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Natural Killer (NK) cells represent a specialized lymphoid population that mediate innate immune responses against tumor or virally infected cells. NK cell cytotoxicity is regulated by inhibitory and activating receptors. Activating receptors include the Natural Cytotoxicity Receptors (NCRs), 2B4, and NKG2D. The NCRs play a key role in recognition and killing of tumor cells and include the receptors NKp30, NKp46, and NKp44. The ligands for the NCRs are not yet known. NKp44 is of particular interest because it is only expressed on activated NK cells, and is implicated in increased cytotoxicity and HIV infection.

To identify and clone the ligand for NKp44, a recombinant fusion protein containing the extracellular domain of NKp44 was constructed and used to identify a cell line, DB, expressing a ligand for NKp44. A directional complimentary DNA (cDNA) library was constructed from this cell line and screened by mammalian expression cloning, resulting in the isolation of several putative cDNA clones of NKp44 ligands.

APPROACHES TO CLONING AND IDENTIFICATION OF THE LIGAND FOR

NATURAL CYTOTOXICITY RECEPTOR,

NKp44

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APPROACHES TO CLONING AND IDENTIFICATION OF THE LIGAND FOR NATURAL CYTOTOXICITY RECEPTOR, NKp44

THESIS

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By

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CHAPTER I

INTRODUCTION TO STUDY

Natural Killer Cell Function

The immune system is comprised of specialized cells and organs that defend the body against foreign invaders and abnormal cells. Developing from hematopoietic stem cells within the bone marrow, the Natural Killer (NK) cell is a specialized lymphocyte that surveys for and kills abnormal or virally infected cells through cell cytotoxicity (31). Cytotoxic action involves the release of granzymes and perforin stored as granules within the NK cell and requires the activation of the NK cell, which can be achieved in several manners (23). First, cytokines such as Interleukin (IL) 2, IL-12, IL-15, and IL-18 activate and stimulate proliferation, cytotoxicity, and Interferon Gamma (IFN- γ) release (31). These cytokines are released by other cells of the immune system in response to infection and inflammation (31). NK cells can also be activated through a phenomenon called Antibody-Dependent Cellular Cytotoxicity (ADCC) by utilizing a surface receptor, CD16, which binds the constant heavy chain portion of antibodies known as the Fc fragment (31). In this event, the NK cell recognizes a target cell covered in antibody. This recognition results is NK cell activation and subsequent release of cytotoxic agents against the target cell. Finally, NK cells can be activated through direct cell-cell contact with target cells. Direct recognition of target cells depends on specific receptor/ligand

interactions to stimulate or inhibit NK cell action (22). KIRs (killer cell Ig-like receptors) recognize Major Histocompatibility Complex (MHC) I molecules presenting self antigens on the target cell surface and represent the inhibitory signal to the NK cells (23). Activating receptors include the Natural Cytotoxicity Receptors (NCRs), NKG2D, and 2B4 (23). These receptors induce cytotoxicity when activated by their ligands and result in target cell death when inhibitory signals are absent. It is the sum of signals received from both inhibiting and activating receptors that determines whether the NK cell remains inactive or becomes activated. Cell-cell interaction is vital in regulating NK cell immune surveillance especially against MHC Class I deficient tumor cells.

Natural Cytotoxicity Receptors

Among the activating receptors, the Natural Cytotoxicity Receptors play a key role in recognition and killing of MHC Class I deficient cells and include the receptors NKp30, NKp46, and NKp44. Binding of one or more of these receptors with a specific ligand induces strong NK cell activation and cytotoxicity (4). While little is known about the identity of the NCR ligands, studies since their discovery of the NCRs ten years ago yield some deductions regarding their nature and expression. Studies utilizing monoclonal antibodies against individual and combinations of NCRs have shown the receptors produce stronger cytotoxicity as a team rather than individually, suggesting simultaneous ligand expression (4, 15). Studies also show the lack of NCR ligand expression on normal cells; however, under pathological conditions expression is

observed (21). Furthermore, the ligands for the NCRs are differentially expressed on numerous tumor cell lines but still remain unidentified.

NKp44

Among the NCRs, NKp44 is of particular interest because it is only expressed on activated NK cells and is implicated in increased cytotoxicity and in Human Immunodeficiency Virus (HIV) infection (1, 18). NKp44 is a transmembrane glycoprotein of the Immunoglobulin (Ig) superfamily encoded on chromosome 6 (1). The structure of NKp44 consists of a 169 amino acid extracellular domain followed by 23 and 63 amino acid sequences in the transmembrane portion and the cytoplasmic tail respectively (1). Crystallography of the receptor demonstrates a surface groove made by two facing β hairpin loops extending from the Ig fold core stabilized by a disulfide bridge between Cystine 37 and Cystine 45 (3). The Ig domain contains an arrangement of positively charged residues at the groove surface, suggesting NKp44 ligands are anionic (3). The groove also appears wide enough to host a sialic acid or elongated or multi-branched ligand (3).

NKp44 surface expression and signaling physically requires the transmembrane accessory protein DAP12 which bears the Immunoreceptor Tyrosine Activation Motifs (ITAM) (1). NKp44 activates NK cells through DAP12 linked directly to Lysine 183 in the transmembrane domain (5). Interestingly NKp44's cytoplasmic domain also contains a tyrosine sequence that matches the context of tyrosine-based inhibitory motif (5). The function of this sequence is unknown as it does not contribute to any known signaling,

receptor down regulation, or internalization (5). Cross-linking of the ligand to the receptor is required for NK cell triggering which leads to calcium mobilization and the release of cytotoxic agents, Tumor Necrosis Factor-α, and IFN-γ (2). Cross-linking of NKP44 and the other NCRs has also been shown to up regulate the transcription, synthesis, and release of the Fas ligand (FasL) (4). FasL then interacts with Fas at the NK cell surface to induce NK cell suicide through activation of caspase 3, possibly representing an intrinsic regulatory mechanism to switch off cytolytic activity (4).

NKp44 expression is not limited to NK cells. Recent discoveries have shown that NKp44 is expressed on isolated polyclonal γδ T cells when cultured for 2 weeks in IL-15 and IL-2 (17). These cells showed marked cytotoxicity against myeloma cells, which could be reduced with monoclonal antibody against NKp44 (17). NKp44 has also been implicated in cross talk between the innate and adaptive immunities through its expression on natural interferon-producing cells (IPCs) located in the T-cell zone in lymph nodes draining a site of viral infection (6). In this case, NKp44 expression is induced by IL-3, possibly secreted from activated memory CD8 T cells, and cross-linking NKp44 in this environment leads to the down regulation of innate responses of IPCs to cytosine-phosphate-guanosine rich DNA (6).

NKp44 Ligands

Currently there is an intense search to identify ligands corresponding to the NCRs. Cytotoxicity studies have shown that numerous tumor cell lines express ligands for the NCRs. In fact NKp44 is implicated in the recognition and cytolysis of

nueroblastomas, glioblastomas, and choriocarcinomas (15, 16). However, no definitive ligand has yet to be isolated and characterized. Recent studies have demonstrated that cell surface heparan sulfate proteoglycans (HSPGs) are involved in the recognition of tumor cells by NKp44 and an increase in INF- γ production (10). HSPGs are found in the extracellular matrix of most mammalian cells and participate in events during cell adhesion, migration, proliferation, differentiation, and tumorigenesis (19). It is known that during tumorigenesis alterations in HSPG expression, targeting, and processing lead to malignant proliferation and differentiation (9, 19). NKp30 and NKp46 have also been shown to bind HSPGs; however, they recognize different HSPGs than NKp44 (10). Therefore, NCRs could be recognizing these uncommon HSPGs through modified glycosylations in a form of "transformed cell pattern recognition" (9, 10). However, HSPGs role in lysis is unclear as it is postulated that they serve as possible co-ligands similar to heparan sulfate interactions with growth factors and growth factor receptors (10).

NKp44 Immune Function

NCRs are also responsible for recognizing virally infected cells. Functional interactions between NKp44 and the hemagglutinin of influenza and hemagglutinin-neuraminidase of Sendai viruses have recently been reported (7, 8). These interactions depend on the sialyation of NKp44; however, viral ligand binding has been shown to involve separate receptor epitopes on the NCRs from cellular ligands, which is not dependent on sialic acid (7, 8, 10). Additionally, recent work show all three NCRs are

needed for NK cells to recognize vaccinia virus infected cells dependent on the expression of an early viral gene (14).

In addition to viral and transformed cell recognition, NK cells have been shown to have a controversial role in bacterial immunity. While the current thought is that the majority of this role is due to NK cell interaction with accessory cells, such as macrophages and dendritic cells, there is an increasing body of evidence to suggest direct interactions between bacteria and NK cells (29). This is supported through recent discoveries implicating NKp46 and NKp44. NKp46 has been shown to directly bind and lyse *Mycobacterium tuberculosis*-infected macrophages through the recognition of vimentin (30). A more recent report describes NKp44's direct binding to the surface of *Mycobacterium bovis* (28). NKp44 bacterial ligand expression was also proven to be conserved among the *Mycobacterium* genus as well as *Pseudomonas aeruginosa* (28). Whether the bacterial ligand is similar to the viral or cellular ligand, as well as its identity, remains to be seen.

NKp44 in Disease

NKp44 has also been implicated in numerous diseases. Reports show that disease can modulate not only the expression of the NCRs but their ligands as well. For instance, NK cells of Acute Myeloid Leukemia (AML) patients show decreased expression of NCRs over healthy individuals (13). NK cells removed from these patients exhibited decreased killing of autologuous AML blasts even after blocking inhibitory signaling receptors on the NK cells (13). Systemic Lupus Erythematosus (SLE) patients not only

demonstrate a decreased number of NK cells, but also a decrease in DAP12 expression (11). DAP12 deficient humans show reduced expression of NKp44 on activated NK cells as well as increased systemic bone cyst formation (11).

More importantly, NKp44 has very strong implications in HIV infection. Similar to AML, HIV down regulates the expression of NCRs and overall decreases NK cell activation (12, 20). NK cells collected from HIV infected individuals show a functional impairment to kill tumor cells, possibly explaining increases in opportunistic tumors in HIV patient (12). HIV infection has also been proven to induce the expression of the cellular ligand for NKp44 (NKp44L) in infected CD4 T cells (18). NKp44L is induced by a highly conserved motif of the gp41 viral envelope protein and correlates with the progression of CD4 T cell depletion and an increase in viral load (18). High expression of NKp44L make infected CD4 T cells extremely sensitive to NK lysis; however, after antiretroviral therapy, CD4 T cell killing is reduced in association with reduced NKp44L expression (18).

Significance

The NCRs represent an emerging field in NK cell work. Since the NCR's discovery nearly ten years ago there has been an intense effort to further understand their function in NK cell immune surveillance. Identifying their ligands as well as understanding why their ligands are expressed and on what cell types is a key area to comprehending overall NK cell function. It is evident that NKp44 and the NCRs play a vital role in recognizing and killing tumor and virally infected cells. Identification of

their ligands will also enable further modeling of NK cell activation and killing of target cells. Additionally, complete characterization of these ligands will provide vital information that can be used to identify markers for disease onset and progression as well as develop novel immunotherapies and medicines.

NKp44 is of particular significance since its expression is confined to activated NK cells and results in a large increase in cytotoxicity (1). In addition, NKp44 is significant in HIV infection as its ligand is inducible by a portion of the HIV envelope protein (18). Isolating and identifying the ligand for NKp44 is paramount to understanding NKp44's specific function in the immune system. Knowledge of this function as well as harnessing ligand expression will be a powerful tool to modulate and understand NK cell activity.

Therefore, we hypothesize that NKp44 recognizes a cellular ligand on target cells and interaction between NKp44 and its ligand regulates NK cell cytotoxicity. In order to evaluate this hypothesis, a recombinant fusion protein containing the extracellular domain of NKp44 will be constructed. This fusion protein will then be used to identify a cell line expressing a ligand for NKp44. Finally the ligand can be isolated using mammalian expression cloning techniques.

CHAPTER II

IDENTIFICATION OF CELL LINE EXPRESSING THE LIGAND FOR NKp44

The search for cells lines expressing ligands for the Natural Cytotoxicity Receptors (NCR) has been a major area of NCR research within the past five years. This work was augmented by the advent of the recombinant fusion protein. Today's cloning techniques has enabled researches to isolate and clone the extracellular portions of surface receptors. The DNA encoding this portion of the receptor can easily be ligated to DNA encoding the constant heavy chain of Immunoglobulin γ (IgG), also called the Fc fragment. This construct [Figure 2.1], when placed in a plasmid vector, can be used to produce fusion protein containing the extracellular portion of the receptor of interest fused to the Fc fragment of IgG, which can easily be tagged with a monoclonal antibody. By simply transfecting the plasmid construct into mammalian cells, milligrams of fusion protein can be produced, concentrated, and analyzed in a matter of days. Fusion proteins are now widely used in research, especially for identifying expression of NCR ligands. Previous work within the laboratory utilizing a fusion protein containing the extracellular portion of NKp44 has identified a cell line expressing a putative ligand for NKp44. Flow cytometry analysis demonstrated binding of the NKp44 fusion protein to a cell line (DB), isolated from a patient with Diffuse Large B Cell Lymphoma (DLBCL), that could be abrogated with monoclonal antibody against NKp44 [Figure 2.2].

Diffuse Large B Cell Lymphoma

Lymphoma is a cancer of the lymphocytes composing the immune system and is the most common blood cancer among adults (32). There are more than 30 types of lymphoma, 5 being classified as Hodgkin lymphoma and in excess of 25 types of the more common non-Hodgkin lymphoma (NHL), including DLBCL (32). Lymphoma occurs when B or T lymphocytes grow abnormally and turn malignant (32). Lymphocytes are found throughout the body; thus, lymphomas can arise in all areas ranging from lymph nodes to bone marrow and other tissues and organs. DLBCL is the most common NHL, accounting for 30 % of new cases, and is a very aggressive, fast growing form of lymphoma requiring immediate treatment (33). DLBCLs frequently exhibit a wide range of genetic abnormalities: gene translocations, aneuploidy, somatic mutation, as well single DNA point mutations (33). While none of these defects represent the exact cause for the disease, which remains unknown, they do allow for evaluation of prognostic factors on a case by case basis as these defects are not consistent in every instance of DLBCL (33). Current treatment methods include a combination of chemotherapy and anti-CD20 monoclonal antibody, Rituxan, which can lead to remission if not a cure (32, 33).

Figure 2.1 Fusion Protein Plasmid Construct. The extracellular portion of NKp44 (V-like domain) fused to human IgG1 constant heavy chain 2 and 3 (CH2, CH3).

