REPORTER GENE FUSIONS OF THE GREEN FLUORESCENT PROTEIN AND *ASP24* FROM *BRUCELLA ABORTUS*

A Senior Thesis

By

Neesha Devaraj

1996-97 University Undergraduate Research Fellow

Texas A&M University

Group: BIOCHEMISTRY/VAPH

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Neesha Devaraj
University Undergraduate Fellow, 1996-1997
Texas A&M University
Department of Veterinary Pathobiology

APPROVED

Undergraduate Advisor Thomas A. Ficht

Exec. Dir., Honors Program [signature]

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Abstract

Brucellosis is a serious infection in swine, goats, and cattle that may also infect humans through contact with infected animals. The bacteria, known as *Brucella,* that causes this disease survive within the host by withstanding the host's defense mechanisms. The activation of virulence genes aids in their survival. *Asp24* has recently been sequenced and identified as the calcium-binding protein in *Brucella abortus*. As a virulence gene, it is expressed under conditions of low pH and specific intracellular calcium levels. The construction of reporter gene fusions can be used to characterize signals controlling the calcium-binding protein expression within the intracellular environment. This project is aimed at making constructs that can be used in further experiments to study interplay between in vivo conditions and the bacterial factors required for intracellular survival of *Brucella*.

Introduction to Brucellosis

Brucellosis is a zoonotic infection caused in humans by a group of related organisms that infect various species of animals. Human brucellosis was first accurately described by J.A. Marston in 1859 (26). As a surgeon in Malta during wartime, he reported his experience with the "Malta fever" or "Mediterranean gastric remittent fever" (5). The actual cause of the disease did not come to light until David Bruce isolated the organism in 1886 from the spleen of an infected soldier. In the early nineteen hundreds, the establishment of the Malta Fever Commission by the Royal Society of London and the Governor of Malta identified goats as carriers of the disease (3,26). With this insight, the practice of pasteurization decreased the number of cases. The bacteria evolved as variants adapting to different hosts. In 1895, a Danish veterinarian by the name of Bernard Bang linked *Brucella abortus* to the spread of abortions in cattle, but not until 1920 was it recognized that “Bang’s disease” and the Malta fever were related (26). This breakthrough was the work of Alice Evans, a bacteriologist in America, who renamed the bacteria *Brucella* in honor of Bruce. The early 1900s brought the isolation of *B. suis* from aborted swine by Traum, and in 1966 Carmichael discovered *B. canis* as a cause of abortions in dogs. Although non-pathogenic to man, two other pathogens of the genus *Brucella* have been identified: *B. ovis* and *B. neotomae,* which infects sheep and rats, respectively.

The recording of Malta fever and its connection with a microscopic organism by David Bruce opened doors to a study of *Brucella* that is continuing today.

Brucellosis is a serious infection in swine, goats, and cattle that can also spread to humans through contact with infected animals and to a lesser degree by drinking infected milk. Due to successful efforts in the vaccination of cattle against *B. abortus,* drinking unpasteurized goat's milk (and related products) has become the major cause of Brucellosis in the U.S., usually found in Hispanics or people who have visited Mexico. This infection is caused by *B. melitensis*. Generally, human contraction of the disease is caused by *B. abortus*, naturally found in cows, *B. melitensis* from goats, or *B. suis* transmitted from pigs.

In Malta in 1887, when Bruce first studied *Brucella*, he contacted the strain of *B. melitensis* found in goats. Areas with a large number of sheep and goats showed different epidemiological patterns of disease. For example, the 1930s left France with a high incidence of disease because of the herds of sheep and goats used in farming. This disease is consistently characterized more as one contracted occupationally, through direct contact with the animals and feces, than as a consumer disease. Contaminated milk and cheese do, however, play a small part in the number of cases (3). *B. melitensis* has been found in countries around the Mediterranean, India, China, South Africa, and South America.

The different types of *Brucella* organisms cause infection throughout the world depending on the relationship of various areas to the animals which are the natural hosts. The widespread development of the pasteurization of milk has decreased incidences of the infection throughout the latter half of the twentieth century. Many countries have almost

completely eradicated the disease, especially illnesses caused *B. abortus*. The low pathogenicity of the disease may create difficulty in completely assessing *B. abortus*. The U.S. has almost completely eliminated *B. abortus* in cattle by vaccination and killing infected animals, but it is still a problem in wildlife (bison and deer). *B. abortus* seems the most important, followed by *B. suis* and then *melitensis*.

B. abortus causes bovine brucellosis, manifested in abortion, decreased milk production, birth of deformed calves, and difficulty in breeding. If the cattle are used for food, the risk of passing the infection on to humans is also relevant, not to mention the loss of the livestock itself (22). Usually those who work with the cattle are the most at risk for contracting the disease. The natural host of *B. suis* is the pig. Handlers of infected carcasses, like slaughterhouse workers and most packers, usually contract the disease. *B. suis* is the least common of the three main species to infect man. Disease has been seen mainly in the midwestern part of America, Brazil, and Argentina where raising pigs is an integral part of the economy (25). Humans usually contract the diseases through skin lesions, but the bacteria can also be airborne. Many laboratory acquired infections, especially in medical labs processing "unknown" specimens, have occurred with *Brucella* because it can be contracted via inhalation of aerosols. For this reason, work with this organism is restricted to a BL3 laboratory.

Upon entering the body, *Brucella* spreads through the lymphatic system. The lymph nodes swell in regions of infection and when dissected contain the organism. The

spleen and liver may also enlarge due to infection. The slow response of the immune systems of animals and humans to *Brucella* causes disease with persistent infection and a long duration. Clinically the disease traditionally manifests itself in a fever that falls and rises continuously, lasting anywhere from ten to thirty days, hence the name "undulant fever". Symptoms commonly following infection are weakness, muscle pain, nocturnal sweats, anorexia, chills, and nervous irritability. The liver and spleen are enlarged also. Symptoms usually appear two to eight weeks after inoculation (26). Infections left untreated cause death in about 3% of cases (3).

Brucellosis is a systemic infection that can involve many organs and tissues, from the gastrointestinal tract to the cardiovascular system. A localized or focal form of the disease manifests itself in a specific organ. A prolonged recovery time lends to the possibility of a relapse not due to antibiotic resistance. It is difficult to distinguish acute from chronic forms of the disease. If symptoms last longer that one year, the disease is usually termed chronic (3). A vaccine for *B. abortus*, known as Strain 19, has been developed for cattle but is pathogenic for humans. Diagnosis of the disease in man may be difficult due to the non-specificity of the symptoms. A detailed history, comprised of the patient's job description, exposure to animals, and a travel log may suggest Brucellosis. Elimination of this disease in natural animal hosts is the way to prevent the spread of this disease entirely. Infected animals must be separated from the herd and vaccines may be used to prevent spread of disease.

The Bacteria

Brucellosis is caused by several species of a bacteria known as *Brucella*. *Brucella* are Gram negative, small, non-motile aerobic rods. They are 0.5 - 0.7 X 0.6 - 1.5 µm in size and commonly grow individually, sometimes in pairs, groups, or short chains. They are non spore-forming. As aerobic bacteria, they have a respiratory type of metabolism and cytochrome based Electron Transport System with a final electron acceptor of oxygen or nitrogen. Optimally, the organism can be grown at a temperature of 37° C and a pH between 6.6 and 7.4 . Carbon dioxide supplementation is sometimes required for growth (13). Chromosomal exchange among the *Brucellae* by transformation, conjugation, or transduction is unknown and no plasmids or temperate bacteriophages have been found (26). *Brucellae* are heat-sensitive and can be killed through approximately thirty minutes of exposure to a temperature of at least 60° C, depending on the density of the suspension (25).

With the identification of the organism, Bruce's discovery did not end Malta fever immediately, but opened a world of discovery and investigation to begin the quest of the disease. *Brucella* is an extremely infectious pathogen capable of passing through unbroken skin, making the study of this organism fairly difficult. The microbacteria *Salmonella* is commonly used as a guide to study *Brucella*. Like *Salmonella*, *Brucella* invades tissue culture cells and survives in macrophages in vitro (4).

Pathogenicity and Virulence

Intracellular pathogenic bacteria including *B. abortus* face various host defense mechanisms when invading. These include acid shock, oxidative stress, temperature change, and nutrient deprivation. In order to cause disease, bacteria must survive these changes in the environment by sensing and adjusting to the new conditions. In response to changes in the extracellular environment, bacterial pathogens are able to activate genes which promote infection of the host. Virulence is the ability of bacteria to cause infection. A virulence factor contributes to virulence or pathogenicity by promoting bacterial colonization or causing damage to the host (23). It is important to study virulence factors to understand how bacteria survive and to limit invasion. Genes and operons encoding virulence factors are often co-regulated in response to environmental conditions. The regulated expression of virulence traits is essential for the survival of pathogenic microbes. Specific environmental stimuli turn on expression for certain genes in two steps, through the use of a two-component regulatory system. One protein senses environmental stimuli and then stimulates another protein which acts as a regulator of one or more genes (23). Changes detected may be in temperature, pH, or differing levels of iron and calcium. It is possible to identify new virulence genes through their coordinate regulatory properties. Checking for gene expression under various conditions similar to those within the host cell is one approach used to identify virulence genes.

