted to the required 1 pM/µl and sent to the SUNY DNA Sequencing Core Facility for sequencing. In Figure-3.4, generated by BioEdit version 7.0, the adenines are labeled in green font, cytosines are labeled in blue font, guanidines are labeled in black font, and thymidines are
labeled in red font. In addition, nucleotides labeled ‘N’, are of unknown identity and can be one of the four DNA bases.

![DNA Sequence Image](image)

Figure-3.4 Fluorescent DNA sequence of the purified plasmid DNA was obtained from the SUNY DNA Sequencing Core Facility at SUNY Upstate Medical University and generated by the BioEdit (version 7) program.

Additionally, the DNA sequence obtained from the SUNY Sequencing Core Facility demonstrates the correct orientation of the bovine rhodopsin.
gene within the pBAD TOPO plasmid. Had the gene been inserted in the reverse direction, the generated DNA sequence (from sequencing) would have been the complement of the DNA sequence shown in Figure-3.5.

Figure-3.5 DNA sequence of the bovine rhodopsin gene obtained from the purified plasmid DNA sequenced at the SUNY DNA Sequencing Core Facility.

The obtained DNA sequence was compared to the nucleotide sequence records on the National Center for Biotechnology Information (NCBI) website using the Basic Local Alignment Search Tool (BLAST) program (Altschul et al., 1990) and Figure-3.6 shows the results. The purified plasmid DNA sequence generated a 96% match with NM_001014890, Bos taurus rhodopsin, due to gaps or mismatched bases, shown in Figure-3.6. All of the errors were after the 531\textsuperscript{st} nucleotide and therefore probably not significant. Most DNA sequencing facilities can accurately sequence DNA molecules up to 500 bp in length. DNA fragments larger than 500 bp are usually sequenced with inconsistencies beyond the 500\textsuperscript{th} base.
Figure 3.6 Results of query generated by the BLAST program on the NCBI website.
3.4 Transformation

After determining the correction orientation of the bovine rhodopsin gene in the pBAD TOPO plasmid, the purified DNA (from the DNA purification step) was used to transform *E.coli* cells strain UT5600, and the resulting colonies were expected to contain the recombinant DNA molecule. The presence of an ampicillin resistant gene within the pBAD TOPO plasmid assured the transfer of ampicillin resistance to the *E.coli* cells by uptake of the recombinant DNA molecule. Consequently, only the *E.coli* cells with the recombinant DNA molecule could survive on the LB/ampicillin plates.

![Figure-3.7 Colonies from the transformation reaction with the recombinant DNA molecule and E.coli cells strain UT5600.](image)

3.5 Protein Expression

As stated previously, when retinal is added to rhodopsin a red to purple color is emitted. Regrettably, the cells were not the anticipated red color; instead, they were an orange-brown color as shown in Figure-3.8. The lysate exhibited an orange-brown in color instead of the expected red, which
we believed to be metarhodopsin III, a semi-stable intermediate formed by the linkage between all-trans retinal and the opsin apoprotein (Bauman, 1972; Blazynski and Ostroy, 1984; Chabre and Breton, 1979; Ebrey, 1968).

![Image](image.png)

Figure-3.8 *E.coli* cells lysed with the cell lysis solution. The resulting supernatant was an orange-brown color.

The metarhodopsin III intermediate absorbs light in the 470 nm region, and to test for the presence of metarhodopsin III in the lysate, a spectrum was taken between 190 and 700 nm to confirm its presence. The spectral results are shown in Figure-3.9. Although no defined spectral peak at 470 nm was observed, there is broad absorbance throughout the region above 400 nm. We believe this could be due to the presence of a mixture of different photostates and/or of partially folded states of rhodopsin in our sample.
3.6 Conclusions

The bovine rhodopsin gene has been successfully cloned into *E. coli* cells strain UT5600. Evidence garnered from the restriction enzyme digest, sequence of the purified DNA sample, BLAST results, and the successful transformation of the *E. coli* cells supports this conclusion. However, the ability of the cells to express the bovine rhodopsin protein in a native or near-native form is inconclusive and needs further investigation.

Future work on this experiment surrounds refinement of the protein purification process and possible attainment of crystals from the purified bovine rhodopsin protein. We want to modify the rhodopsin gene to include parts of the proteorhodopsin gene that permit citrate induced precipitation. Ultimately, we want to crystallize the structure of bovine rhodopsin expressed
in the bacteria in order to compare it to known crystal structures of the bovine rhodopsin protein derived from cow eyes.
References


Capstone Summary

G-protein coupled receptors (GPCRs) make up a total of ~950 genes in the human genome (Fredriksson and Schioth, 2005; Mirzadegan et al., 2003; Takeda et al., 2002). GPCRs are important because they play a vital role in cell signaling in that they relay extracellular messages to a cell’s interior; they detect and mediate hormones (Heideman and Bourne, 1990); and they are involved in electron transfer and ion transport, (Trabanino et al., 2004) critical functions for nerve impulses and other biochemical processes.