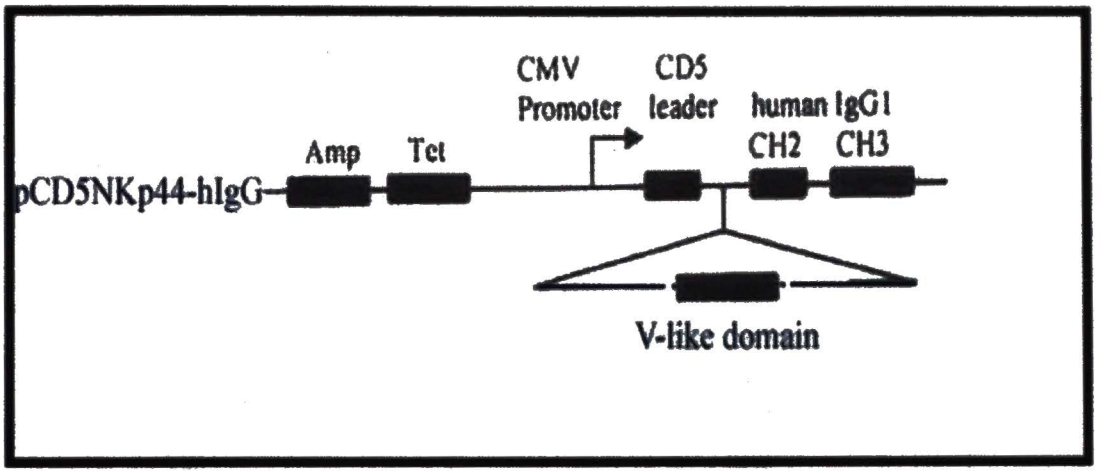
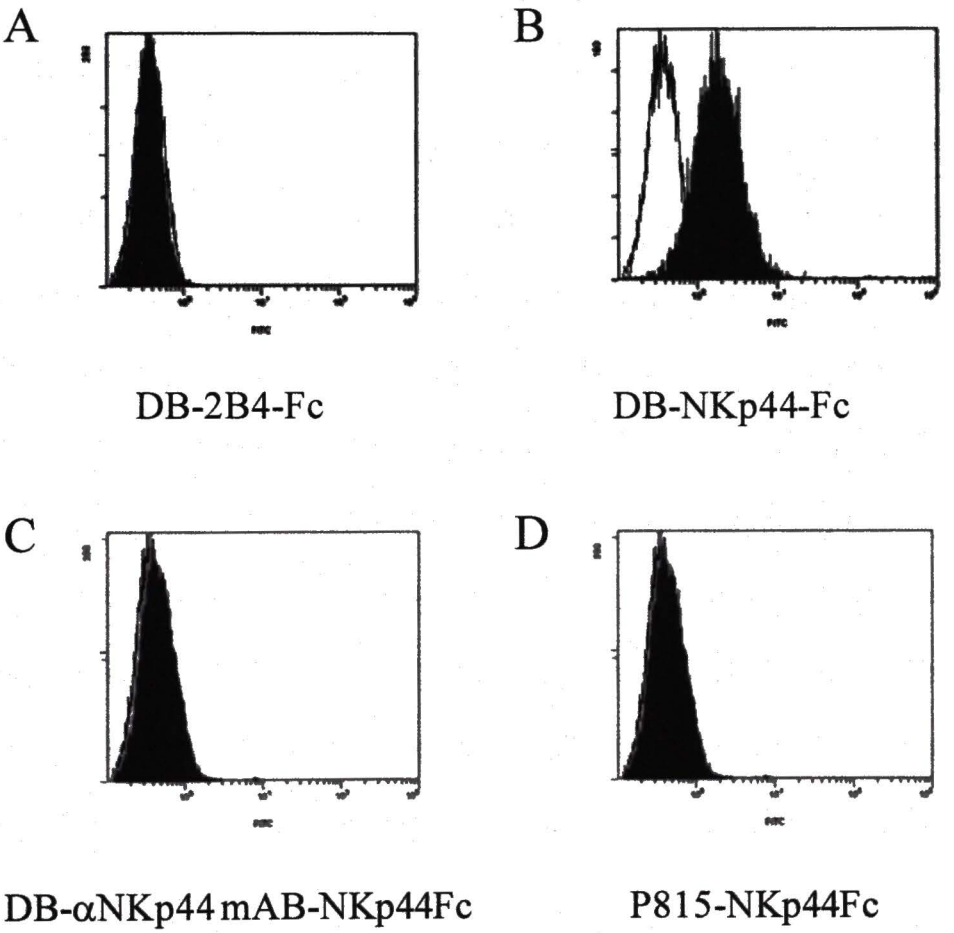


Figure 2.2 Flow Cytometry Analysis of Cell Lines Expressing a Ligand for NKp44.

A. Isotype control 2B4 fusion protein B. 100 microgram (ug) NKp44 fusion protein binding DB cells C. 100 ug NKp44 fusion protein binding blocked by anti-NKp44 monoclonal antibody D. P815 tumor cell line not expressing the ligand for NKp44



CHAPTER III

CONSTRUCTION OF DIRECTIONAL cDNA LIBRARY

The key to isolating the ligand for NKp44 from the DB cell line lies in constructing a high quality complementary DNA (cDNA) library. A cDNA library is a collection of DNA that is reverse transcribed from messenger RNA (mRNA). During normal cellular function, genes encoding proteins are transcribed from DNA to mRNA. The mRNA is then translated into protein by the ribosome and the protein is expressed. Once the mRNA is isolated it can be reverted back to DNA using a modified reverse transcriptase from the Moloney Murine Leukemia Virus. The resulting cloned DNA is complementary to the mRNA and represents the genes being expressed by the cells at the time of mRNA harvesting. The cDNA can then be directionally ligated into a plasmid vector containing a Cytomegalo Virus (CMV) promoter enabling expression of the cloned cDNAs in eukaryotic cells. Directional ligation of the cDNAs into the plasmid vector is highly favored over random insertion. Utilizing specific adapters, the cDNA is cloned into the vector in a specific orientation relative to the transcriptional polarity of the original mRNA. In other words, every cDNA insert is cloned into the vector so it can be properly expressed by the CMV promoter. In a random library, some of the clones would contain cDNA oriented properly for expression while others would contain cDNA inserted backwards yielding nonsense protein and greatly decreasing the efficiency of the library.

A superior cDNA library starts with the extraction of quality mRNA from cells in log phase growth. To this end, total RNA was first extracted from DB (Human B cell Lymphoma) cells. mRNA was purified from the total RNA and analyzed for quality. cDNA was then constructed from the mRNA, ligated into pCMV SPORT 6 vector, and amplified in *E. coli*.

Specific Aim I

Isolate mRNA from cells expressing a ligand for NKp44 and construct a directional cDNA library.

Material Methods

Cell Culture

DB cell line was maintained in RPMI 1640 supplemented with 10% FetalPlex Animal Serum (FPX) (Gembio, West Sacramento, CA), 2 millimolar (mM) glutamine, 100 Units per Mole (U/mol) penicillin, 100 U/mol streptomycin, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10mM non essential amino acids at 37°C in a humidified 5% CO2/95% air environment.

Isolation of Messenger RNA

To obtain high quality mRNA, total RNA was first extracted from DB cells using RNA STAT-60 according to manufacturer's protocol (Tel-test Inc, Friendswood, TX). mRNA was purified from total RNA using Invitrogen's FastTrack 2.0 kit (Invitrogen, Carlsbad, CA). The FastTrack kit allows for the purification of mRNA by hybridizing the poly Adenosine (poly A) tail of mRNA to a deoxy Thymidine (dT) oligomer coupled to a solid-phase matrix under high salt conditions. After removing other contaminating RNAs, mRNA is released from the dT oligomer by lowering the ionic concentration during eluting. Both total and mRNA were electrophoresed on 1% agarose gel in a denaturing loading buffer at 100 volts for 50 minutes to analyze quality. Denaturing loading buffer consisted of 2 microliters (ul) 3-(N-morpholino) propanesulfonic acid (MOPS), 3 ul Formaldehyde, and 10 ul Formamide per ul of Total RNA. Samples were incubated with buffer for 10 minutes at 65°C.

cDNA Construction

cDNA was constructed from mRNA using the Superscript Plasmid System for cDNA Synthesis and Cloning kit (Invitrogen, Carlsbad, CA) [Figure 3.3]. An oligo dT primer linked to a *Not I* restriction enzyme site adapter is used to prime the first strand synthesis reaction by binding the poly A tail of mRNA. DNA is reverse-transcribed using a modified Moloney Murine Leukemia virus reverse transcriptase lacking RNase, which degrades RNA. After completing first strand synthesis, RNase H is used to nick the RNA bound to the first strand. The nicked RNA serves as primers for DNA

polymerase I facilitated second strand construction. *Sal I* adapters are added after second strand completion to allow for unidirectional ligation in to the pCMV-SPORT 6 vector [Figures 3.3-3.5]. cDNA fragments were then size fractioned by column chromatography to ensure residual adapters do not enter the library. This process also increases the probability of cloning large cDNA fragments. Fractioned cDNA was then ligated into the pCMV SPORT 6 vector utilizing T4 DNA ligase [Figures 3.4, 3.5]. cDNA ligated vectors were transformed into Electromax DH10B competent cells by electroporation. Following one hour of recovery in Super Optimal Catobolite (SOC) medium at 37°C, 1 and 10 ul were removed for serial dilution plating on Ampicillin Lysogeny Broth (LB) plates. Remaining transformants were amplified in 450 milliliter (ml) 2x LB with 1.35 grams Sea Prep agarose at 30°C for 45 hours. Sea Prep agarose forms a semi-solid media which allows for three-dimensional amplification of transformants, greatly reducing the loss of slow growing clones. After incubation, amplified transformants were collected and glycerol stocked for storage purposes. Individual aliquots were amplified in 1 liter (L) of LB and plasmid DNA was extracted. Plasmids were digested with *Not I* and *Sal I* for 4 hours at 37°C and electrophoresed on a 1.2% agarose gel for 50 minutes at 100 volts followed by Ethidium Bromide staining to determine presence and size of inserts.

Results

DB cells were grown until cells reached log phase growth, attaining 100 % confluency 24 hours after splitting. 1.5×10^8 DB cells where collected for Total RNA extraction. Total RNA fractions were analyzed for ribosomal RNA subunits by gel

electrophoresis [Figure 3.1]. Total RNA from fractions containing all 28s, 18s, and 5s ribosomal subunits was combined into one sample totaling 1.3 milligrams (mg) for mRNA extraction.

mRNA extraction yielded 35 ug of high quality mRNA. 5 ug of mRNA, starting amount for cDNA synthesis, was examined by gel electrophoresis for size and brightness of Ethidium Bromide staining. mRNA collected exhibited a bright band with a size range of 3 kb to .5 kb [Figure 3.2], both aspects of high quality mRNA. mRNA was divided into 5 ug aliquots for storage and cDNA construction.

cDNA synthesis begins with 5 ug of mRNA. cDNA was constructed, fractionated, and ligated into the pCMV-SPORT 6 vector as described above. Following transformation of the vector into DH10B *E. coli* cells, serial dilutions were made to quantitate the library. Plating the dilutions yielded a library containing around 1.22 $x10^6$ clones. After amplifying the library, plasmid DNA was extracted and 3 ug were digested as described above. Gel electrophoresis of the plasmid DNA showed a strong streak of inserts ranging in size from 4kb to .5 kb [Figure 3.6 B]. Additionally, individual colonies from serial dilution plates were analyzed for size and presence of cDNA inserts [Figure 3.7].

Figure 3.1 Electrophoresis of Total RNA. Ribosomal RNA fragments indicated. Each lane represents 1ul of Total RNA.

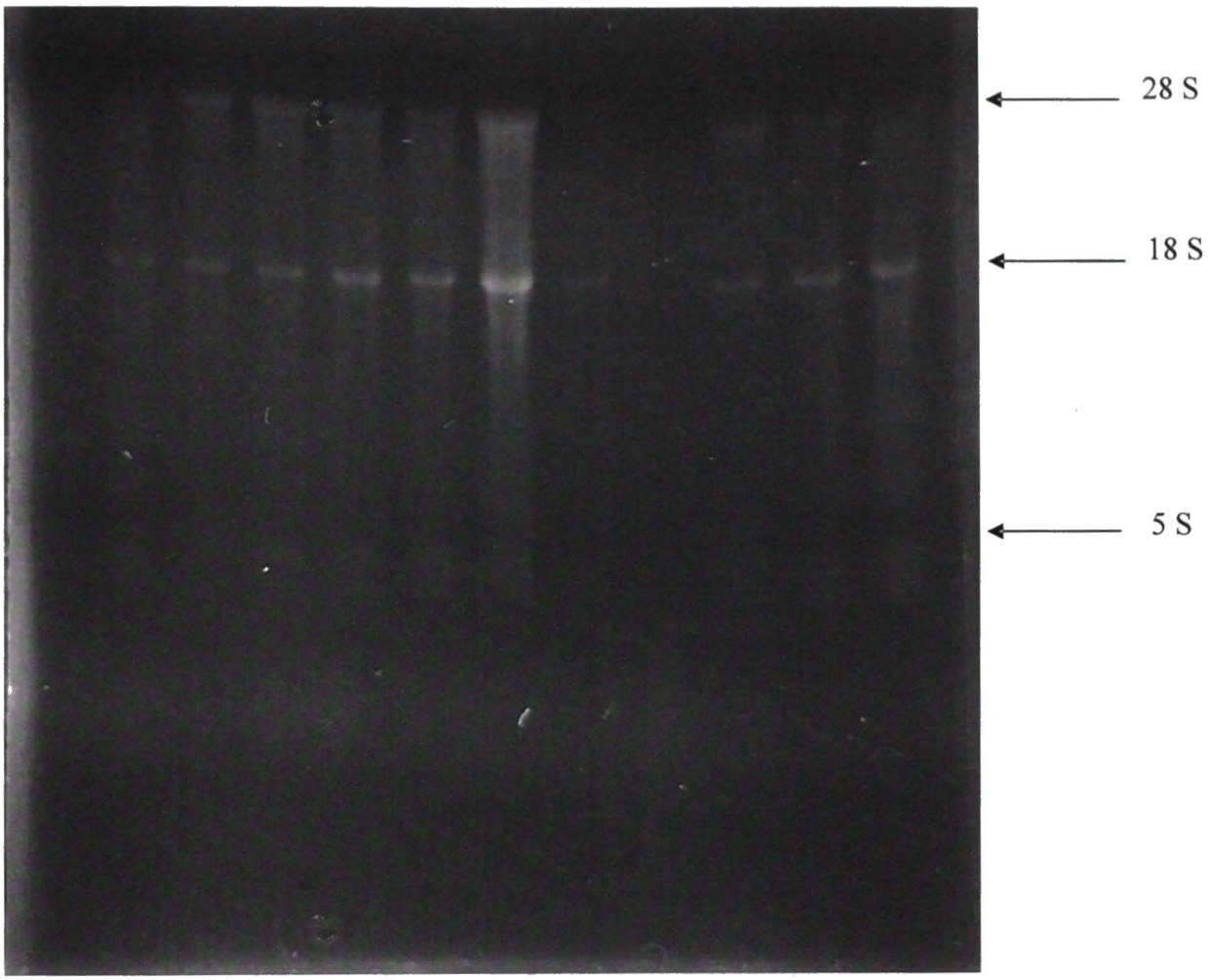


Figure 3.2 Electrophoresis of mRNA.