Virulence Factors

B. abortus is a pathogenic bacteria that causes disease through its ability to multiply within macrophages of the host organism. This ability to replicate has been linked to virulence in the host. Virulent strains that are smooth have the ability to survive within phagosomes. Rough bacteria are non-virulent and are sensitive to the destructive conditions or the enzymes within the cell. The most important virulence factor identified so far in *Brucella* is lipopolysaccharide (LPS). The organisms *B. abortus*, *B. suis*, and *B. melitensis* all have smooth LPS variants that are virulent due to the O-polysaccharide and rough strains lacking the O-polysaccharide of the lipopolysaccharide structure that attenuated. *B. canis* and *B. ovis* have virulent rough strains that lack the O-polysaccharide (12). In order for *Brucella* to survive, they must prevent fusing with the phagolysosomes or resist bacterial mechanisms of those cells. Research has not defined the exact mechanism used by *Brucella* for survival intracellularly in phagosomes.

Asp24, the Calcium-Binding Protein

Few of the genes that participate as virulence factors for *Brucella* have been identified. Lipopolysaccharide structure (LPS), two stress response proteins, and one other gene product have successfully been linked to aiding *Brucella* in intracellular survival and maintaining life in the host environment (7, 8, 16). *HtrA*, a stress response protein, is a serine protease that degrades damaged proteins before they can build up to toxic levels within the cell. A mutation in the *htrA* gene diminishes survival of *Brucella* in mice. *DnaK* is another stress response protein, also known as a chaperone, due to its

involvement in the folding, assembly and transport of proteins essential for replication of *Brucella*.

In studying bacteria, often what is known about other microorganisms can be a useful tool for discovery. The expression of virulence factors is regulated according to environmental stimuli. By examining genes that are transcribed when phagocytosis begins, possible virulence genes can be identified. As defenders against invasion, phagocytes place many strains on bacteria, such as oxygen-dependent killing mechanisms and other mechanisms involving lysosomes, lowering the pH, and defensins. Reduced pH and calcium levels, which are thought to occur in macrophages, are very important to the following research.

When an organism enters the host, it may be exposed to hostile environmental changes, such as acidification of the macrophage vacuole which contains bacteria. Research has shown that *Salmonella* can survive in this acidic environment by sensing the change in pH and allowing that to activate expression of certain virulence eradicating genes. Acid-inducible genes have been studied in many microorganisms. In *Salmonella* studies have shown that the acidic conditions of a macrophage are essential for the survival and replication of the bacteria. This shows that low pH is an essential environmental stimulus for transcribing genes necessary for intracellular survival. The eight acid-inducible promoters new found in the study hold homology to promoter regions of genes encoding stress proteins and cell-surface-maintenance enzymes (24). This may

also be true of *Brucella* and this acidificaiton may go so far as to aid the replication of *Brucella.*

Just as a pH change may act as a signal to turn on certain genes, so to may calcium levels. Through investigations with the bacteria *Yersinia pestis*, the possibility of a calcium binding protein becomes relevant (14). A low intracellular calcium level may signal the bacteria to phagocytosis. During growth within macrophages, *B. abortus* expresses high levels of stress response proteins. The calcium binding protein in *Brucella* is known as Asp24. Its expression is controlled by low pH, chelation of calcium extracellularly, and upon entry into a macrophage (9). Thus, *Asp24* expression is regulated by environmental signals, similar to those found in the host. Since many gene products of bacteria that are important in causing disease are regulated environmentally, the *Asp24* gene of *B. abortus,* which is important to the survival of the organism, is similar in its regulation to other known virulence factors. In vivo experiments in mice have proven *Asp24* is an important virulence factor.

Reporter Gene Fusions

The gene *Asp24* has been targeted as an important virulence gene not only because it is acid-inducible but also because of the findings of the ability of calcium- binding proteins to regulate the expression of virulence genes (23). Since these signals are also thought to exist in the macrophage, a determination of whether *Asp24* is expressed intracellularly tells its involvement with the virulence of *Brucella* . Because *Asp24* does

not have an activity which can be readily assayed, we constructed a transcriptional fusion between the *Asp24* promoter and a reporter gene, *gfp*$_{uv}$. A reporter gene is one that codes for a protein that can be measured quantitatively. Reporter genes are very useful in designing experiment to study virulence. Since the *Asp24* promoter has been identified and sequenced, reporter gene fusions can be used to characterize signals controlling calcium binding protein expression within the intracellular environment. These fusions show the interplay between in vivo conditions and bacterial factors required for intracellular survival of *Brucella*. Performing a transcriptional fusion involves joining the promoter-operon of a virulence gene to a structural gene encoding some easily assayable enzyme, or a reporter gene (23). The ribosome binding site is provided in the reporter gene. RNA polymerase begins transcription from the regulated promoter-operon region of the virulence gene. The reporter gene is translated because it is expressed where the virulence gene would have normally been produced (Figure 1).

Green Fluorescent Protein

Bioluminescent systems are useful as molecular probes for analysis of gene expression and cell biology markers in different organisms. Firefly luciferase, bacterial luciferase, b-galactosidase, and enzymes can be used to monitor gene expression in bacteria. The reporter gene used in this project is known as the green fluorescent protein (GFP). It is a fairly new reporter gene used for monitoring gene expression and protein localization in living organisms. Taken from the jellyfish *Aequorea victoria*, this protein emits green light at 509 nm when excited with UV or blue light at 395 nm. It is a small

protein of only 238 amino acids (5). Unlike other bioluminescent reporters, the chromophore, a molecular group capable of selective light absorption, is intrinsic to the primary structure of the protein and the green fluorescent protein fluorescence does not require a substrate or cofactor to be added to assay expression. GFP fluorescence is stable, species-independent, and can be seen in living cells and often in animal itself if it is transparent.

Luminescence in the jellyfish itself is produced by an energy transfer. Calcium binds to the photoprotein aequorin and excites it. The green light produced by the jellyfish is a result of a second protein that is excited by aequorin, known at the green fluorescent protein (15). The full length *gfp* is necessary for fluorescence but a hexapeptide chromophore is minimal for light emission. The region with the Ser-dehydro Try-Gly trimer cyclizes and emits light through an unknown mechanism. Mutations at different sites within this hexapeptide produce functional GFP mutants with various fluorescence spectra. Cloning and sequencing of the GFP gene allows expression of this protein in various systems. GFP can be expressed in prokaryotic and eukaryotic cells (5). An important advantage of GFP as in vivo marker of gene expression is the ability of the protein to generate a fluorescent signal without requirements for additional factors from *A. victoria,* and fluorescence can be detected by the eye with the use of UV light. Quantitatively, it can be measured using a fluorimeter.

The discovery of the green fluorescent protein has led to its use as a marker for gene expression. Cell growth does not change when containing the protein and the protein extracted from these cells was no different from the purified native form (15). GFP has many advantages over other biological systems. Besides not requiring a substrate for activation, it also allows gene expression to be monitored and protein localization in the cell to be viewed.

Important Research using GFP

The development of GFP in experimentation aided in its use as a new expression marker in mycobacteria (18). The uses and advantages are portrayed for reporter molecules involving GFP and mycobacteria. Promoter-probe plasmids and transposons have been developed and successfully used in a number of microorgansims to identify genes and to understand gene regulation in response to environmental changes.

Although it is fairly new, GFP has been used in research with many different types of bacteria. According to Valdiva and Falkow, *Salmonella typhimurium* survive and replicate in a macrophage due to the low phagosomal pH (24). The necessity of an acidic condition suggests that the low pH is an important environmental stimulus for the transcription of genes necessary for intracellular survival. GFP was used in this study to help conclude that low pH is a relevant inducer of intracellular gene expression, essential to protecting the bacterium against the phagocyte's killing mechanisms.

The green fluorescent protein has been useful in the study of in vivo gene expression and protein localization in eukaryotic systems (15). Bacterial combinations present different problems that may be solved with time. The experiments reveal that creating a mutant with GFP provides a unique measurement tool for gene expression in individual bacteria in response to a complex environment such as the phagosome. Also in bacteria, *gfp* fusions have been used successfully to localize a protein within the bacterial cell. In this work, translational fusions of *gfp* to *Bacillus subtilis* proteins were constructed. The hybrid protein was readily detected by fluorescent microscopy and its synthesis correctly localized to one end of the cell. In this expression GFP was used to determine compartmentalization of gene expression (19).