Additionally, properly functioning GPCRs are imperative for normal body function. Minor mutations in GPCR structures result in diseases such as diabetes and hypertension, dwarfism, obesity, and retinitis pigmentosa (Wilson, et al., 1998). Other manifested conditions resulting from deficient GPCRs include: allergies, anxiety, asthma, congestive heart failure, migraines, Parkinson’s, and ulcers (Wilson and Bergsma, 2000). As you can imagine, a malfunctioned GPCR is potentially deadly.

However, much of what is currently known about GPCR structure was based on X-ray crystallographic measurements of rhodopsin. This was the only GPCR with a known structure until the recent determination of the crystal structure of the human β2 adrenergic G-protein coupled receptor (Rasmussen et al., 2007). Of the presently available therapeutic drugs, approximately 40% of them target GPCRs (Muller, 2000; Sautel et al., 2000). The goal of finding new pharmaceuticals would be aided if the identity and structures of more GPCRS were known.
Rhodopsin, the source of investigation in our experiment, has been extensively studied because it is readily available and accessible for manipulation, plays an important role in vision, and its active and inactive states are easily quantified (Ebrey and Koutalos, 2001; Hargrave, 2001; Okada et al., 2001; Okada and Palczewski, 2001; Filipek et al., 2003(b)). Further, earliest crystallized structures of rhodopsin have provided information its helical arrangements and interactions, chromophore binding site, and extracellular ligand binding interactions in other types of GPCRs (Palczewski et al., 2000).

In the current experiment, the gene for bovine rhodopsin protein, a mammalian gene, was cloned into Escherichia coli (E.coli) cells strain UT5600. The main goal was to assess the capability of a GPCR to be expressed in large quantities as a functional protein in bacterial cells. Successful expression of bovine rhodopsin in E.coli could pave the way for cheaper and less complex of GPCR proteins suitable for crystallization trials.

The pBAD TOPO TA Expression kit purchased from Invitrogen was used to conduct the cloning process. PCR (Polymerase chain reaction) was used to amplify the prospective gene to be cloned, cDNA of bovine rhodopsin. The gene, in the form of bovine rhodopsin cDNA, was contributed by Jeremy Nathans, et al. (1989).

Cloning is the process whereby a gene is manipulated and inserted into an organism in which it is not naturally found (Brown, 1990). In the TOPO® Cloning reaction, the bovine rhodopsin gene was inserted into the pBAD
TOPO plasmid to form a recombinant DNA molecule. The recombinant DNA molecule, when uptaken by the *E. coli* cells strain XL-1 blue, made copies of itself along with the bovine rhodopsin gene. As a single *E. coli* cell divided, copies of the recombinant DNA molecule were passed on to its progeny. The *E. coli* cell continued to divide until it formed a colony, or cluster of cells.

A single colony resulted from the TOPO® Cloning reaction and was subsequently grown in a 40 ml flask containing LB media (food for the bacteria). One and a half milliliters were taken from the culture and DNA was extracted from the cells and purified using a Wizard® Plus SV Minipreps DNA Purification System.

The purified plasmid DNA containing the cloned PCR product was treated with restriction enzymes and then analyzed on a 0.8% agarose gel to determine the correct orientation of the bovine rhodopsin gene. Restriction enzymes cut DNA at specific sequences. So treating the DNA sample with restriction enzymes produced DNA fragments of varying sizes based on the location of the restriction enzyme sites in the DNA sample. The agarose gel aided in the process of separating the DNA fragments and created a visual depiction of the gene’s orientation. Based on the results of the gel, we determined the bovine rhodopsin gene to be inserted in the correct direction in the pBAD TOPO plasmid. Furthermore, the DNA was sequenced and compared to the nucleotide sequence records on the National Center for Biotechnology Information (NCBI) website using the Basic Local Alignment
Search Tool (BLAST) program (Altschul et al., 1990) and was determined to have the correct orientation.

The purified plasmid DNA was used to transform *E. coli* cells strain UT5600, and the resulting colonies were grown in growth media containing ampicillin. To induce expression of rhodopsin, the sugar L-arabinose was added to the cells along with retinal (which causes the opsin protein to turn a red color).

The entire culture was centrifuged and the supernatant was removed. Cell lysis solution was added to the cells to extract the protein. The lysate exhibited an orange-brown color, which we believed to be the result of all-trans retinal (present as one of many isomers in the retinal stock solution that was added to the cells) combining with the opsin apoprotein (part of the rhodopsin protein) to form an intermediate called metarhodopsin III (Heck et al., 2003).

In summary, the bovine rhodopsin gene was successfully cloned into the bacterial cells, although the expressed protein was not completely refolded to the native rhodopsin form. The sequenced DNA obtained from the SUNY DNA Sequencing Core Facility, BLAST query results, and restriction enzyme digests proves that the bovine rhodopsin gene was not only inserted into the *E. coli* cells but inserted with the correct orientation. Also, the ability of the cells to express the bovine rhodopsin protein is inconclusive and needs further investigation. Future work on this experiment surrounds refinement of the protein purification process and possible attainment of crystals from the
purified bovine rhodopsin protein. We want to crystallize the structure of bovine rhodopsin expressed in the bacteria in order to compare it to known crystal structures of the bovine rhodopsin protein derived from cow eyes.