Lane 1. 100 base pair (bp) DNA ladder

Lane 2. 5 microgram (ug) mRNA

Lane 3. 1 kilobase (kb) pair DNA ladder

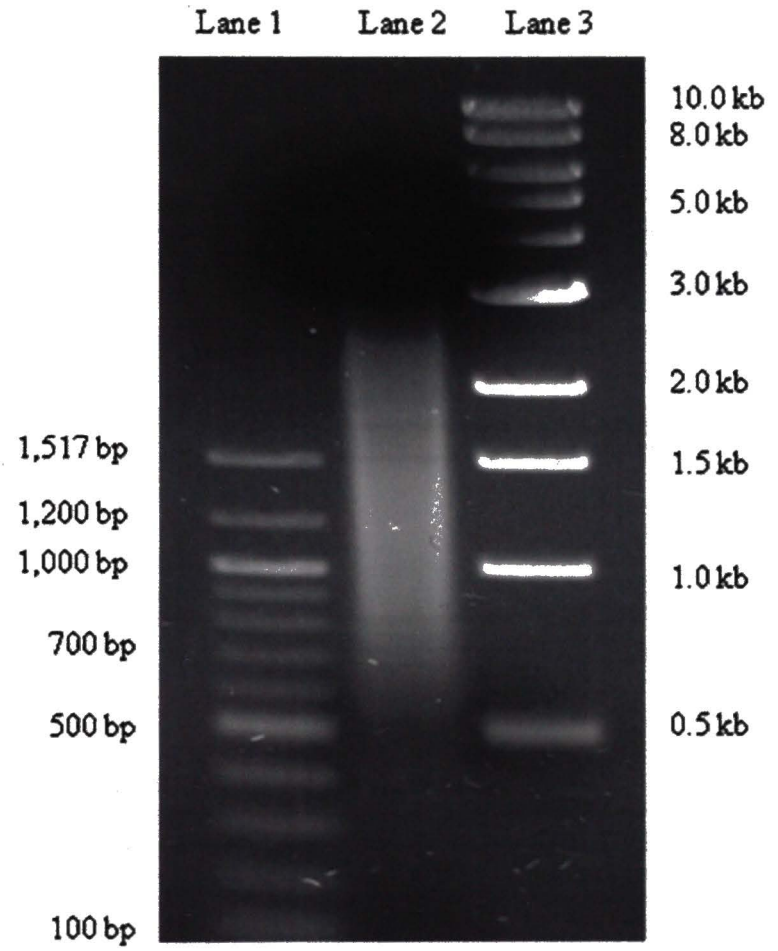


Figure 3.3 Complementary DNA Construction Overview.

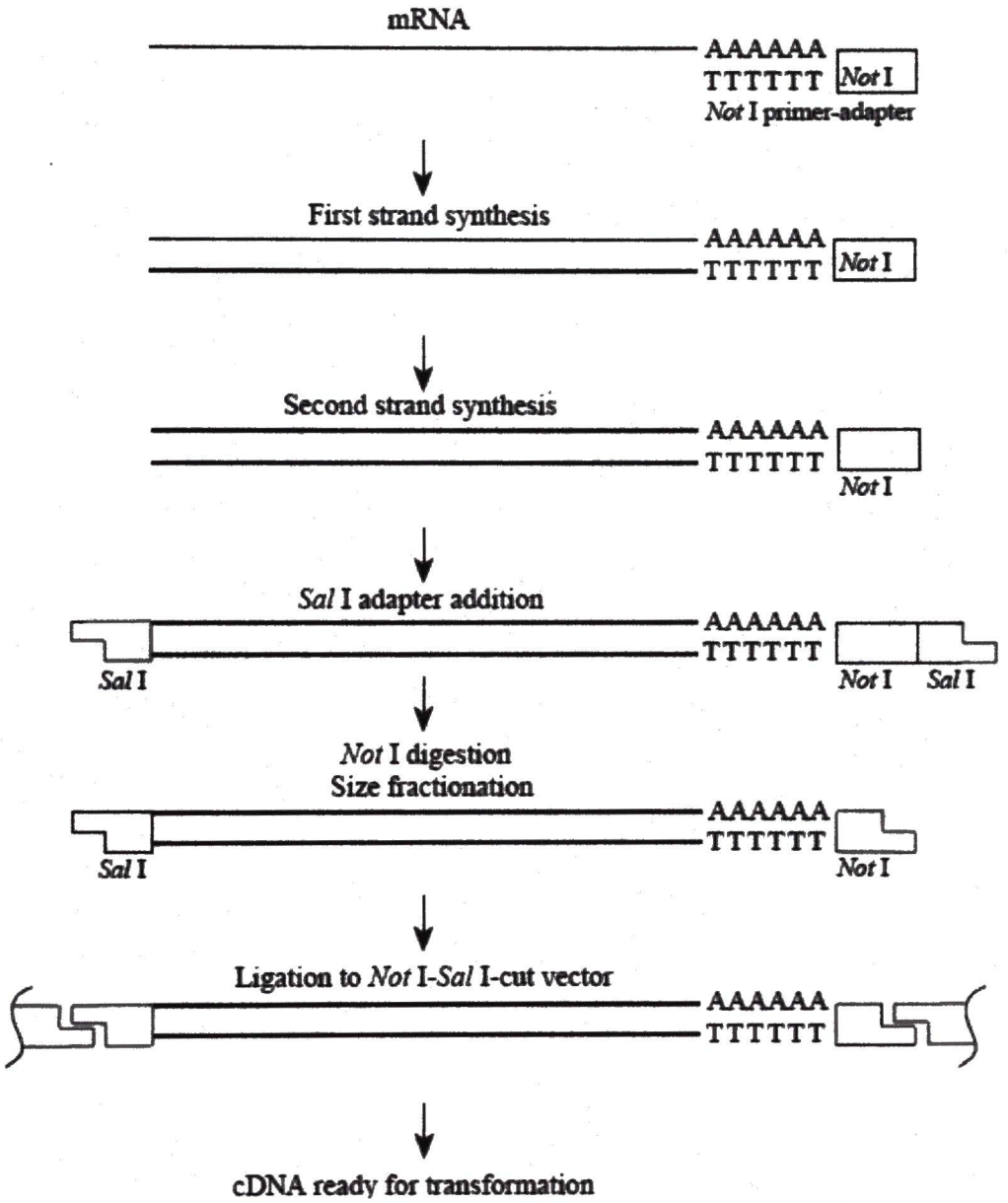


Figure 3.4 pCMV-SPORT 6 Vector Map.

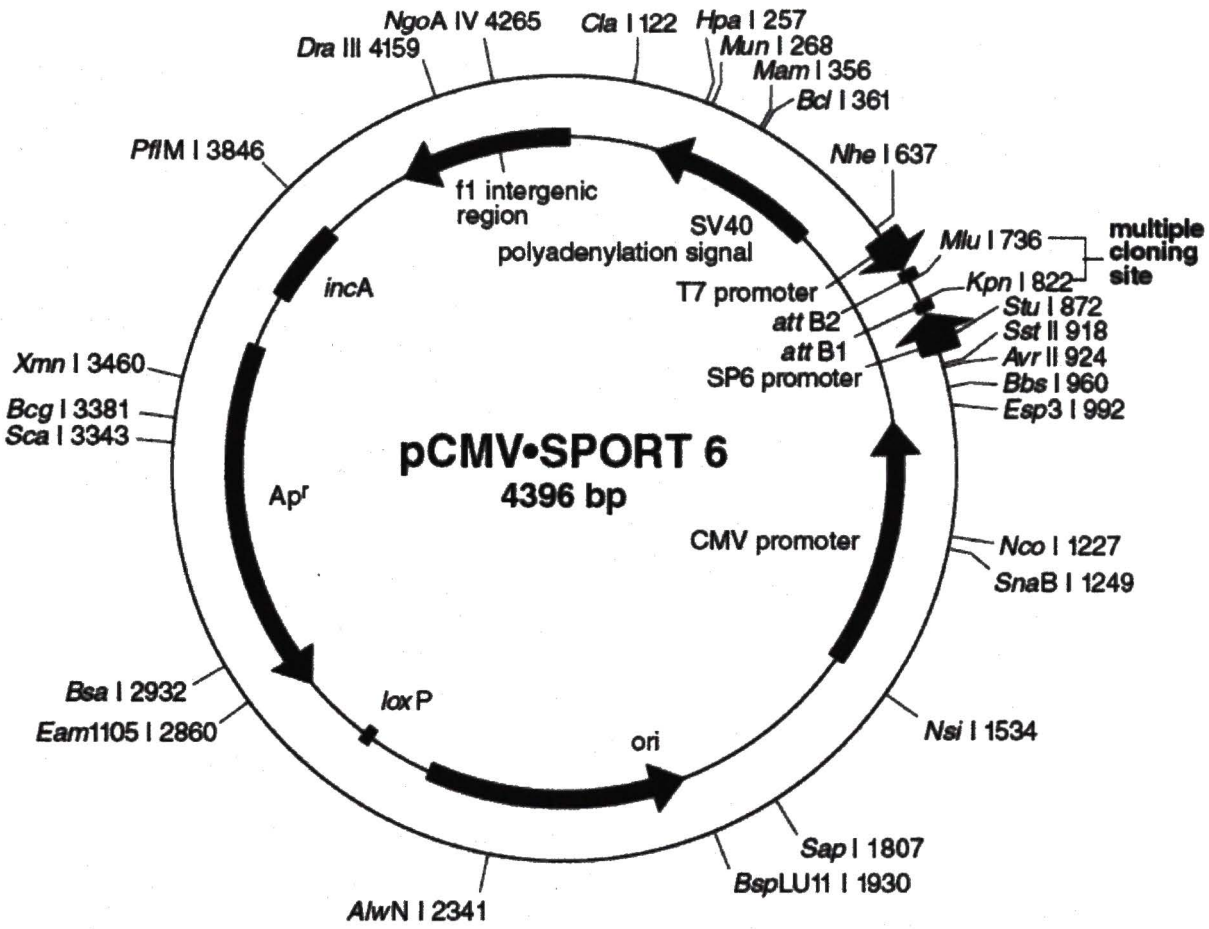
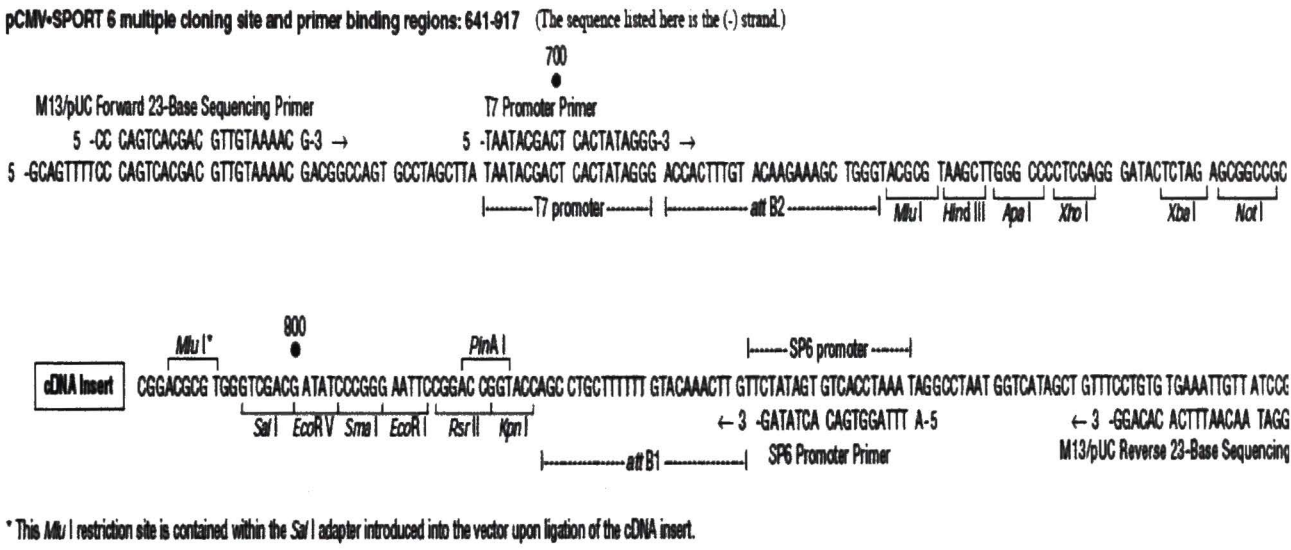


Figure 3.5 Multiple Cloning Site of pCMV-SPORT 6 Vector.



* This *Mlu* I restriction site is contained within the *Sal* I adapter introduced into the vector upon ligation of the cDNA insert.

Figure 3.6 Digestion of cDNA. A. 2 hr *Eco R I and Xho I* digestion of 10 individual clones from serial dilution LB plates of a cDNA library constructed with Stratagene kit next to 1 kb DNA ladder. B. 4 hr *Not I* and *Sal I* digestion of 3 ug of amplified cDNA library constructed with Invitrogen kit next to 1 kb DNA ladder.