Dhandayuthapani and others report that the GFP can be used as a marker for gene expression and cell biology of mycobacterial interactions with macrophages (6). This expression employed the construction of a mycobacterial shuttle-plasmid vector carrying *gfp*, that was used to generate transcriptional fusions with specific promoters and then expression was examined in *smegmatis* and *bovis*. Fluorescence microscopy and spectroscopy were among the tool used to analyze and quantify expression within populations of bacterial cells. Through the work presented, GFP can be viewed as a useful tool for analysis of mycobacterial gene expression.

Project Goal

Because of the great interest in the calcium-binding protein as related to the virulence of *Brucella*, there was an idea to combine this promoter with a reporter gene to detect what conditions increase production of the protein. The purpose of this project was to complete constructs of the *Asp24* promoter and *gfp* within a plasmid that could be introduced into *Brucella*. With this recombinant *Brucella* strain, expression of the *Asp24-gfp* construct will be tested under different conditions for bacterial growth and then checked for growth in macrophages.

Because *Asp24* has only recently been sequenced and named a virulence gene, some uncertainty surrounding its activity exists. *GroE* and *PhoP* are promoters for other virulence genes in *Brucella* that are induced within macrophages and will serve as positive controls.

Vectors

Clonetech has developed a vector known as pGFPuv which is used in this project (15). It is eighteen times brighter than the wild type because it has a UV optimized GFP gene, due to 3 amino acid substitutions at Phe-99 to Ser, Met-153 to Thr, and Val-163 to Ala. These substitutions do not interfere with the chromophore. The emission and excitation wavelengths are the same as in the wild-type GFP. It has many unique restrictions sites at the 5' and 3' ends and also Ampicillin resistance. The vector is intended primarily as a source of the GFPuv coding sequence. It is ideal for GFP

expression when using a UV light and can be detected by the eye when it is excited by standard long wave UV. This modified gene codes for proteins with increased solubility than the wild-type resulting in a lower toxicity to the host cell.

Another important vector used in this project is pBBR1.mcs (17). It is a broad-host-range cloning vector developed for genetic analysis of gram negative bacteria, such as *Brucella*. It contains the pBluescript ® II KS-*lacZα*-polylinker that provides sixteen unique restriction sites that interrupt the fragment containing lacZα. Rather than resting solely on antibiotic sensitivity, this allows for screening of recombinants using chromogenic β–galactosidase substrates. The vector contains B-gal and T3 and T7 promoters for inducible expression of cloned genes both in vitro and in vivo. Considered small for a broad-host-vector, it is easy to manipulate and also grows in *Brucella*.

Materials and Methods

Bacterial strains, plasmids, media, and growth conditions.

The strains and plasmids used are listed in Table 1.

Media. *E. coli* was grown in Luria-Bertani Broth (LB). Solid media contained 15 g/liter of agar. Antibiotics used to maintain plasmids were used at the following concentrations: Chloramphenicol (Cm) 30 mg/liter, Ampicillin (Ap) 100 mg/liter, Kanamycin (Km) 100 mg/liter. Bacteria were cultured aerobically at 37°C.

Recombinant DNA techniques

Isolation of plasmid DNA. Plasmid DNA was isolated by using ion-exchange columns from Qiagen, according to the manufacturer's instructions. The TENS prep for isolating plasmid DNA was also used (16).

The Qiagen Plasmid Maxi kit was used to obtained a large prep of plasmid DNA. The rapid purification protocol was based on modified alkaline lysis procedure, followed by binding of plasmid DNA to Qiagen anion-exchange resin under low salt and proper pH conditions. A medium salt wash removed RNA, proteins, and low molecular weight impurities. A high salt buffer then eluted the plasmid DNA and it was then cleaned by isopropanol precipitation.

First cells were harvested and resuspended. They were then lysed in NaOH/SDS with RNAse A and the lysate was neutralized with a potassium acetate solution. A spin then separated the plasmid DNA in the supernatant from impurities of RNA and protein that were bound within the tip. The tip was washed to remove contaminants with 1M NaCl. The DNA was then eluted with a buffer (pH of 8.5) of 1.25 M NaCl and collected in a falcon tube. An isopropanol precipitation and wash desalted and concentrated the DNA. After being air-dried, the DNA was resuspended in TE and stored at 4°C.

Restriction digests of plasmid DNA. Usually, 2 µl of DNA were digested in a volume of 30 µl using buffers supplied by the manufacturer (Boehringer Mannheim) for 1 hour at 37°C.

Heat inactivation of digestion. After the first digestion, the sample was placed at 65°C for 20 minutes, put on ice, and then spun quickly.

Agarose gel electrophoresis. Agarose gels, containing ethidium bromide, were run at 80 volts and all digests were run with molecular weight standards.

Isolation of DNA from agarose gels. The procedure was done according to the Qiaex (Qiagen) manufacturer's instructions. The Gel Extraction Kit extracts and purifies DNA fragments based on the solubilization of agarose and the adhesion of the DNA to glass particles in the presence of high salt. The DNA was eluted with a low salt solution.

Ligation of DNA. According to the reaction, varying volumes of vector and insert DNA were ligated in a total volume of 15 µl using 1 U T4 Ligase (Boehringer Mannheim) and the buffer supplied by the manufacturer.

Transformation of DNA. Frozen competent cells were thawed on ice. One µl of each plasmid was placed in 100 µl of competent cells. A control of only competent cells was aliquoted. 1 ml of LB broth was added and incubated for 1 hour at 37°C. Samples

were then plated out on petri dishes with LB agar and appropriate antibiotics and grown overnight at 37°C.

Polymerase Chain Reaction (PCR). PCR was performed using Taq polymerase (Boehringer Mannheim), and buffer supplied by the manufacturer. Primers used are described in the Results section (Genosys).

Results

Isolation of plasmid DNA

Since we had received only small aliquots of plasmid DNA, it was necessary to purify large amounts for cloning experiments. The plasmids pGFPuv and pBBR1mcs-6 were transformed into *E. coli* DH5α MCR competent cells, according to the Materials and Methods section. Colonies were inoculated into LB with the appropriate antibiotic and grown overnight at 37°C and the Qiagen Plasmid Maxi Kit was used to extract the DNA. 2μl of the samples were digested with *Eco*R1 and run out on a 1% agarose gel. A large prep of pBBR1.mcs was then done to obtain more DNA. This DNA was then separately digested with BamHI and ApaI. pGFPuv was also digested with ApaI.

Construction of a pBBR1mcs derivative for insertion for promoter sequence

The first construct that was necessary for this experiment combined the GFP gene from pGFPuv with pBBR1.mcs (Figures 2, 3, 4). pBBR1.mcs and pGFPuv were digested with ApaI and the linearized bands of DNA were excised from the gel and the

DNA recovered through the Qiaex protocol. Purified DNA was run out on a gel to check its concentration and purity. The DNA was then digested with HindIII. A gel was run and the 800 bp GFP fragment excised and the vector pBBR1.mcs were extracted. The gfp_{uv} was inserted into pBBR1.mcs opposite the *lacZ* gene to make a promoterless *gfp* vector. 2 μl of the insert and 2 μl of the vector were then ligated overnight and transformed into DH5α competent cells. 12 colonies were isolated and grown in LB with Cm for TENS mini-preps to check for the insert in the vector by digestion with EcoRI and XbaI. Colonies were selected for a large prep. This construct was named pNAD1 and represented gfp_{uv} in pBBR1.mcs (Figure 5).

Amplification of promoter sequences by PCR

The next step in the project involved using PCR to amplify the promoter sequences of *Asp24*, *GroE*, and *PhoP*. Primers were selected using the MacVector program. They were designed by examining the sequences of the virulence genes. The promoters for these genes were usually located around -35 to -10 basepairs (bp) upstream of the start codon. The primers were selected to include that site, create a PCR fragment of at least 500 bp, only anneal at one site, and not fold on itself. The expected product sizes of the PCR products were *Asp24*: 491 bp, *GroE:* 410 bp, and *PhoP*: 397 bp (Figure 6). PCR reactions were performed by combining dNTPs, primers. PCR products are run out on an agarose gel. The product size was visible on the gel and the DNA extracted from the gel.

Because a 1.2% agarose gel was used, bands were cut precisely, as excess agarose will inhibit the ligase reaction.

Cloning PCR products into pCRII

The promoters were then cloned into the pCRII vector in the TA cloning kit, using 1 μl of the vector and μl of the insert (Figure 7). The control ligations contained only the vector. The ligations were then transformed into frozen DH5α competent cells and plated out on LB with Ampicillin and X-gal as a substrate to differentiate white and blue colonies. Because of the instability of Ampicillin, it was important to remove the plates after incubation overnight. Plates were then stored at 4° C until colonies were selected to be prepped. This incubation at 4° C also aided in differentiating white and blue colonies. The white colonies contained the insert. A TENS mini-prep was done on the DNA, then digested with *Eco*R1, and run out on a 1.2 % gel.

Construction of pNAD2

Orientation of the fragments within the vector must then be checked by digesting with enzymes that will cleave different size fragments depending on the orientation of the PCR fragments (Figure 8). Enzymes were chosen so that one site is within the promoter and the other is in the vector itself. The *Asp24* product was located at 930-1420 bp in the vector. Digestion with XbaI and ClaI gave either a 121 bp or 425 bp product, depending on the fragment's orientation. *GroE* had a product located at 139-548. A digestion with *Eco*RV gave a 326 bp or a 105 bp fragment. The *PhoP* product was at 56 - 452 bp.

Digestion showed a corrects clone of *GroE* with a 326 bp fragment. This vector was named pNAD2 (Figure 9). Next a ligation of pNAD1 with the promoter of *GroE* was performed. Two colonies of large prepped DNA of pNAD1 and *GroE* were digested with HindIII to begin ligations.

Cloning promoter from pNAD2 into pNAD1

Because PCR fragments degraded slightly during the semester break, PCR was redone to obtain new promoters that were then cloned into pCRII. A Qiaex gel extraction on the PCR products was performed and pNAD1 and pNAD2 were digested with HindIII and run out on a agarose gel. The bands were cut out, the *GroE* fragment from pNAD2 and the linearized vector pNAD1. The same DNA was then cut with BstXI at 55°C. The enzymes both cut upstream from the *gfp* gene in the pNAD1 vector. Because these enzymes work in different optimal temperatures, the first digestion was done, run out on a gel, and the DNA purified. That DNA was digested by the second enzyme. The gel that was run did not show the *GroE* insert. Because these enzyme combinations did not seem to be cutting the plasmids, new enzyme combinations were attempted.

pNAD1 and pNAD2 were redigested with HindIII and heat inactivated according to the procedure described in Material and Methods. Then the DNA was digested with PvuI. A 2% gel was run and the vector and insert bands excised. Qiaex gel extraction was performed and a ligation attempted to combine the *GroE* promoter from pNAD2 with pNAD1. White colonies were selected and colonies were inoculated for mini-preps. The

DNA was transformed into DH5α competent cells and plated out on LB plates. White colonies were selected and used to inoculate media for mini-preps. The DNA was prepped and digested with XhoI to check for the insert. This site flanked the area of the insert in the vector.

Another method used for checking for inserts involved digesting the clones with an enzyme and digesting the original vector only with the same enzyme and then comparing fragment sizes in a gel. If the ligation worked correctly, the clones should be larger size due to the addition of the insert. This method was employed using HindIII as the enzyme. Upon running the gel however, it was found there was no DNA from the clones. The digests were reattempted. The original vectors pNAD1 and pNAD2 were digested with HindIII. The samples were then heat inactivated, digested with PvuI, and run on a 2% gel.

Construction of pNAD3

Fresh DNA from PCR of *Asp24* was also ligated into the pCRII vector. These ligations were transformed using the TA cloning kit. A TENS mini-prep was performed on the selected colonies. The clones were digested with *Eco*RI to check for the insert. This construct was named pNAD3 and consisted of the *Asp24* fragment within the pCRII cloning vector (Figure 10). The vector was digested with ClaI and XbaI to check the direction of the insert.

Conclusion

Many vectors were constructed that would be helpful in further experiments in studying the virulence genes in *Brucella abortus*. The construction of the vector pNAD1 was completed. This vector placed the gfp_{uv} gene into pBBR1.mcs. Any promoter of interest can be inserted into the vector and then put into *Brucella*. Other constructs made were pNAD2 and pNAD3. With the promoters of the GroEL and Asp24 genes cloned into the pCRII vector, vectors pNAD2 and pNAD3 have the potential to be used in other experiments. Future experimentation in the lab may be aided by the constructs completed in this experiment.

Future projects could use these vectors to complete constructs of the promoter fragments upstream to the *gfp* fragment within pBBR1.mcs. This vector could then be put into *Brucella*. The microorganisms would be tested under different conditions for growth. The use of a fluorimeter would quantitatively measure promoter activation in the different conditions. Growth of the bacteria in macrophages would also be monitored to detect how the conditions within a macrophage affect *Asp24* expression.

The loss of DNA with each purification procedure may have affected the success of ligations. In making constructs, enough DNA must be present to create many clones that can be screened for correct orientation of the insert within the vector. Part of the difficulty with the experiments done involves the loss of DNA from beginning digests, through purification, to ligations.

Asp24 is a very interesting gene because it is activated only under specific environmental conditions. These conditions are what the bacteria faces when it enters a host to attack. Since the connection of the gene to the virulence of the bacteria is so strong, research on this gene can lead to the ending of Brucellosis.

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Table 1. Bacterial strains and plasmids used

Strain	Characteristics	Reference
E. coli DH5α	*endA1hsdR17* (r_K - m_K-)	
Plasmid		
pBBR1.mcs		Kovach (17)
pGFPuv		Clonetech
pCRII	Vector for TA cloning	Invitrogen
pNAD1	pBBR1.mcs with GFPuv	This study
pNAD2	pCRII with GroEL promoter	This study
pNAD3	pCRII with Asp24 promoter	This study

Figure 1: Mechanism of Reporter Gene

A reporter gene is cloned immediately downstream of the promoter of the virulence gene (VIR). When the promoter is activated, the reporter gene is expressed and can be quantitatively measured to determine the degree of activation of the promoter.

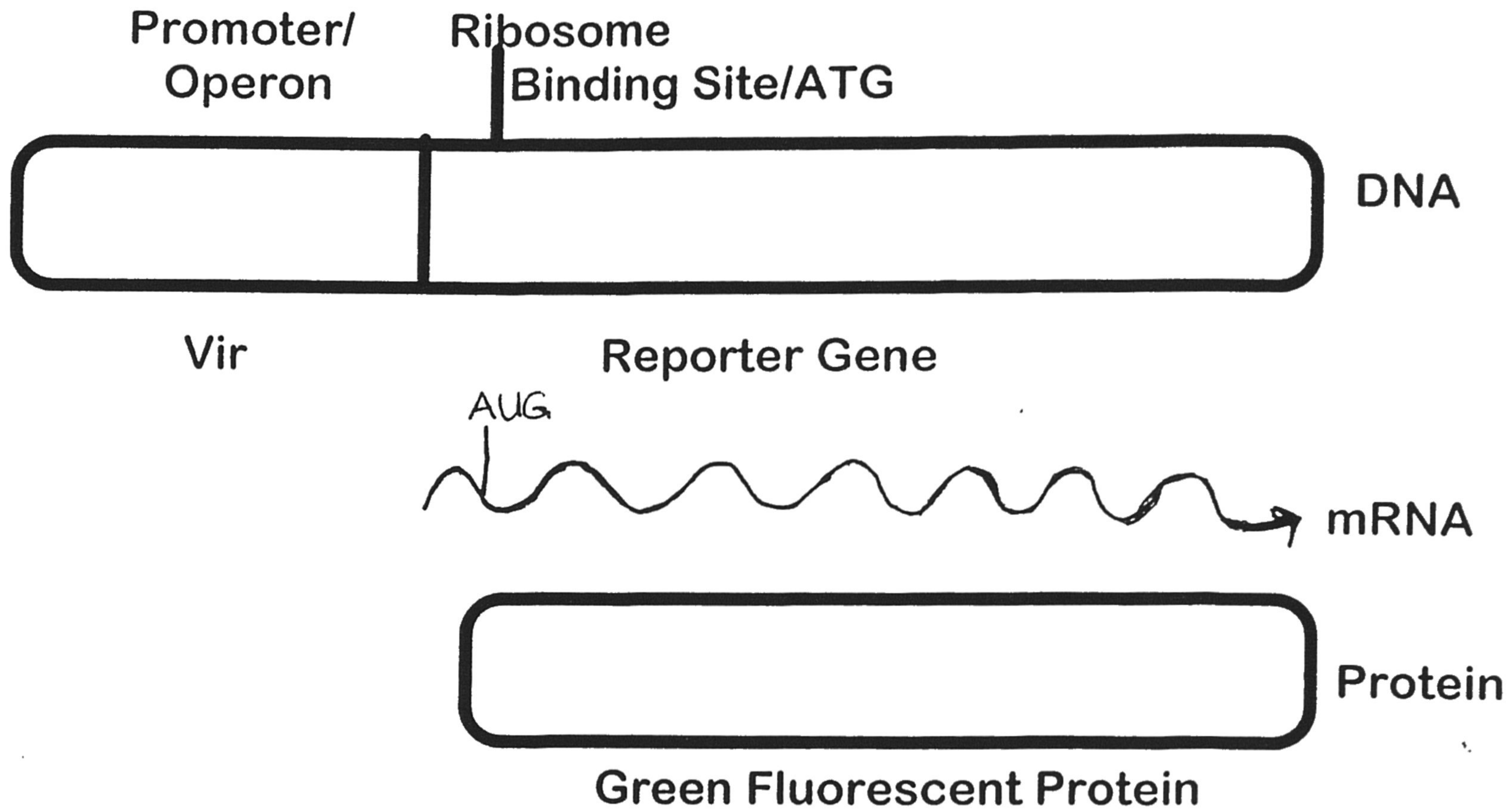
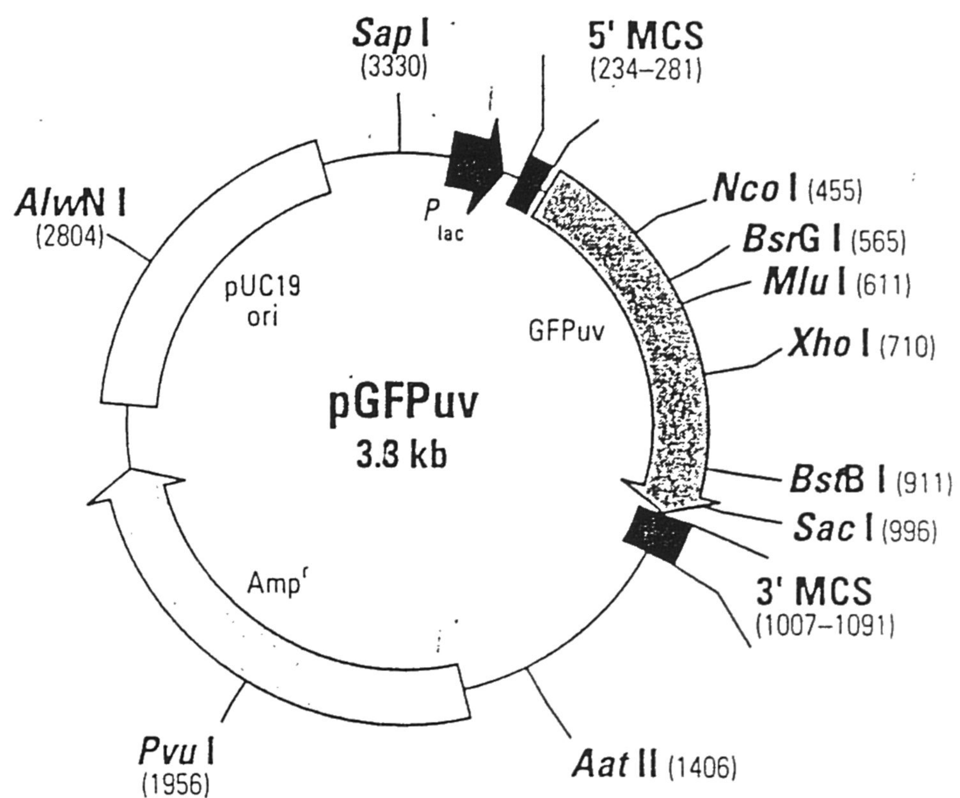