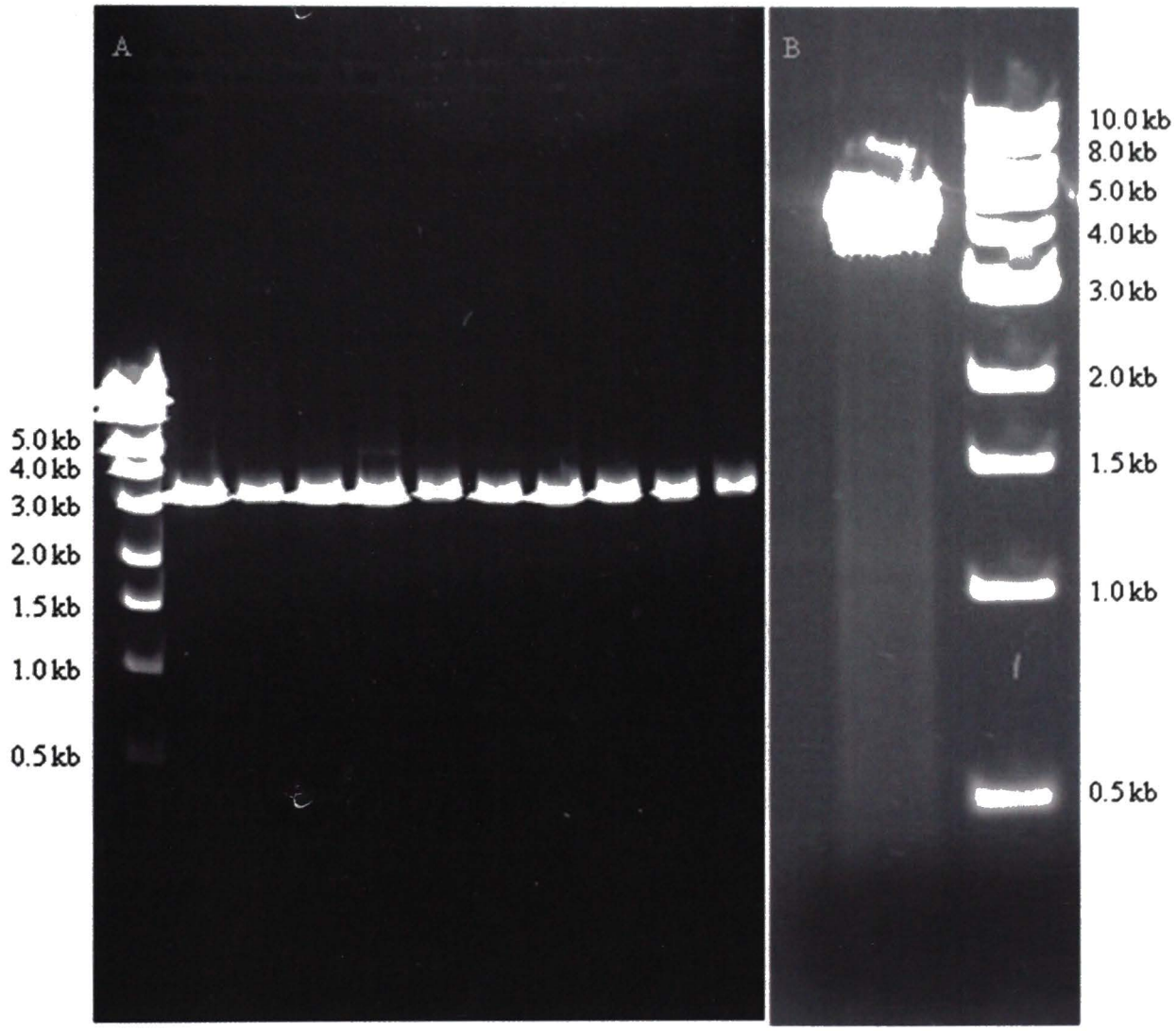
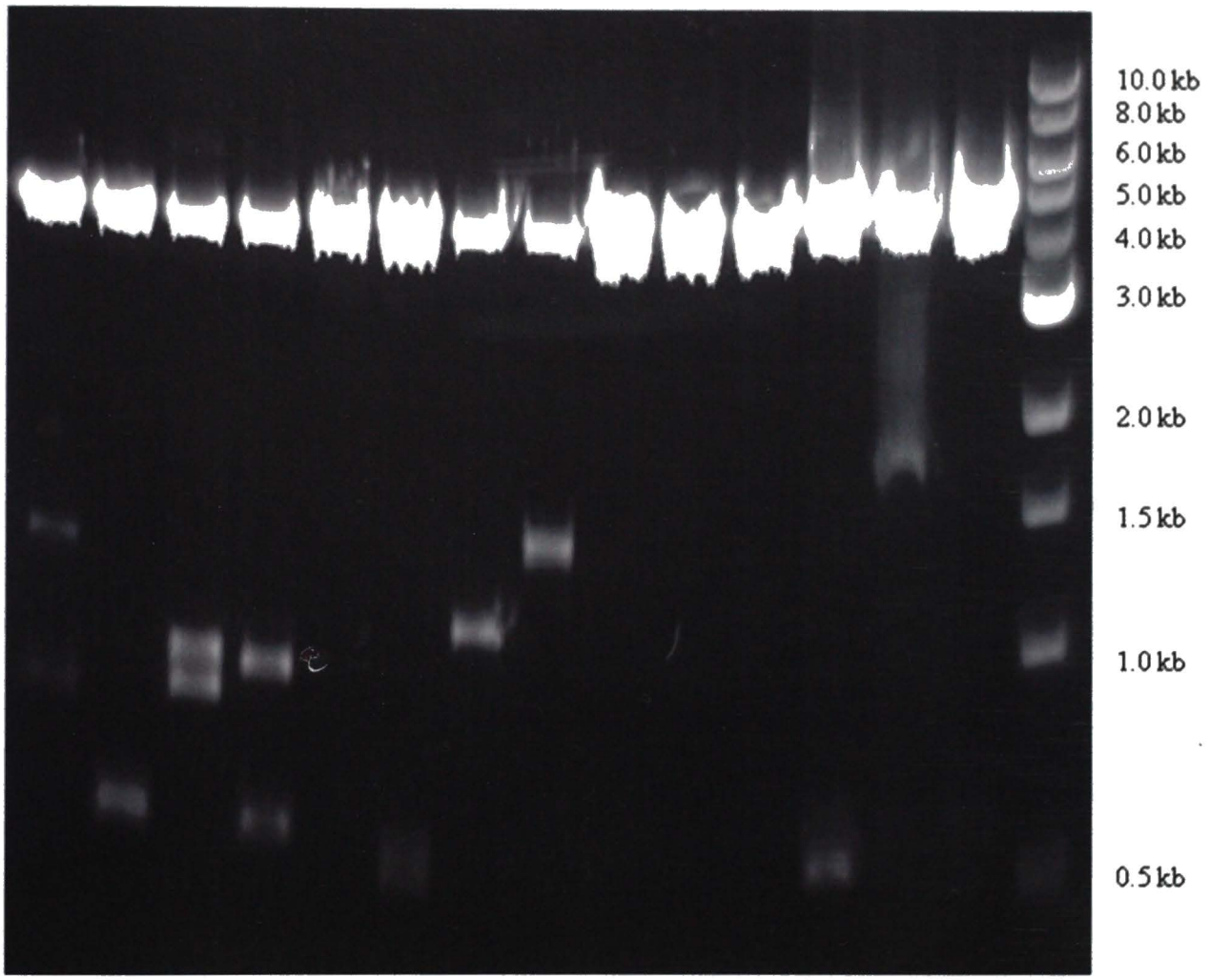


Figure 3.7 Digestion of Individual cDNA Clones. 4 hr *Not I* and *Sal I* digestion of 14 individual colonies next to 1kb DNA ladder.



CHAPTER IV

MAMMALIAN EXPRESSION CLONING

Mammalian expression cloning involves the detection of protein expressed from individual complementary DNA (cDNA) clones first popularized by Brian Seed in 1987 to isolate the CD2 antigen on T cells (35). This form of cloning involves expressing a cDNA library in mammalian cells and selecting cells expressing a cDNA clone of interest. cDNA expression in mammalian cells is facilitated by the Cytomegalo Virus (CMV) promoter present in the pCMV-SPORT 6 vector. In this setting, cells transfected with DB (Human B Cell Lymphoma) cDNA expressing the NKp44 ligand will be selected utilizing the NKp44-Immunoglobulin γ (IgG) fusion protein. The mammalian expression method has two important advantages over bacterial expression systems. First, the selection method detects protein in its native state as opposed to the denatured state in bacteria. Second, the mammalian system provides for glycosylations, which are extremely important in NKp44 recognizing its ligand. However the system does have one steep disadvantage, it is very tedious and has limited success. Another pitfall of the system is the use of the fusion protein. Little is known about the binding strength of the fusion protein to the ligand as it is certainly not as strong as a monoclonal antibody or full receptor binding. Despite these disadvantages this method remains the best viable option to clone the ligand to NKp44.

Mammalian expression cloning begins by transiently transfecting aliquots of the DB cDNA library into B16 (mouse melanoma) cells using the Fugene 6 Reagent. Two days after transfection, cells are gently detached from the culture dishes and incubated with NKp44-IgG fusion protein. The labeled cells are then incubated in petri dishes coated with anti-human IgG Fc, referred to as panning plates. This procedure positively selects for cDNA transfected cells expressing possible NKp44 ligands through two linkages. Transfected cells expressing the NKp44 ligand will be bound by the NKp44 extracellular domain portion of the fusion protein. In turn, the IgG Fc region of the fusion protein will be bound by the anti-human IgG coated to the bottom of the panning plate [Figure 4.2]. The panning plates are washed to remove cells not secured by anti-IgG. Remaining cells are then lysed and plasmid cDNA is recovered. This process will enrich for plasmids carrying cDNA encoding the ligand for NKp44 and by repeating the protocol several times [Figure 4.1], ligand cDNA can be resolved from the whole pool of DB cDNA. Confirmation of the cDNA encoding the ligand can be made through flow cytometry analysis.

Specific Aim 2

Isolate cDNA clones encoding the ligand for NKp44 utilizing mammalian expression cloning

Methods

Cell Culture

B16 (mouse melanoma) cell line was maintained in RPMI 1640 supplemented with 10% FetalPlex Animal Serum (FPX) (Gembio, West Sacramento, CA), 2 millimolar (mM) glutamine, 100 Units per Mole (U/mol) penicillin, 100 U/mol streptomycin, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), and 10mM non essential amino acids at 37°C in a humidified 5% CO2/95% air environment.

Fusion Protein Production

NKp44 fusion protein was produced by transiently transfecting B16 cells with NKp44 fusion protein construct using Fugene 6 Reagent (Roche, Indianapolis, IN) at a ratio of 3 microliter (ul) Fugene to 1 microgram (ug) DNA in a 100 millimeter (mm) tissue culture plate. Transfected cells were incubated for 24 hrs in Opti-MEM (Invitrogen, Carlsbad, CA) followed by collection of supernatant and replacement of media every 24 hours for 2 days. Following collection supernatants were concentrated on an Amicon Ultra 30k (Millipore, Billerica, Ma). Concentrated fusion protein was analyzed by western blot and flow cytometry for functionality.

Mammalian Expression Cloning

Mammalian expression cloning was carried out by transiently transfecting B16 cells with the DB cDNA library using Fugene 6 Reagent at a ratio of 3ul Fugene to 1 ug DNA in a 100 mm tissue culture plate. Transfected cells were incubated for 36-48 hrs in

Opti-MEM followed by gentle collection with phosphate buffered saline (PBS)/1milliMolar (mM) ethylene diamine tetraacetic acid (EDTA). Collected cells were resuspended in PBS/1mM EDTA/5% FPX with 50 ug/milliliter (ml) NKp44-IgG fusion protein and incubated on ice for 45 min. Cells were spun through PBS/1mM EDTA/2% Histopaque 1077 (Sigma-Aldrich St Louis, MO) for 4 min at room temperature and resuspended in .5ml PBS/1mM EDTA. Cells were then incubated for 3 hrs in Anti-human IgG Fc (Rockland Gilbertsville, PA) coated panning plates containing 3ml PBS/1mM EDTA/5% FPX.

Panning plates were made by adding 3 ml of 10 ug/ml anti-human IgG Fc in 50 mM Tris HCl pH 9.5 to 60 mm bacteriological petri dishes. Dishes were left at room temperature for 1.5 hours after which the solution was removed to a second dish for another 1.5 hours followed by a third dish for 1.5 hours. All plates were washed three times with 0.15 Molar (M) NaCl and incubated overnight with 3 ml 1 mg/ml Bovine Serum Albumin (BSA) in PBS. PBS/BSA solution was removed the following day and plates were stored at -20^{o}C until needed.

Following 3 hours incubation in panning plates, unbound cells in solution were aspirated and panning plates were washed gently with PBS/5% FPX to further remove unbound cells. Plasmid DNA was recovered from adherent cells by Hirt's Method (Hirt, 1967). Briefly, Hirt's method uses .4 ml of .6% Sodium Dodecyl Sulfate (SDS)/10 mM EDTA to lyse bound cells on the pan plate. Following 20 minutes of incubation, the solution is removed to a microcentrifuge tube and .1 ml of 5 M NaCl is added. The mix

is kept at -20°C overnight. The next day the mix is centrifuged at 10,000 g for 4 minutes. The supernatant is removed and extracted with phenol/chloroform. Finally the plasmid DNA is precipitated. The concentration of plasmid DNA may be very low; therefore, 10 ug of yeast tRNA is added to help facilitate precipitation.

DNA recovered by Hirt's method was electroporated into Electromax DH10B *E. coli* at the listed specifications: 25 uF, 200 Ω and 1.8kV, time constants 4.2-4.3 seconds. After recovery in Super Optimal Catobolite (SOC) medium and one hour incubation at 37°C, the resulting colonies were amplified over night in 25 ml Lysogeny Broth (LB) at 37 °C. Plasmid DNA for transfection and analysis was further amplified in 500 ml and harvested. Amplified plasmid DNA was digested after each round of panning with *Not I* and *Sal I* for 4 hours and imaged by gel electrophoresis on a 1.2% agarose gel. The above process was repeated three times with plasmid DNA recovered from adherent transfected cells used in each successive B16 transfection [Figure 4.1]. Repeating this process enriches for clones carrying the ligand cDNA.

FACS analysis and Cell Sorting

5 x 10^5 cells per reaction were washed with 1% PBS-BSA. Cells were resuspended in 100 ul PBS/1% BSA and 2 ul of human IgG Fc fragment blocking solution was added to prevent non-specific binding of PE to Fc receptors on the cell surface. Following 20 minutes of incubation on ice, cells were washed in the same manner as above and resuspended in 100 ul of PBS-BSA. 50 ug of NKp44-IgG Fc fusion protein was added. Cells were washed twice in PBS-BSA following 45 minutes of

incubation on ice with fusion protein. Cells were resuspended in 100 ul of PBS-BSA and 2 ul of anti-human IgG Fc conjugated to PE (Beckman Coulter, Fullerton, Ca) was added and incubated for 20 minutes on ice in the dark. Cells were then washed twice, resuspended in 1 ml of PBS-BSA, and analyzed on the Beckman Coulter Cytomics FC500 Flow Cytometer. Cells were labeled in the same manner and sorted on the Cytopia InFlux Cell Sorter.

Results

After the first round of panning, plasmid cDNA was recovered, amplified, and 3 ug were digested [Figure 4.3]. Digestion yielded an array of inserts ranging between 2 kb and 500 bp. The same procedure was repeated for the second round of panning using cDNA retrieved from the first panning attempt. Digestion of 3 ug of amplified cDNA yielded inserts spanning near the same size region as first panning inserts [Figure 4.4].

Prior to a third panning attempt, cells transfected with cDNA retrieved from the second panning were analyzed by flow cytometry for fusion protein binding [Figure 4.5]. Around 0.1% of cells analyzed were positive for binding of PE-anti-IgG to the Fc portion of the fusion protein. All cells transfected with second panning DNA were then sorted based on the level of PE signal [Figure 4.6]. Plasmid DNA from cells highly positive for PE binding was retrieved and amplified in the same manner as before. Individual colonies were analyzed for cDNA inserts by digestion as previously described [Figure 4.7]. In addition to empty vector, a cDNA insert around 800 bp was retrieved. This

cDNA clone was individually amplified and transfected into B16 cells for flow cytometry analysis of fusion protein binding, which was negative [Figure 4.13 D]

Third panning further eliminated cDNA species. Following amplification of cDNA recovered from third panning, three LB/ampicillin plates were streaked with amplified culture to isolate single colonies. Single colonies were picked and grown over night in 5 ml LB with ampicillin. Gel electrophoresis yielded sizeable inserts in 4 out of 14 colonies analyzed [Figure 4.8]. One colony with a cDNA insert of around 1.5 kb was further amplified for evaluation. After transfection of the cDNA clone into B16 cells, it was found that the 1.5 kb cDNA clone did not facilitate binding of the fusion protein compared to B16 cells transfected with empty vector by flow cytometry [Figure 4.13 C, E]. In addition, bulk plasmid DNA from the third panning was amplified and 3 ug were digested as previously described [Figure 4.8].

Since none of the cDNA clones resolved through three panning attempts elicited binding of the fusion protein, first panning attempt cDNA was revisited. B16 cells were again transfected with cDNA retrieved from the original first panning [Figure 4.3]. As opposed to original panning plates that only received one wash, one group of plates received two washes while the other received three washes prior to lysing any cells attached to the panning plate.

Plasmid cDNA recovered from panning plates receiving two washes was amplified as before. Amplified cDNA was digested for analysis by gel electrophoresis [Figure 4.10]. Digestion yielded a group of cDNA inserts around 800 kb in size. Three

LB/Ampicillin plates were streaked with amplified bacteria from panning plates receiving two washes. Single colonies were amplified, digested, and analyzed for single cDNA inserts [Figure 4.11]. Gel electrophoresis of the digestion product showed all but one of the ten colonies contained an insert of about 700 bp [Figure 4.11]. This cDNA clone was further amplified to analyze fusion protein binding by flow cytometry. The 700 bp cDNA clone did not elicit fusion protein binding compared to B16 cells transfected with empty vector [Figure 4.13 F].

Plasmid cDNA recovered from pan plates receiving three washes was transformed and plated across three LB/ampicillin plates and incubated overnight at 37°C. Plating resulted in the growth of only six colonies, which were individually amplified and plasmid DNA was digested and analyzed by gel electrophoresis [Figure 4.12]. Three of the six colonies contained a cDNA insert about 500 bp in size. Sequence data from all three cDNA inserts contained no open reading frames or homology to the human genome through a BLAST search.

Figure 4.1 Overview of Mammalian Expression Cloning.