Figure 2: Vector map of pGFPuv
From Clonetech



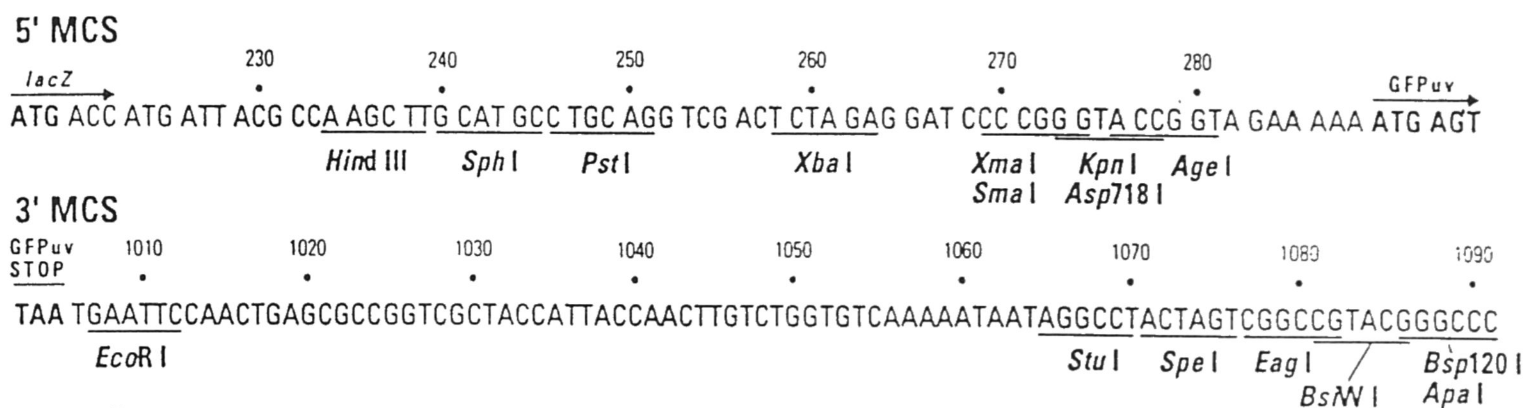


Figure 3: Vector map of pBBR1.mcs
From Kovach, et al. (1994). (17)

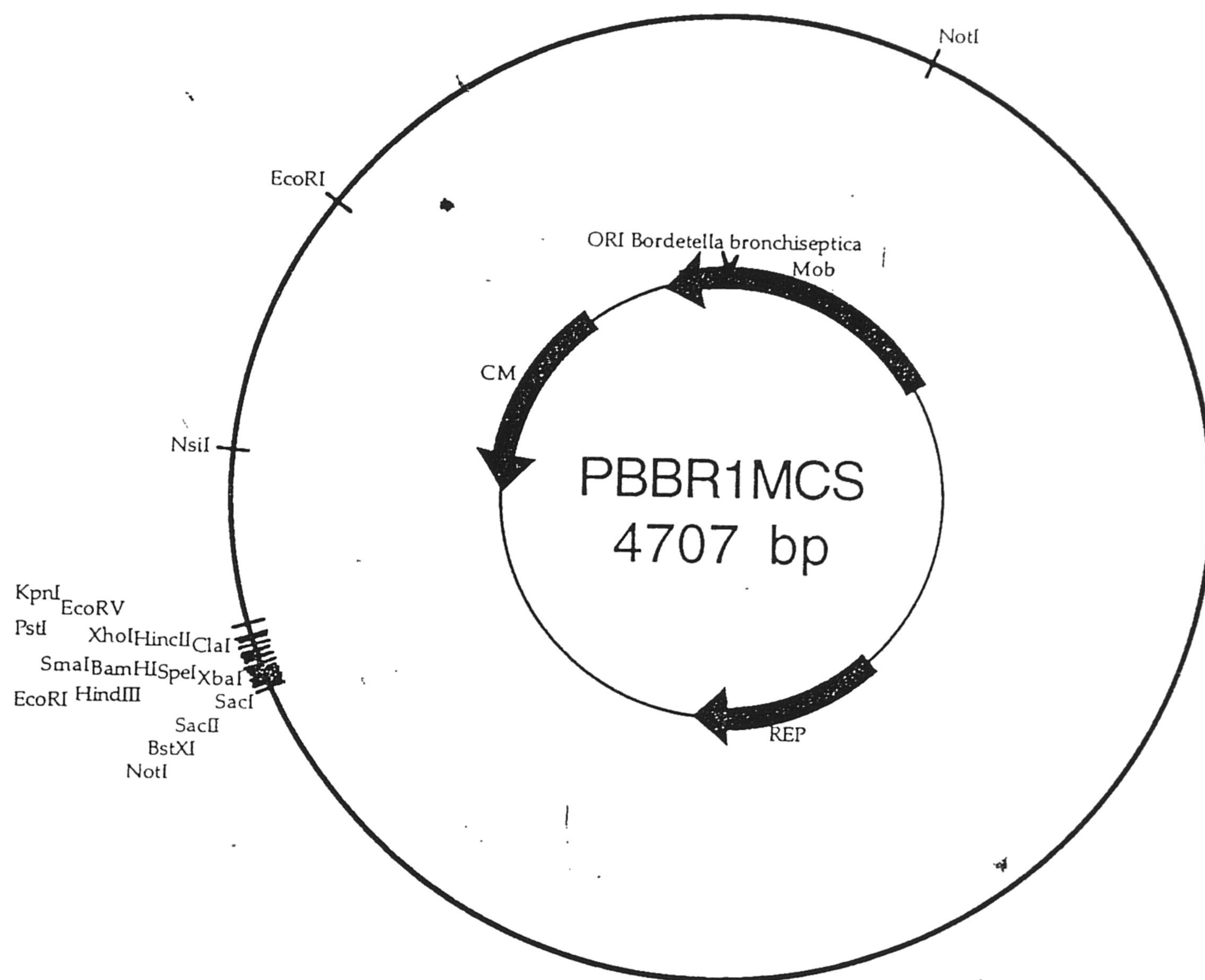


Figure 4: The *gfp*$_{uv}$ gene was cut from the pGFPuv vector and placed in pBBR1.mcs in the opposite orientation to the *lacZ* promoter.

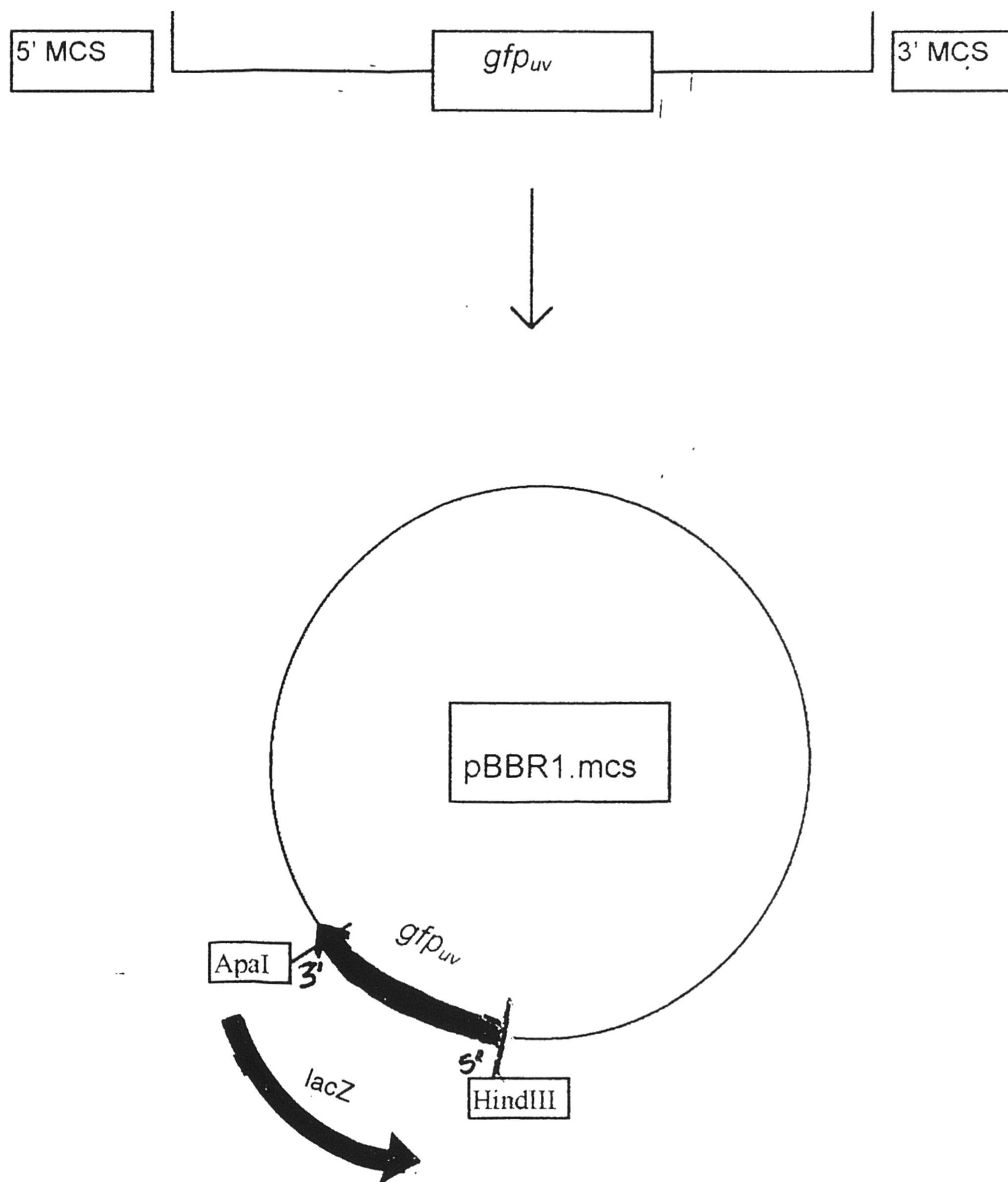


Figure 5: Vector map of pNAD1

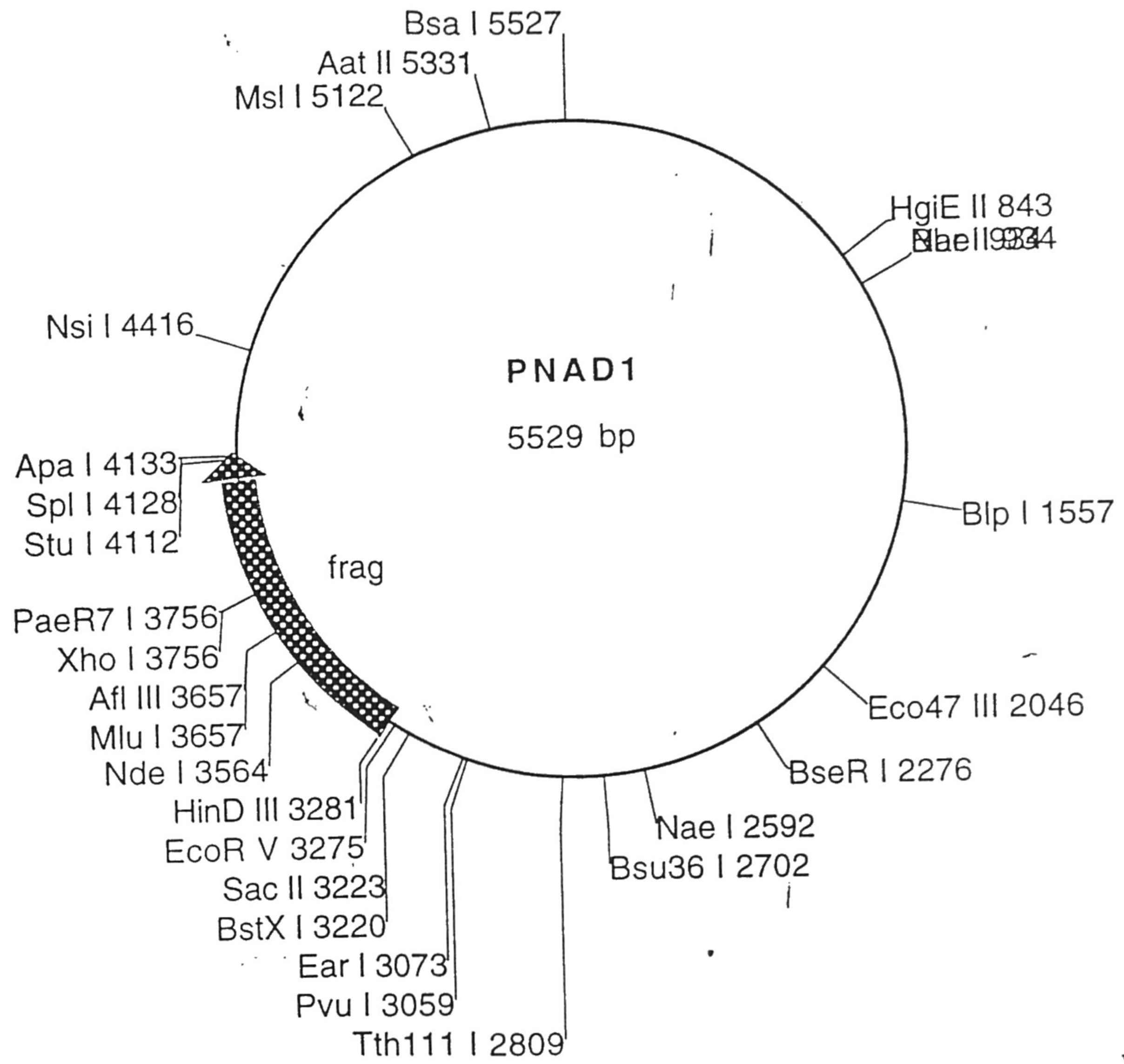


Figure 6: Primers were constructed for PCR to amplify the fragment containing the promoter region of Asp24.