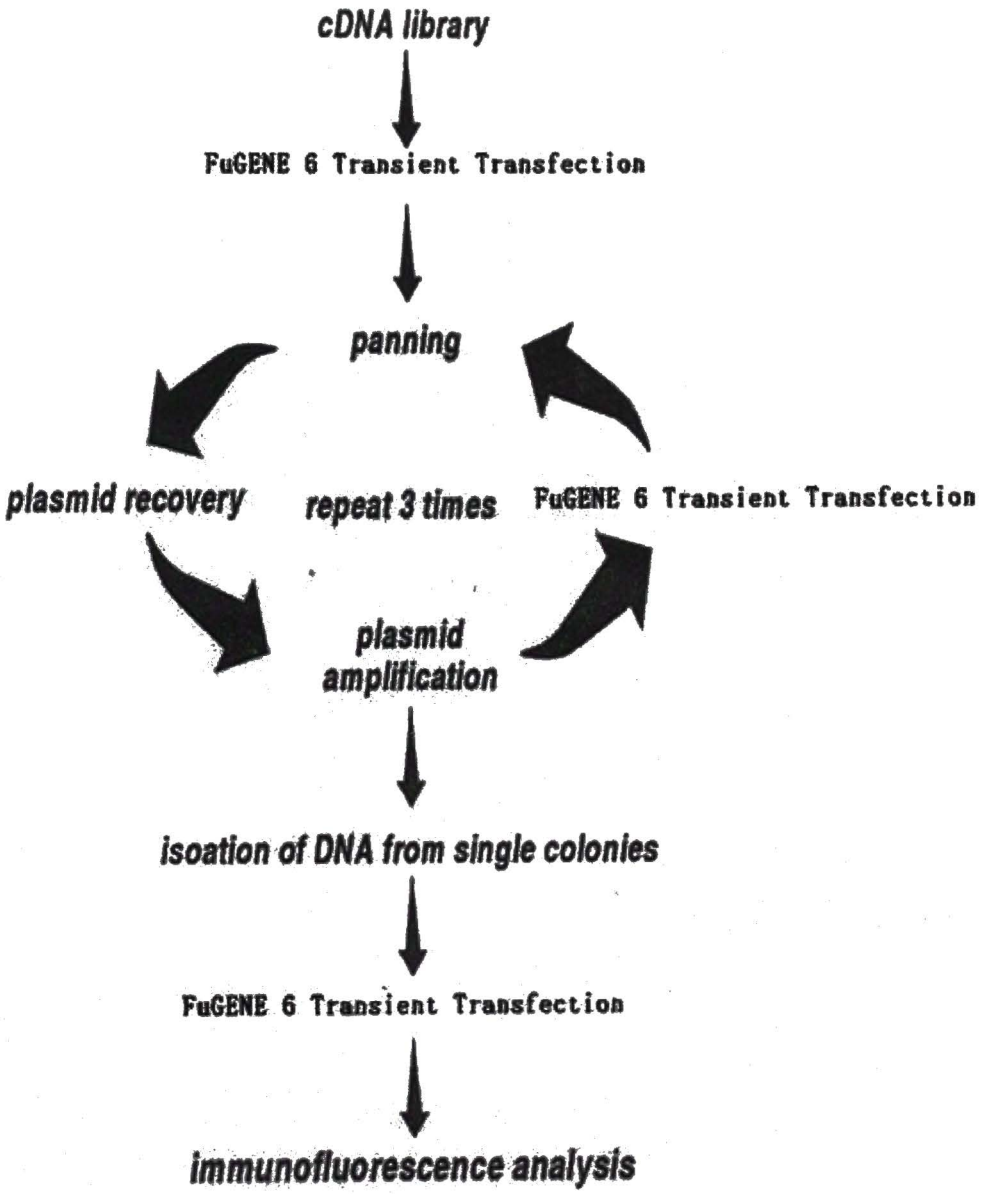


Figure 4.2 Diagram of Panning Plate Procedure. cDNA expressed ligand will be bound by the NKp44 region of the fusion protein. The IgG Fc region of the fusion protein is then bound by Anti-IgG Fc coated to the plate to secure the cell to the panning plate during washes.

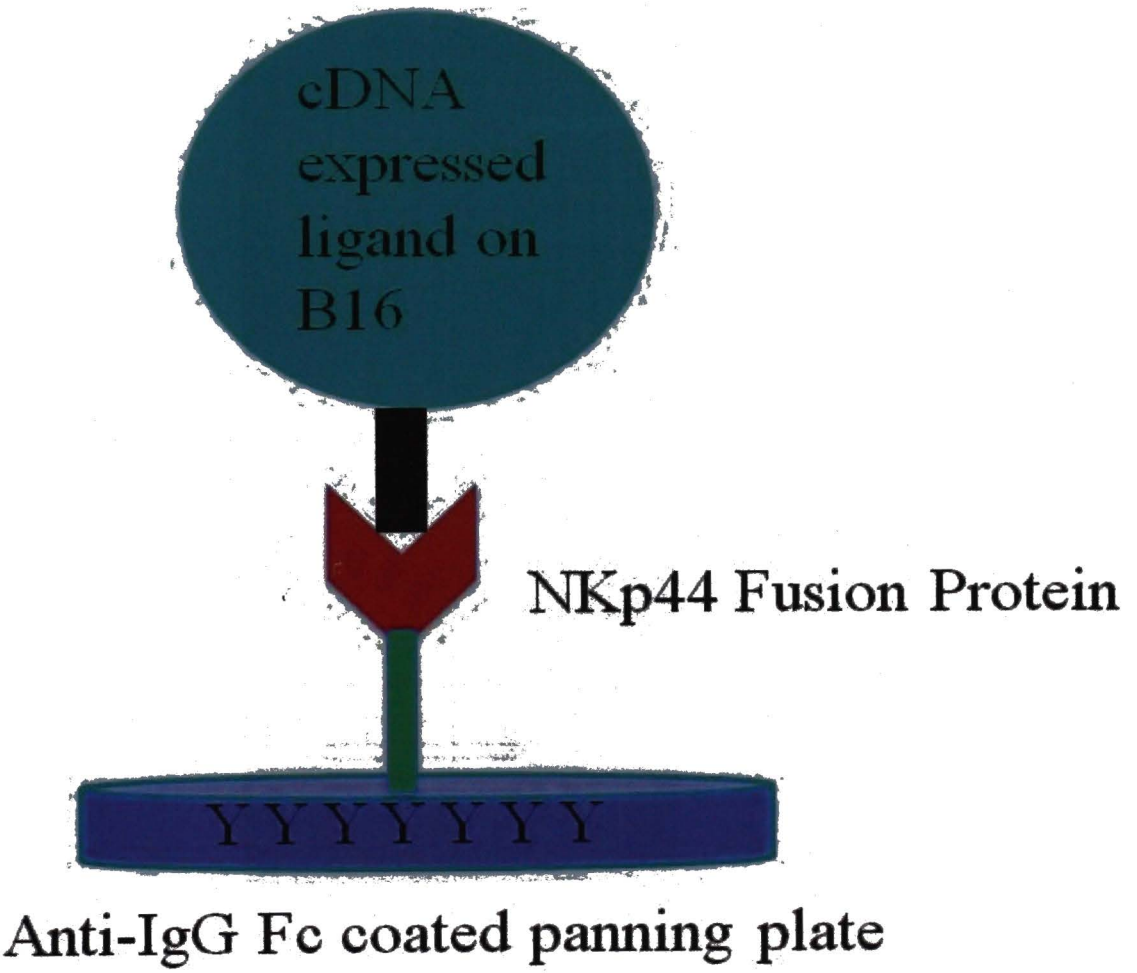


Figure 4.3 Digestion of Amplified First Panning cDNA. 4 hr *Not I* and *Sal I* Digestion of 3 ug amplified first panning cDNA next to 1 kilobase (kb) DNA ladder.

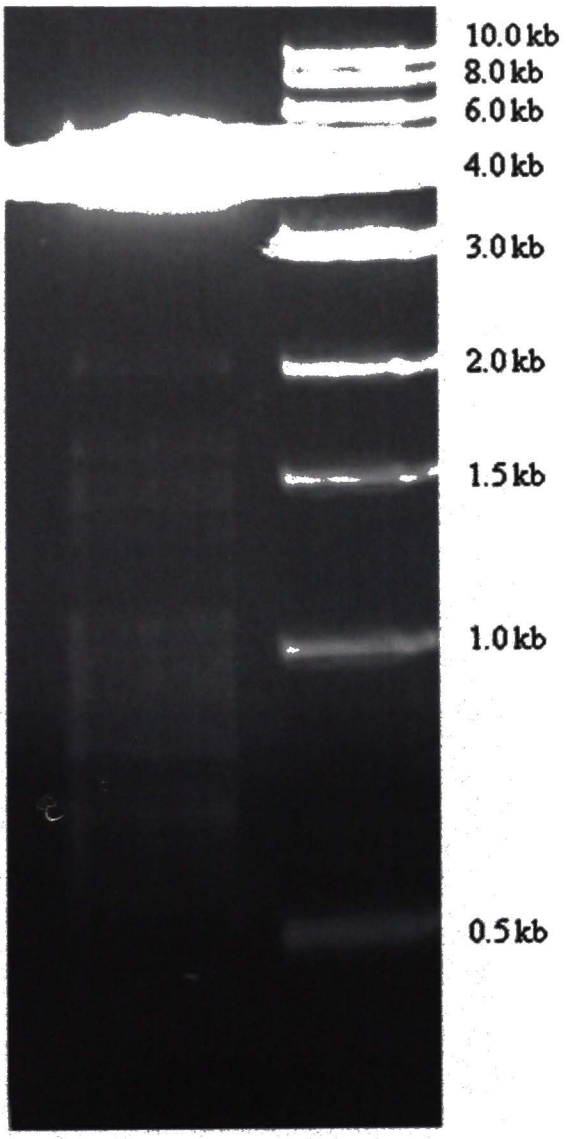


Figure 4.4 Digestion of Amplified Second Panning cDNA. 4 hr *Not I* and *Sal I* Digestion of 3 ug amplified second panning cDNA next to 1 kb DNA ladder.

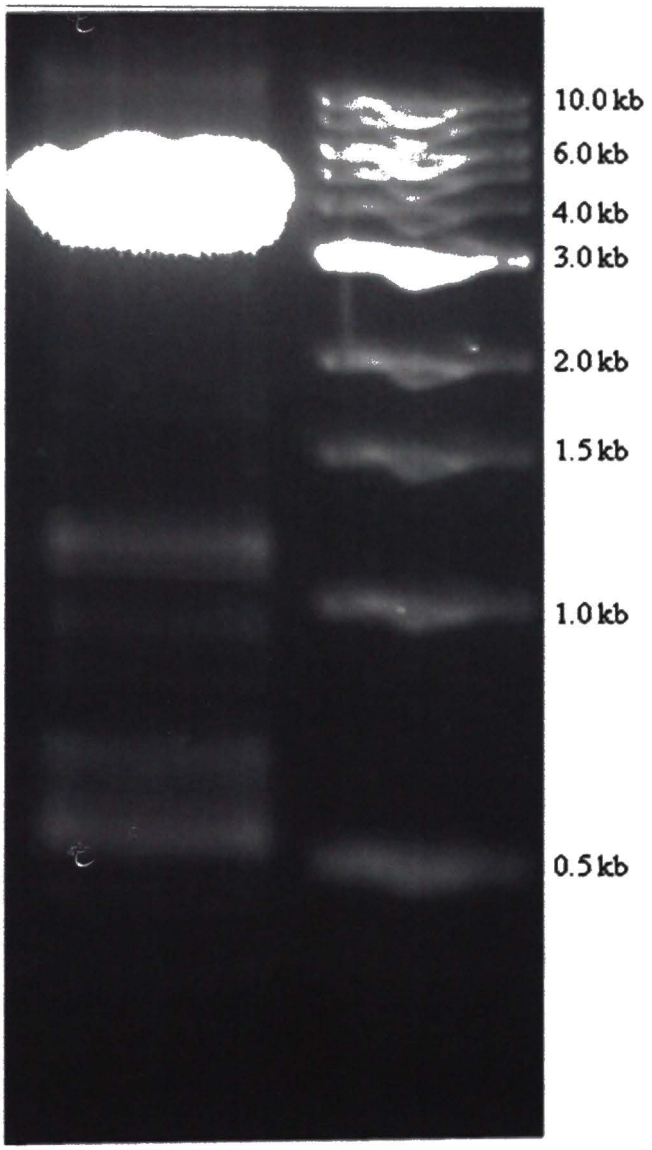


Figure 4.5 Dot Plot Analysis of B16 Cells Transfected with Second Pan cDNA by Flow Cytometry. Red dots represent cells not bound by PE. Green dots are cells binding PE, presumably through binding of fusion protein. FL2 Log represents log intensity of PE staining. FSLin represents forward scatter, or size of each cell.

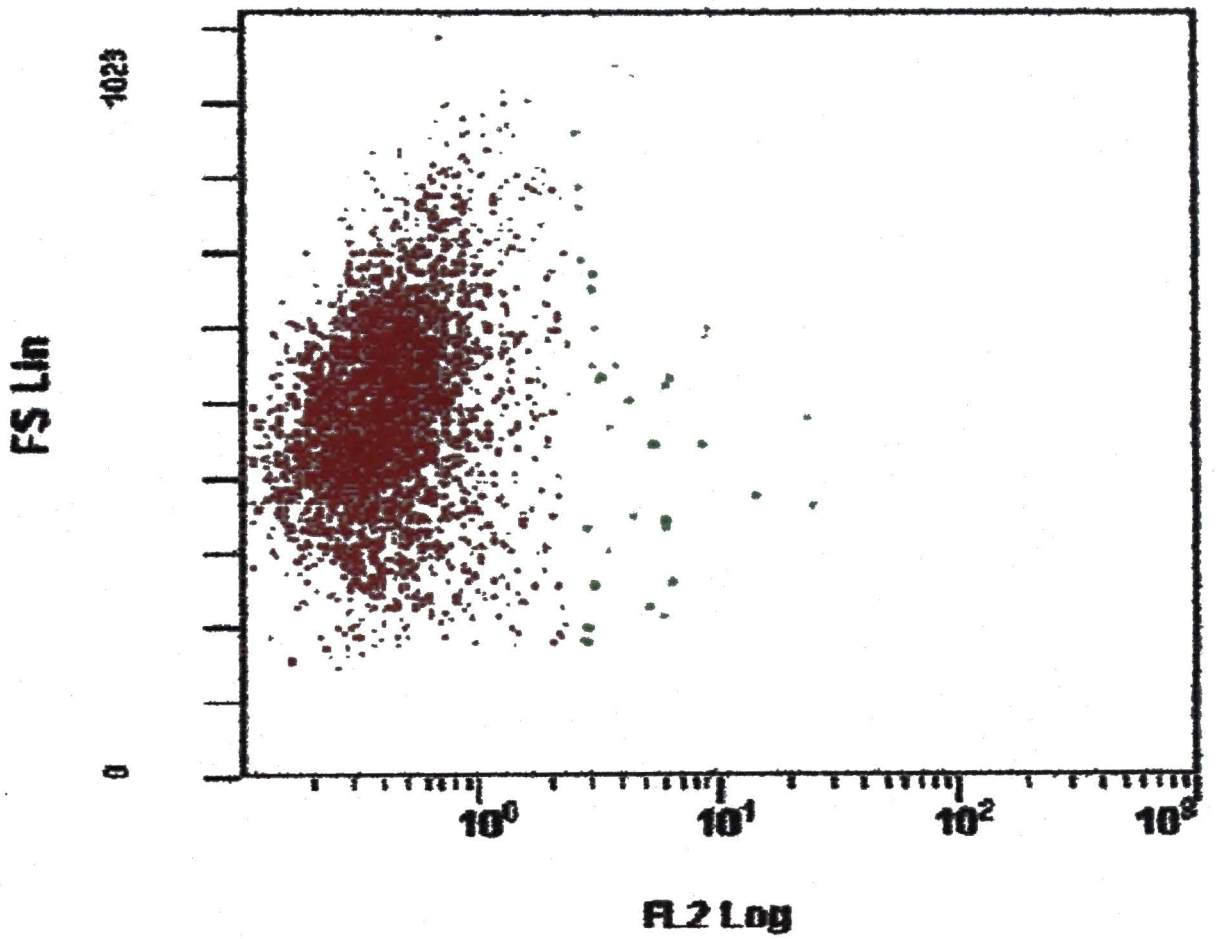


Figure 4.6 Screen Shot from Cytopia Cell Sorter. ADC4 represents log intensity of PE staining. ADC1 represents forward scatter, or size of each cell. Green dots represent cells highly positive for PE staining and those that were sorted and processed.

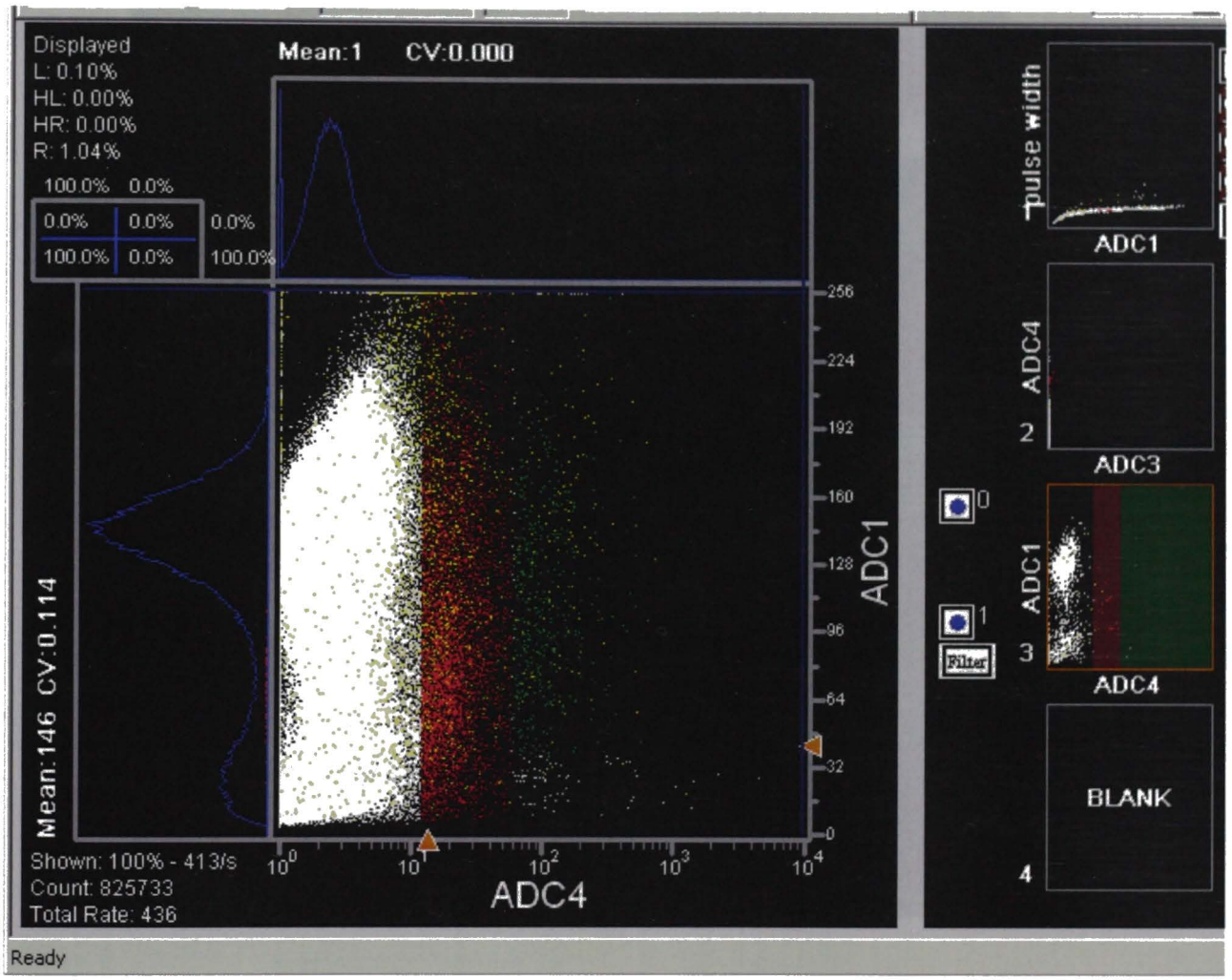


Figure 4.7 Digestion of cDNA from Sorted Cells. 4 hr *Not I* and *Sal I* digestion of plasmid cDNA from single colonies isolated from cells sorted for fusion protein binding.

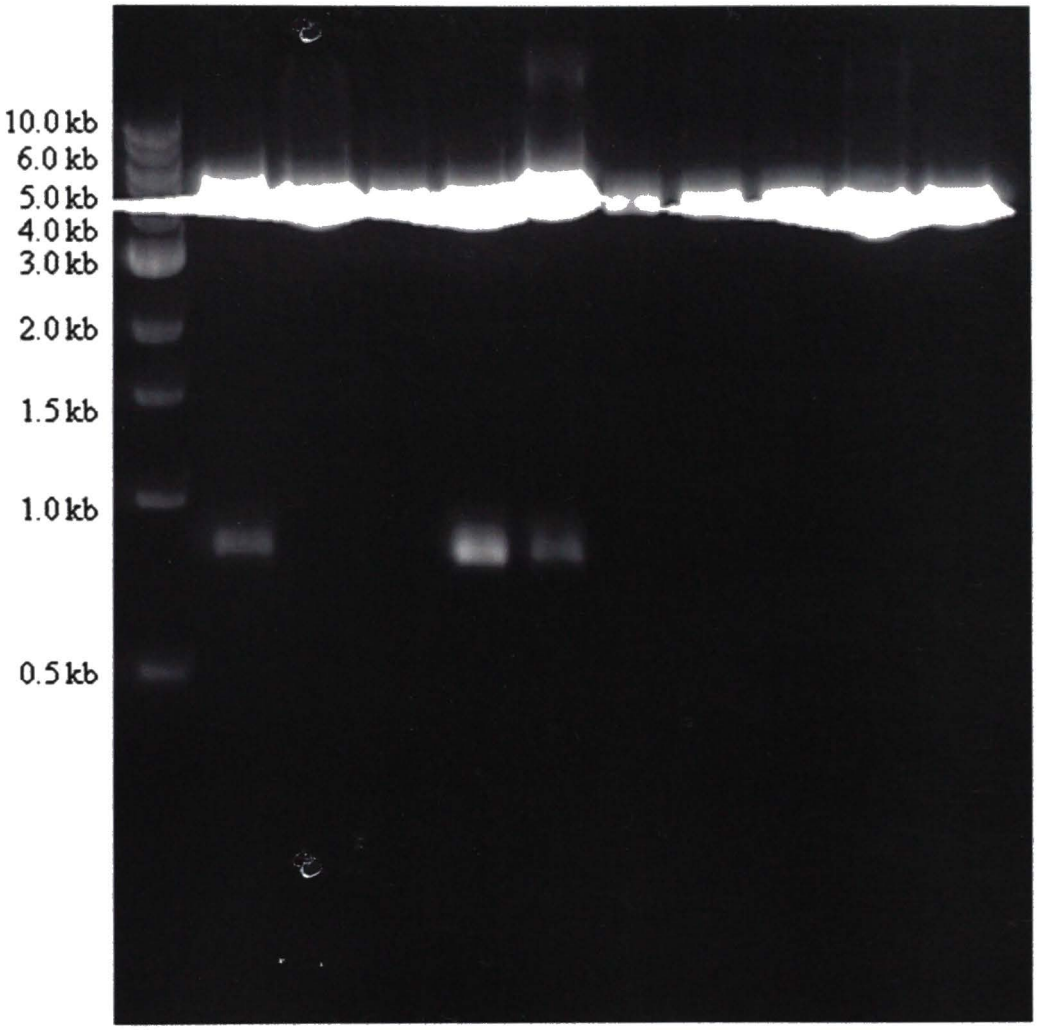


Figure 4.8 Digestion of cDNA Clones from Third Panning. 4 hr *Not I* and *Sal I* Digestion of plasmid DNA from single colonies isolated from amplified transformation of DNA recovered from third panning.

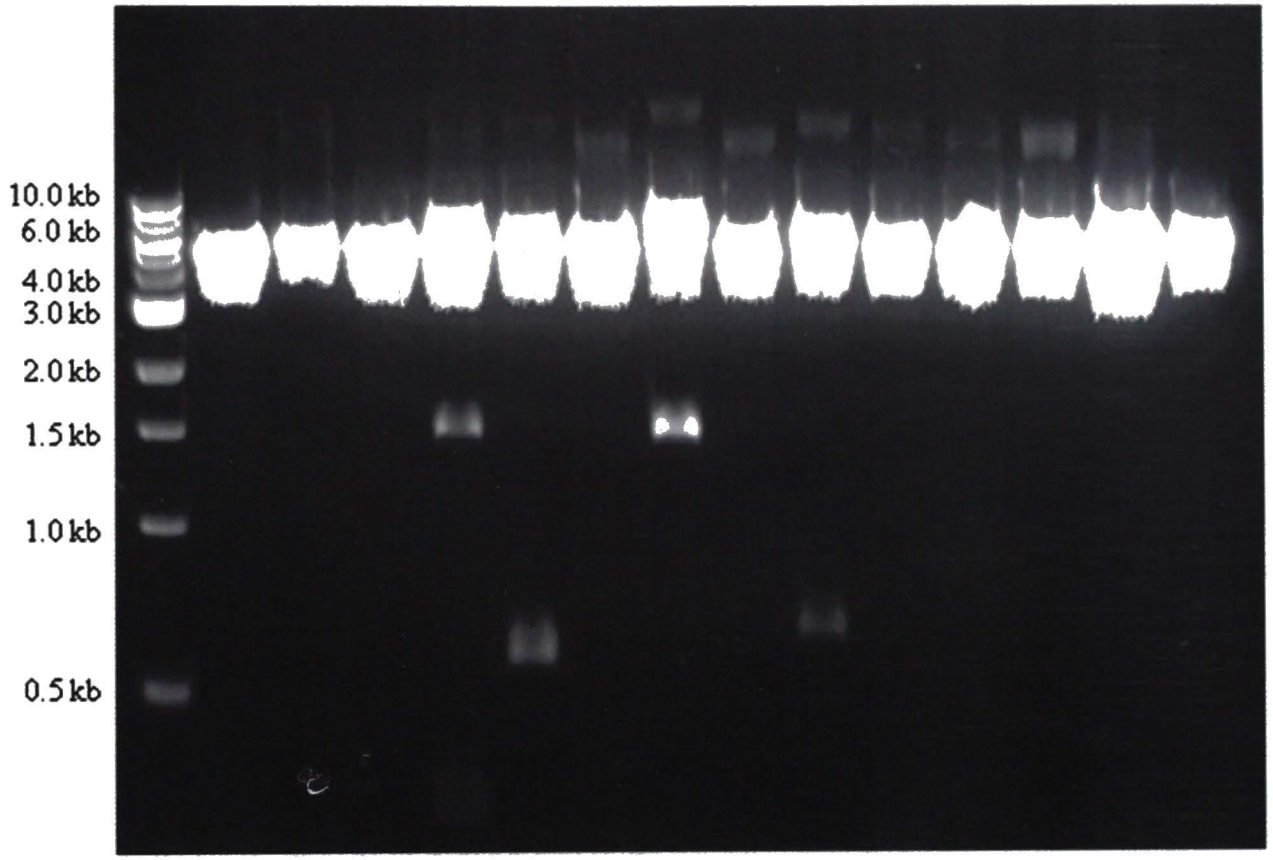


Figure 4.9 Digestion of Amplified Third Panning cDNA. 4 hr *Not I* and *Sal I* Digestion of 3ug amplified third panning DNA next to 1 kb DNA ladder.

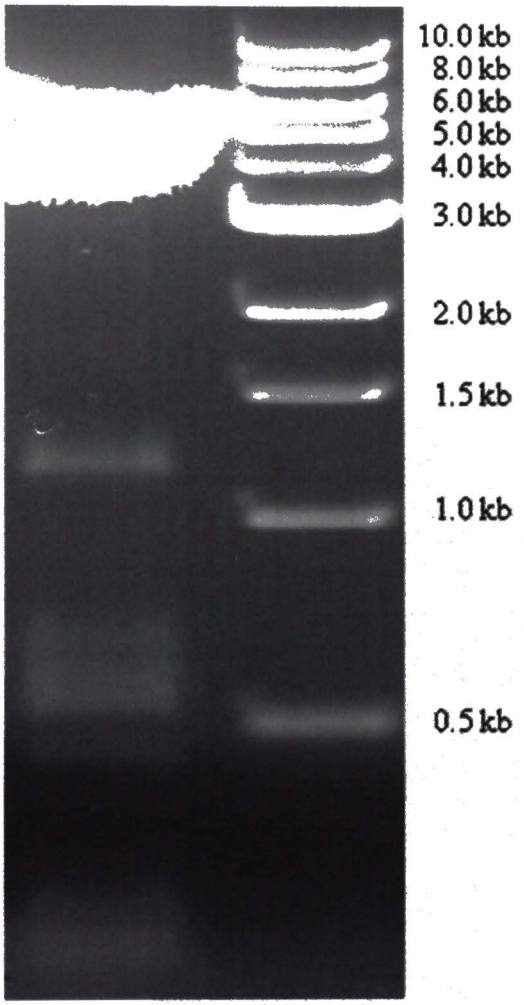


Figure 4.10 Digestion of Amplified cDNA from Panning Plates Receiving Two Washes. 4 hr *Not I* and *Sal I* Digestion of 3 ug amplified DNA recovered from panning plates receiving 2x wash.

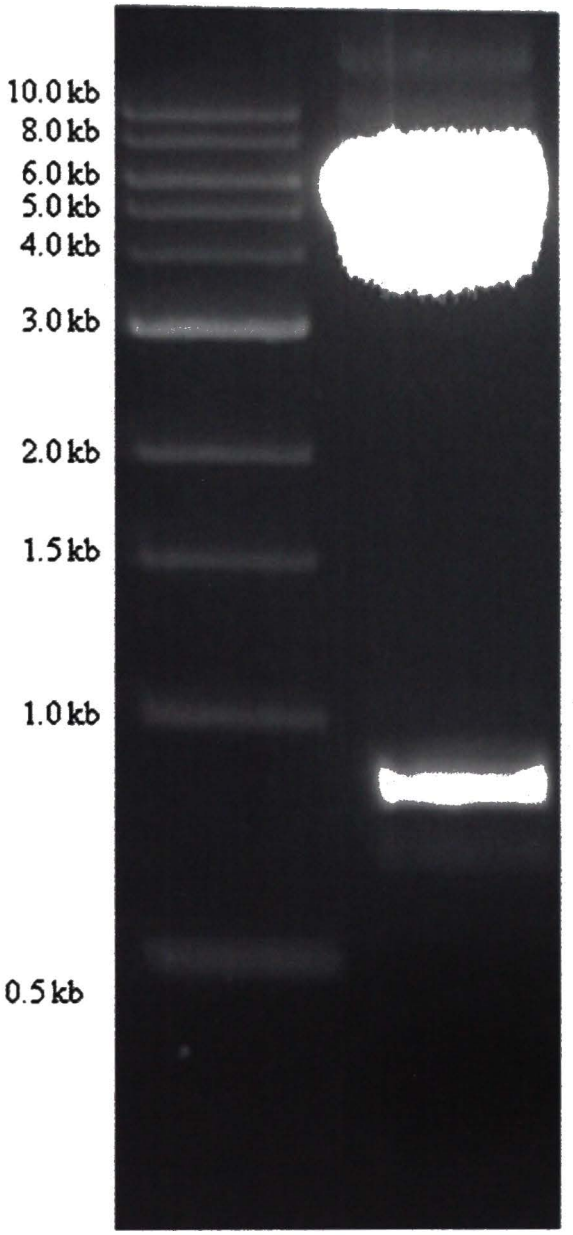


Figure 4.11 Digestion of Single cDNA Clones from Amplified 2x Wash cDNA. 4 hour *Not I* and *Sal* I digestion of single colonies from amplified transformation of DNA recovered from pan plates receiving 2 washes.