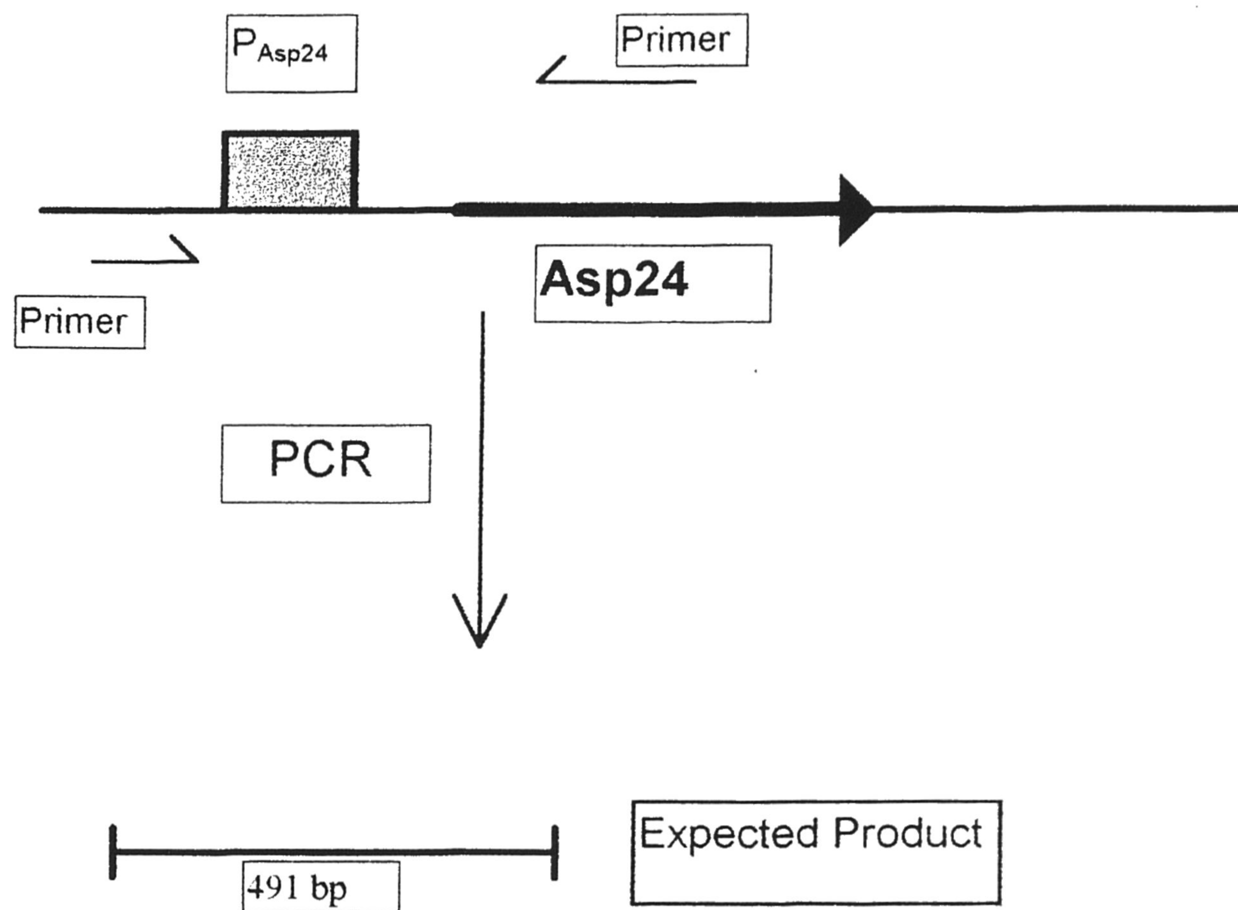
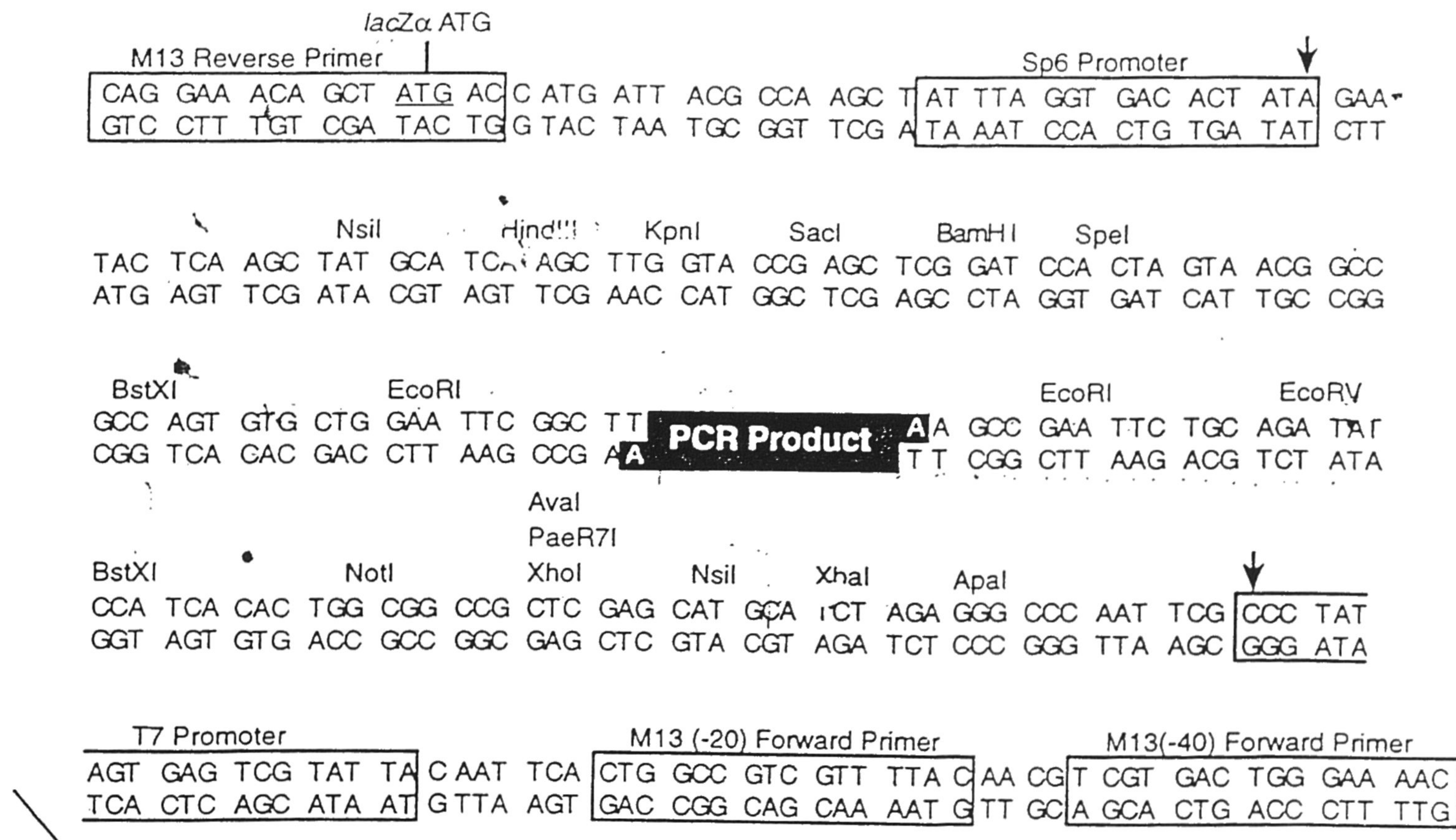


Figure 7: Vector map of pCR™II From TA Cloning



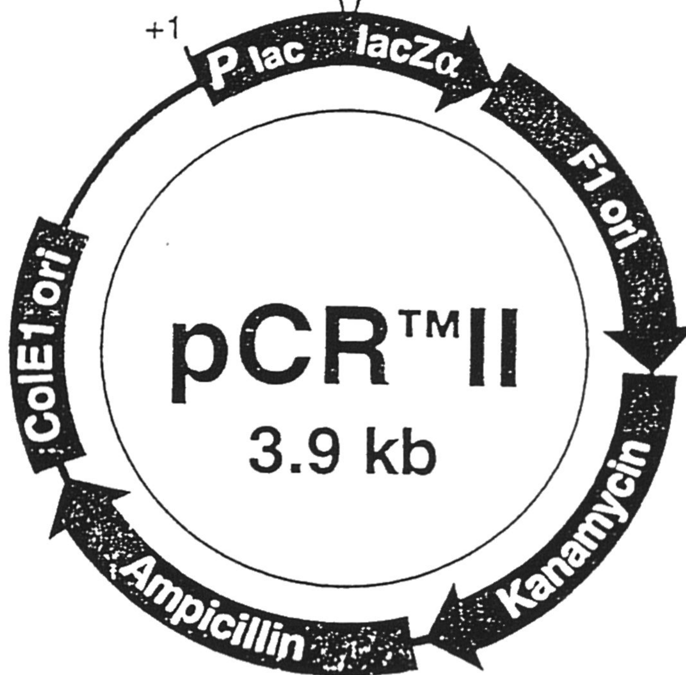


Figure 8: To tell the orientation of a fragment within the vector, the vector should be digested with two enzymes. One that is within the insert and another outside. The size of the resulting fragment will determine the direction of the insert.

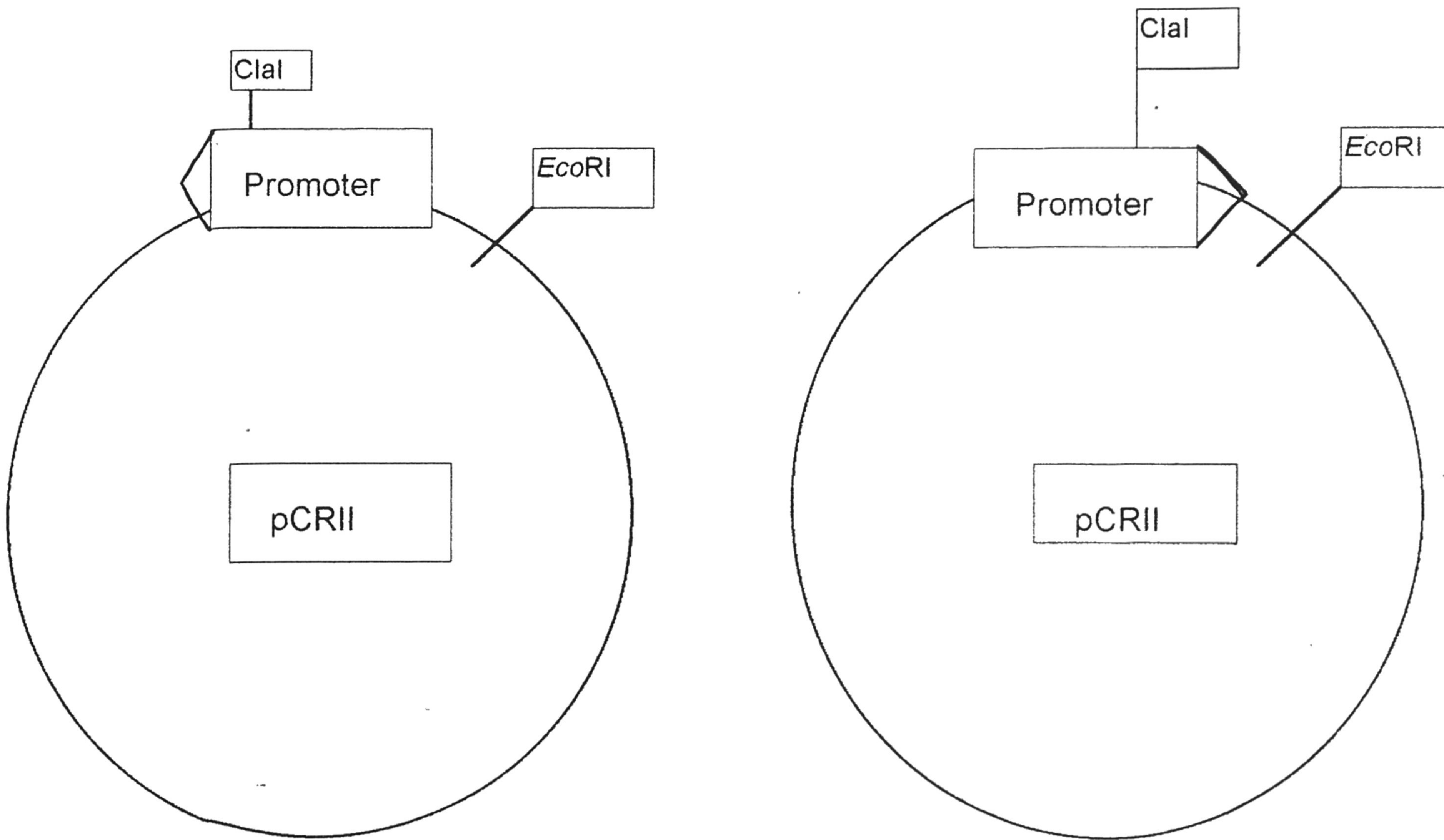
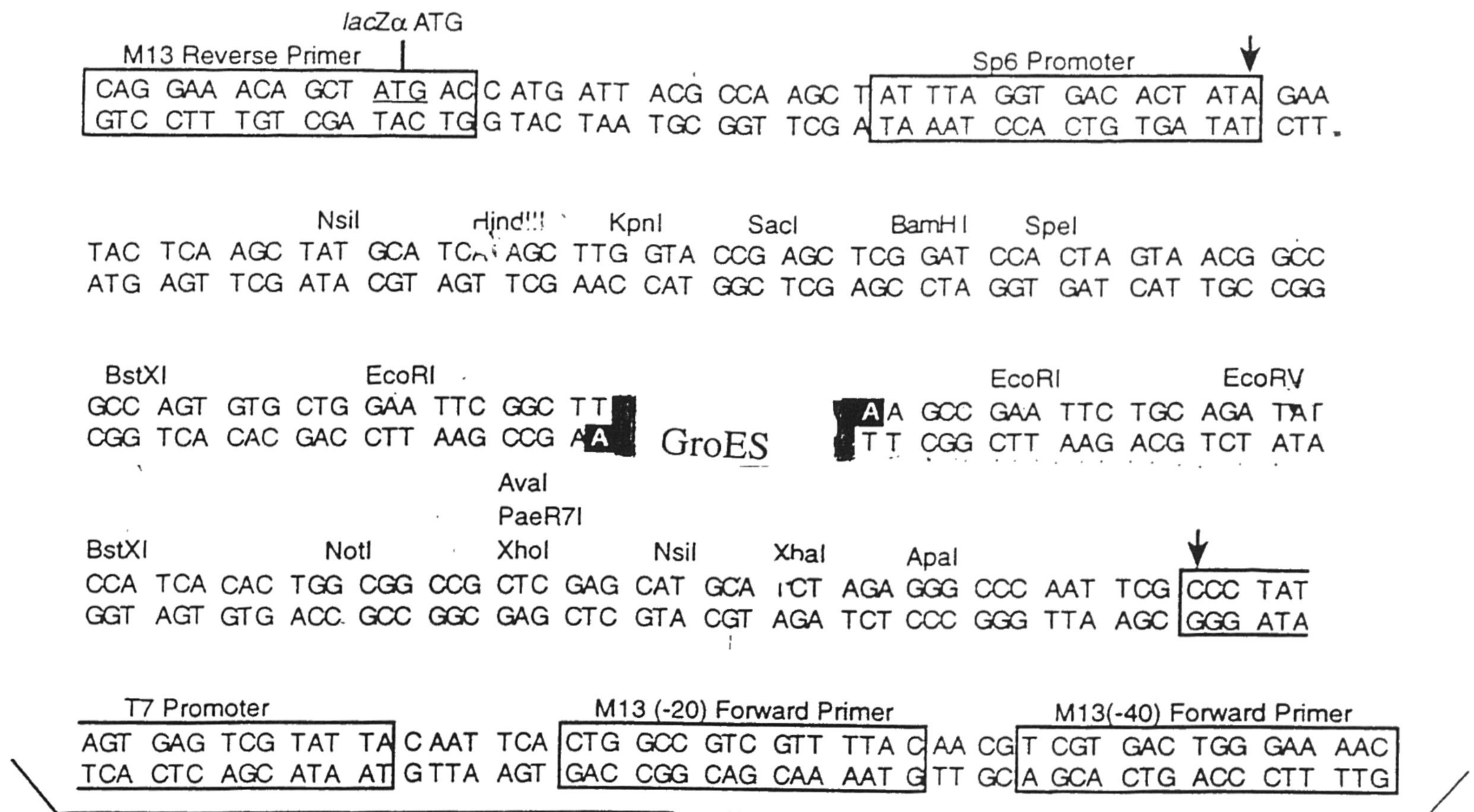


Figure 9: Vector map of pNAD2



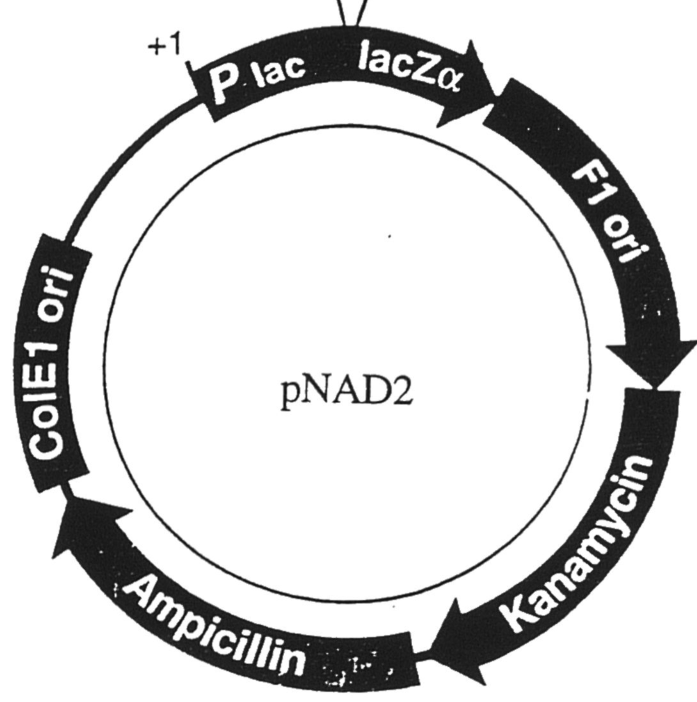


Figure 10 : Vector map of pNAD3