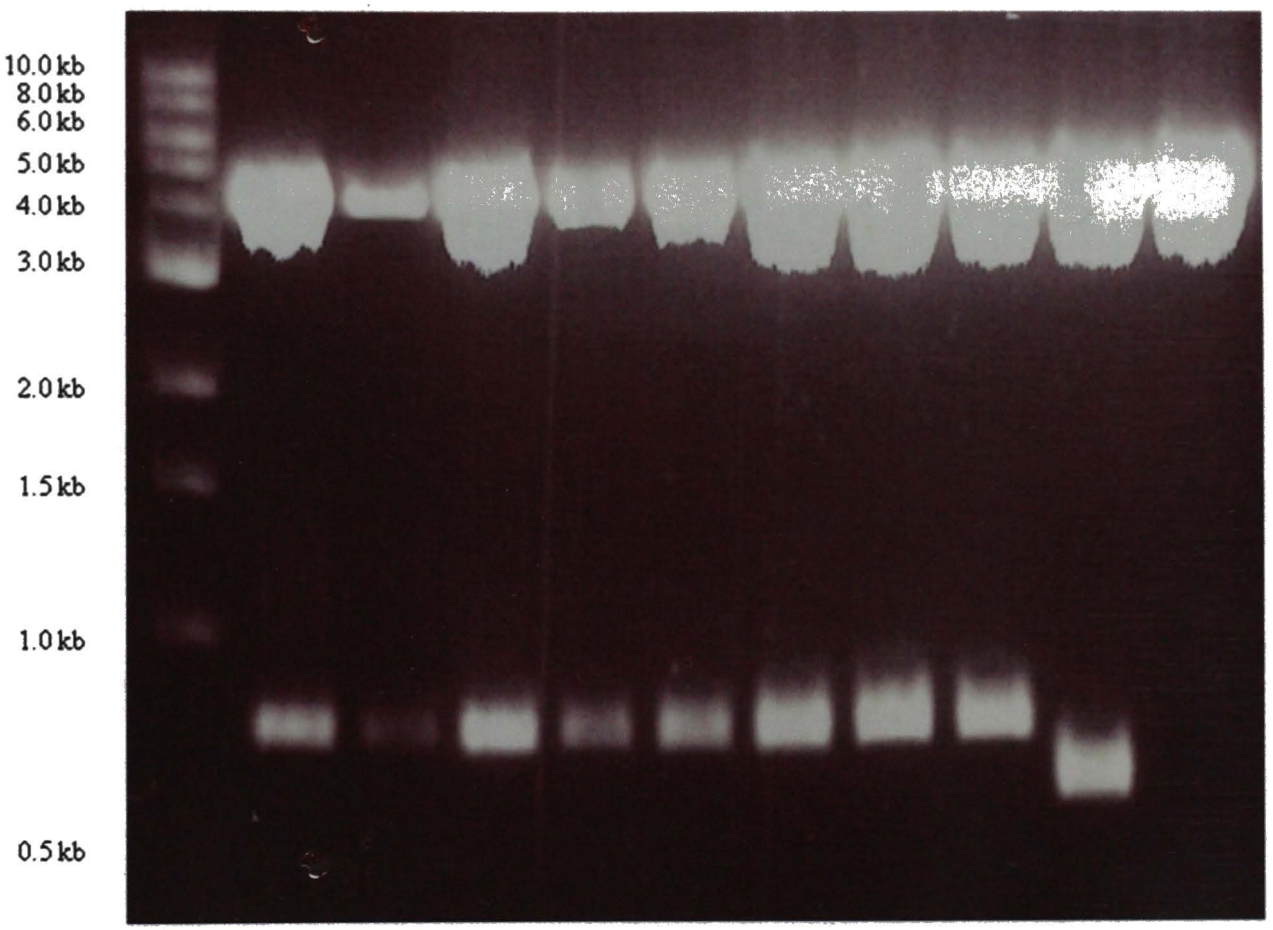


Figure 4.12 Digestion of Single cDNA Clones from Panning Plates Receiving Three Washes. 4 hr *Not I* and *Sal I* Digestion of plasmid DNA from single colonies isolated from amplified transformation of DNA recovered from panning plates receiving 3x wash.

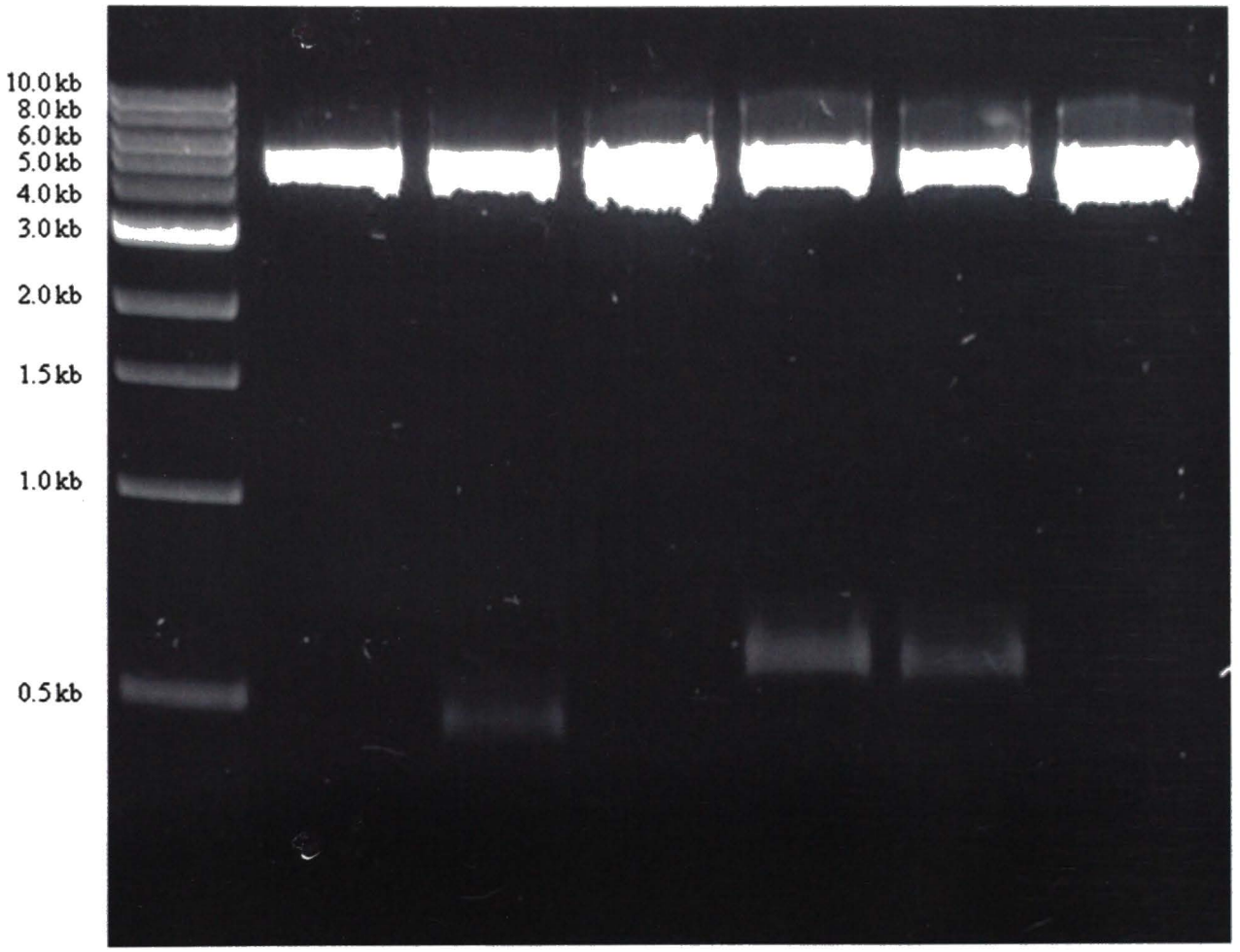
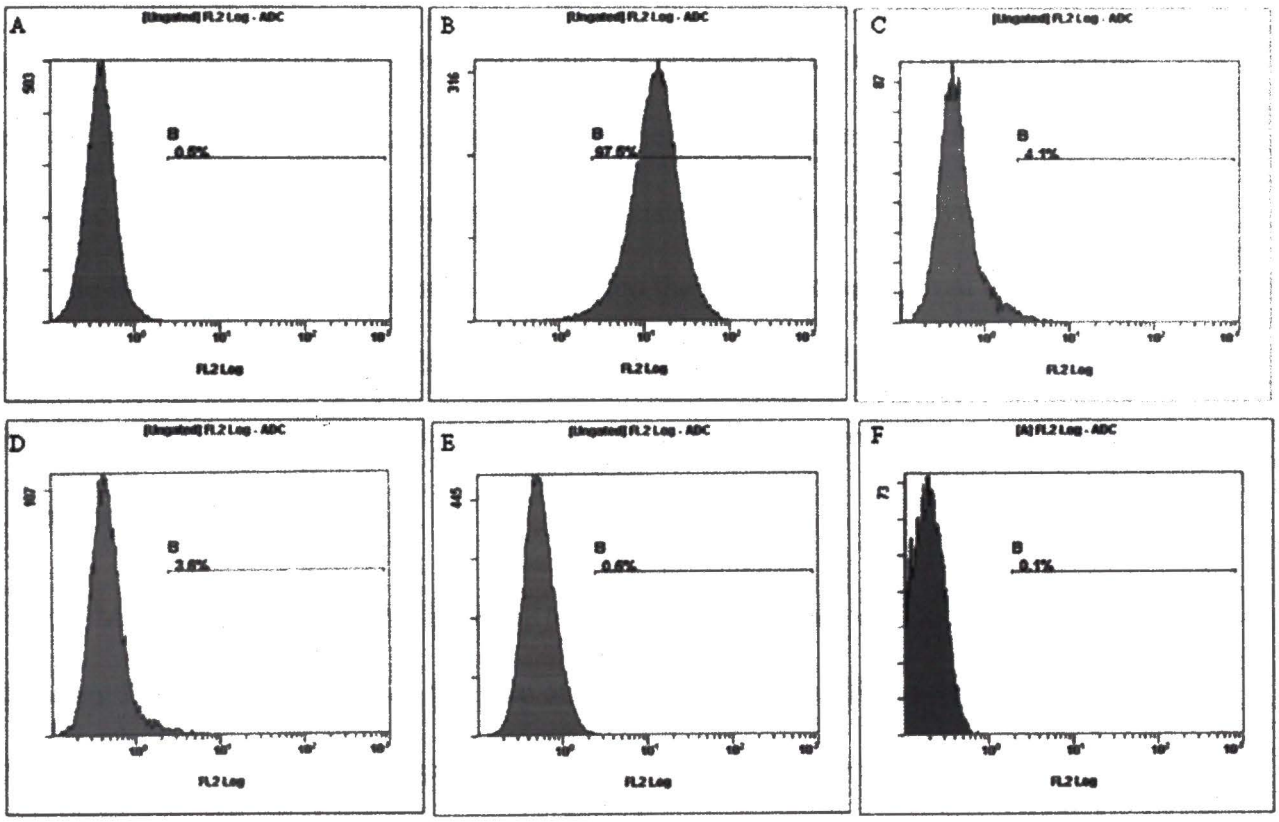


Figure 4.13 Flow cytometry data. A. DB cell negative control incubated with 2B4-IgG fusion protein. B. DB positive control incubated with NKp44-IgG fusion protein. C. B16 transfected with empty vector incubated with NKp44-IgG fusion protein. D. B16 transfected with 800kb cDNA clone isolated from cell sorting incubated with NKp44-IgG fusion protein. E. B16 transfected with 1.5 kb cDNA clone isolated from third panning incubated with NKp44-IgG fusion protein. F. B16 transfected with 700 bp cDNA clone isolated from panning plates washed twice incubated with NKp44-IgG fusion protein.



CHAPTER V

DISCUSSION

Mammalian cells contain several types of RNA all combined totaling about 10^{-11} grams (34). Ribosomal RNA accounts for the majority of Total RNA content at about 80 %. On the other hand, mRNA is the least abundant form of RNA in the cell comprising only one to five percent of the Total RNA population (34). Due to this lack of abundance, large amounts of Total RNA must be extracted in order to obtain a sizeable amount of mRNA for cDNA construction. Another disadvantage accounting to the difficulty of mRNA isolation is the ubiquitous existence of RNases, enzymes that rapidly degrade RNA. RNases are not only found within the cell but also external to the cell and tissue culture environment. Without minimal handling of mRNA samples, exogenous RNases from sources such as skin and sweat can greatly affect the efficiency of mRNA isolation.

Many commercially available mRNA isolation kits work well to circumnavigate these hurdles. This is done with the help of oligo dT cellulose which selectively binds the poly A tail of mRNA and a spin column filter system that binds the cellulose and allows for the removal of contaminating RNAs and proteins. This system is advantageous because it allows for direct mRNA isolation of tissue culture cells. However, the downfall to this system is the filter's lack of functionability with large scale

preparation of cell lysates. When lysing a large number of cells, cellular debri consistently clogged the spin column filter completely abolishing any efficiency in mRNA extraction. Thus it was necessary to first make large scale preparations of Total RNA using RNA Stat reagent. This reagent not only eliminated RNases, but also enabled the removal of cellular debri prior to mRNA extraction effectively stopping any clogging of spin column filters.

For the large scale preparation of Total RNA, DB cells were grown in a T-175 tissue culture flask until cells reached log phase growth, attaining 100 % confluency 24 hours after splitting. 1.5×10^8 DB cells where collected for Total RNA extraction. Extracting Total RNA first also allowed for the qualitative and quantitative analysis of the RNA prior to mRNA removal. Analysis enabled the selection of only the highest quality Total RNA preparations for mRNA extraction. After visual confirmation of the presence of the 28s, 18s, and 5s ribosomal RNA fragments by gel electrophoresis and Ethidium Bromide staining [Figure 3.1], Total RNA preparations containing all three were combined and quantitated. This process resulted in the extraction of 1.3 mg of Total RNA.

Total RNA was then incubated with oligo dT cellulose according to Invitrogen's Fast Track 2.0 mRNA isolation kit and transferred to a spin column. Following removal of contaminating RNA species, mRNA was eluted utilizing a very low ionic strength buffer to release the mRNA from the oligo dT cellulose. This process yielded 35 ug of mRNA, about 2.7 % of the 1.3 mg Total RNA. 5 ug of mRNA was then analyzed on a 1

% gel by electrophoresis [Figure 3.2]. Staining with Ethidium Bromide showed a bright smear of mRNA ranging in size from 3 kb to .5 kb, suitable for cDNA construction.

cDNA construction is a tedious process that harbors several of the same hazards as mRNA isolation, at least until second strand DNA synthesis is complete. Since the starting material is 5 ug of mRNA, samples as well as tubes and equipment used must all be free of contaminating RNases. cDNA construction is also difficult because the library is the end product of many individual reactions. Therefore library quality can be compromised by inefficiency at any point during the construction. The first step of cDNA construction is an innovative step that greatly increases the efficiency of library construction. The poly A tail of mRNA is bound by a poly T primer-adapter with a *Not I* restriction site within the adapter [Figure 3.3]. This addition not only primes for first strand DNA synthesis but also induces directionality into the sequence. Following second strand construction using the first strand as template, *Sal I* adapters are added to both ends of the now double stranded DNA [Figure 3.3]. The addition of the adapter covers the *Not I* adapter from the first step of construction; however, the *Sal I* adapter is easily removed from this side by *Not I* digestion completing the directional construction of cDNA. The cDNA is termed "directional" due to the *Not I* and *Sal I* non-compatible adapters at either end of the cDNA. These adapters eliminate cDNAs being ligated into the pCMV SPORT 6 vector in reverse orientation (3' to 5'). The down side to using such adapters is they can often be carried into the vector ligation step despite size fractionation. This results in the formation of empty vectors containing no insert, only

adapters that enable the vector to ligate without the presence of cDNA. Following vector cDNA ligation, the library was transformed into *E. coli* and amplified.

Initial attempts at cDNA construction were made using a cDNA kit manufactured by Stratagene (La Jolla, Ca). Utilization of this kit met much difficulty and failure. Size fractionation appeared to be a problem area due to the lack of sizeable cDNA inserts and the abundance of empty vectors once the library was completed and analyzed [Figure 3.6 A]. This problem reoccurred in subsequent cDNA construction attempts which exhausted the limiting reagents in the Stratagene kit.

At this point a new cDNA kit was ordered; however, it was decided that the Invitrogen Super Script cDNA kit held several advantages over the Stratagene kit. First, the Invitrogen kit was supplied with preloaded fractionation columns. The Stratagene kit supplied only the cephalose beads for the fractionation column which had to be constructed before use. Second, the Invitrogen kit had numerous citations in literature for successful use and construction of quality cDNA libraries. The Super Script kit from Invitrogen proved to be far superior to the kit from Stratagene. Size fractionation was much easier with preloaded columns and, after two attempts at library construction, a quality library was achieved and amplified.

Amplified plasmid DNA was digested as described above and analyzed by gel electrophoresis [Figure 3.6 B]. Digestion yielded a bright smear of inserts ranging in size from 4kb to .5 kb [Figure 3.6 B], equivalent to the size range of mRNA collected [Figure 3.1]. Single colonies were also isolated from serial dilution plates. These colonies were

individually amplified and analyzed for the presence of cDNA inserts by digestion [Figure 3.7]. Nine out of fourteen colonies examined contained cDNA inserts. Of the nine, six contained cDNA inserts larger than 1 kb. Five of the fourteen colonies also contained empty vector. The library consisted of 1.22 $x10^6$ clones and was considered to be a good representation of the genes expressed by DB cells.

Panning is perhaps the most tedious protocol in isolating the cDNA clone encoding the ligand for NKp44 for several reasons. First, it is almost impossible to discriminate cell attachment to panning plates under a microscope. The only way to measure success of each panning attempt is to analyze amplified plasmid DNA post-panning. Digesting amplified cDNA after panning allows for evaluation of presence and size of cDNA inserts. Second, the strength in which the fusion protein binds to not only the cDNA encoded ligand, but also the anti-IgG coated panning plate [Figure 4.2] is unknown. For this reason, only one wash was performed on panning plates prior to adding the lysing solution. This process initially was met with no success as plasmid DNA could not be recovered from panning plates in earlier experiments. However, utilizing more modern DNA precipitation procedures instead of those described in 1967 greatly increased the efficiency of plasmid recovery. In addition, since the quantity of plasmids remaining to be precipitated is very low, adding yeast tRNA greatly enhances the recovery of plasmid cDNA by acting as a carrier in the precipitation steps. Once the plasmid is recovered it is electroporated into bacteria; thus, the use of tRNA has no effects on downstream applications.

For the first round of panning, twelve 100 mm tissue culture plates of B16 cells were transfected with DB cDNA as described above. Two days post-transfection, cells were removed from the plates, labeled with NKp44 fusion protein, and incubated on 18 panning plates for 3 hours. Following incubation, unbound cells in solution were aspirated and panning plates were washed once with PBS/5% FPX to further remove unbound cells. Plates were only washed once so as not to remove any weakly bound cells. Any remaining cells were lysed and plasmid DNA was recovered. Once the plasmid DNA was transformed into bacteria, amplified, and harvested, 3 ug were digested with *Not I* and *Sal I* and analyzed by gel electrophoresis [Figure 4.3]. The first round of panning yielded a reduction in the number of cDNA clones with inserts ranging in size from 2kb to 500 bp. This not only validated the plasmid DNA recovery procedure but also the integrity of the cDNA library used since larger individual cDNA inserts were visible on the gel. There were also numerous species of cDNA inserts visible, eluding to the fact that more than one wash after incubation in the pan plate may be necessary to further eliminate unbound transfected B16 cells, especially in early panning attempts. This data was viewed as extremely encouraging; therefore a second panning was carried out.

To begin the second round of panning, 8 100 mm tissue culture plates of B16 cells were transfected with amplified plasmid DNA recovered from the first round of panning. Fewer plates were used since the volume of cDNA inserts was reduced during the first panning. Two days post-transfection, cells were removed from transfection plates,

labeled with NKp44 fusion protein, and incubated in 12 panning plates in the same manner as before. Unbound cells were aspirated and removed by a single wash. Plasmid DNA was then recovered and amplified. 3 ug of plasmid DNA was digested with *Not I* and *Sal I* and analyzed by gel electrophoresis [Figure 4.4]. The second round of panning further eliminated cDNA species leaving a cDNA insert of about 1.75 kb as the largest visible fragment on the gel. Smaller cDNA inserts were still visible near the 500 bp region. This data was again encouraging and prompted a small deviation from the original plan.

Two rounds of successive panning had eliminated a large number of cDNA species from the pool of original DB cDNA [Figures 3.6 B, 4.3, 4.4]. In order to see if the project was on the right track, plasmid DNA recovered from the second panning attempt was transfected into 4 100 mm tissue culture plates of B16 cells. Two days after transfection, cells were harvested and prepared for flow cytometry analysis as described above. If any cDNA species contained within the second panning DNA encoded the ligand for NKp44, it would be easily seen by flow cytometry analysis. Four samples of one million transfected cells were prepared and analyzed with the help of Dr. Xiangle Sun in the flow cytometry core facility. Untransfected B16 cells were used as a negative control and DB cells incubated with Nkp44 fusion protein was used as a positive control. Empty vector transfected control was not available at the time of initial flow cytometry analysis. There was no obvious binding of the fusion protein when analyzed; however, Dr. Sun believed there were some cells that appeared to be positive for PE binding by

analyzing the dot plot [Figure 4.5]. These transfected, labeled cells exhibiting a positive PE signal were then sorted on the Cytopia InFlux Cell Sorter by Dr. Sun [Figure 4.6]. Sorted cells were lysed in the same manner as those bound to panning plates and plasmid DNA was recovered and amplified. Unfortunately, only about 0.1% of the cells showed a positive PE signal out of the entire group of 8 million transfected, labeled cells. Once the plasmid DNA was analyzed it was discovered that the majority of the positive signal cells contained empty vector; however, one clone did contain a cDNA insert of about 800 bp [Figure 4.7], which was sent to SeqWright DNA Technology Services for sequencing.

The DNA sequence of the 800 bp insert was compared to the human genome using a BLAST search. This search reported an 89% homology to the 14-3-3 Epsilon protein. 14-3-3 is a family of proteins that are highly conserved and ubiquitously expressed in mammalian cells. They are acidic in nature and can form dimers to associate with over two hundred molecules. 14-3-3 proteins are functionally involved in numerous cellular processes ranging from DNA replication, cell adhesion, as well as masking active sites (36). The Epsilon variety of 14-3-3 has implications in the organization of cell signaling, exocytosis, DNA replication, and cell cycle control (36).

The 800 bp clone was amplified and plasmid DNA was harvested and transfected into 1 100 mm tissue culture plate of B16 cells. Transfected cells were analyzed by flow cytometry in the same manner as above. DB cells labeled with NKp44 fusion protein was again used as a positive control and B16 cells transfected with empty vector was used as negative control. Unfortunately the cDNA insert of about 800 bp

[Figure 4.7] did not exhibit any positive signal for PE, meaning fusion protein did not bind [Figure 4.13 D]. The positive signals received during initial flow cytometry analysis could be explained by the empty vector transfected control exhibiting nearly 5% binding of PE over untransfected B16 cells [Figure 4.13 C].

Despite the negative flow cytometry data, a third pan was executed to hopefully eliminate empty vector species from the second panning DNA. One possible explanation for the negative flow data is the over abundance of empty vector contaminating both the original cDNA library as well as the transfection procedure. If empty vector greatly out numbers the vectors containing sizeable inserts, this could greatly decrease the odds of a B16 cell being transfected with a plasmid containing a cDNA insert. The third panning was carried out by transfecting 4 100 mm tissue culture plates of B16 cells with plasmid DNA recovered from the second panning. As before, cells were labeled with NKp44 fusion protein and incubated in 6 pan plates two days after transfection. Unbound cells were aspirated and plates were washed once before bound cells were lysed and plasmid DNA recovered. Following amplification, 3 plates of LB were streaked for isolation and single colonies were picked, grown up overnight in 5 ml of LB/ampicillin broth, and analyzed for presence of cDNA inserts by digestion and gel electrophoresis. In addition, 3 ug of amplified DNA was digested with *Not I* and *Sal I* as previously described. Fourteen colonies from the streaked LB plates were picked and analyzed [Figure 4.8]. Four out of the fourteen colonies proved to contain sizeable inserts of 1.75 kb and 500 bp, similar to those seen in the 3 ug digestion of amplified third pan DNA [Figure 4.9]. The

clone containing the 1.75 kb insert was individually amplified and plasmid DNA was harvested.

The sequence of this cDNA insert contained 82% homology to the flavoprotein enzyme dihydrolipoamide dehydrogenase (DLD) by a BLAST search. DLD is located in the mitochondria and is involved in oxidoreductase reactions of the pyruvate, alpha-ketoglutarate, and branched-chain amino acid dehydrogenase complexes (37). Within these complexes, DLD uses dihydrolipoic acid and NAD+ to generate lipoic acid and NADH (37). The cDNA clone was transfected into 1 100 mm tissue culture plate of B16 cells and analyzed as before by Flow Cytometry. The clone did not exhibit binding of the fusion protein [Figure 4.13 E] over empty vector transfected control.

Reasons for failure of the panning system could be two fold. First, the cDNA library contains too many empty vectors. This greatly diminishes the efficiency of transfecting B16 cells with clones containing sizeable cDNA inserts. In addition, the use of only one wash to remove unbound cells from the panning plates may not be enough. To explore the plausibility of the later cause, a return to the first panning DNA was in order.

As before, first panning plasmid DNA was transfected into 8 100 mm tissue culture plates of B16 cells. Two days post-transfection, cells were removed from transfection plates and incubated in 12 panning plates. Following incubation, 6 panning plates were aspirated and washed two times while the remaining 6 plates were aspirated and washed 3 times.

Plasmid DNA recovered from plates receiving 2 washes was amplified as previously described. 3 ug of amplified plasmid DNA was digested with *Not I* and *Sal I* and analyzed by gel electrophoresis which yielded a small group of cDNA inserts around 800 bp [Figure 4.10]. Three LB/ampicillin plates were streaked to isolate single colonies from the amplified product of panning plates receiving two washes. Single colonies where individually amplified, digested, and analyzed by gel electrophoresis [Figure 4.11]. Nine out of ten colonies contained an insert of about 700 bp. One colony was selected for further amplification to harvest plasmid DNA for transfection. Transfected cells were analyzed for fusion protein binding by flow cytometry two days post transfection [Figure 4.13 F]. Yet again, the cDNA clone isolated did not produce fusion protein binding.

Plasmid DNA recovered from plates receiving 3 washes was transformed into bacteria and the transformation reaction was plated on 3 LB/ampicillin plates and incubated overnight at 37°C. LB/ampicillin plates yielded 6 colonies which were picked and grown overnight in 5 ml of LB/ampicillin. Plasmid DNA recovered from each amplified colony was digested and analyzed by gel electrophoresis [Figure 4.12]. Small cDNA inserts of about 500 bp were found in 3 of the 6 colonies. cDNA from all three clones were sent for sequencing. None of the three sequences contained any open reading frames. Sequences did not contain any homology to the human genome sequence through a BLAST search as well. Therefore, these clones were stored and not immediately analyzed for fusion protein binding.

CHAPTER VI

SUMMARY AND FUTURE DIRECTIONS

Natural Killer (NK) cells represent the human body's most potent anti-cancer weapon. They can be governed by chemical signals from other cells of the immune system as well as ligand receptor interactions through direct contact with target cells. This direct interaction provides detailed immune surveillance on a single cell level, which still remains poorly understood due to the lack of knowledge about NK cell receptor ligands. One such group of receptors, the Natural Cytotoxicity Receptors (NCRs), provides powerful recognition of tumor and virally infected cells through direct cell contact. Unfortunately their ligands still remain unknown. NCRs are known to recognize and induce NK cell killing of numerous cancer types. For this reason, NCRs represent a powerful immunotherapy tool and the resolution of their ligands remains a highly sought feat by groups worldwide.

NKp44 stands out from the other NCRs due to its specific expression only on activated NK cells as well as its role in the immune system and disease, namely HIV. NKp44 ligand resolution in itself represents an accomplishment that would change the course of NK cell work and immunotherapy. Ligand resolution could produce new disease markers as well as usher in a new class of immunotherapy that merely involves activating a patient's own NK cells against a specific ligand from their own cancer.

Attempts here in to isolate the ligand for NKp44 were met with limited success. Messenger RNA (mRNA) from a Diffuse Large B Cell Lymphoma cell line (DB) expressing a ligand for NKp44 was constructed into a complementary DNA (cDNA) library. This library was then transfected into a mammalian cell line in order to resolve the ligand utilizing a fusion protein containing the extracellular portion of NKp44 fused to the constant heavy chain of Immunoglobulin γ (IgG). Transfected mammalian cells expressing a cDNA encoded ligand were sequestered to a panning plate coated with anti-IgG Fc through a linkage with the fusion protein. cDNA clones were recovered, amplified, and then used in place of the DB cDNA library in successive panning attempts. Following three panning attempts, cDNA clones recovered were analyzed for true fusion protein bind through flow cytometry.

Unfortunately, all clones isolated by the above method did not produce fusion protein binding and the ligand has yet to be resolved. Failure in these attempts is possibly three fold. First, the cDNA library used for mammalian expression cloning must be of the highest quality. This of course relies on quality mRNA and cDNA construction. Despite high quality mRNA, the cDNA library contained numerous empty vectors that greatly reduced the efficiency of transfecting cDNA clones with sizeable inserts for panning. This is a result of residual adapters necessary for the construction of a unidirectional cDNA library contaminating the ligation of cDNA inserts into the pCMV-SPORT 6 vector. While tedious to fix, elimination of residual adapters can be

accomplished through careful phenol/chloroform extraction during cDNA construction as well as proper size fractionation prior to ligation into the vector.

Second, the use of fusion protein in the panning steps is a novel idea. The panning protocol was previously established utilizing monoclonal antibody against the ligand of interest attached to the bottom of the panning plates. This is the first known instance using anti-IgG Fc to bind a fusion protein attached to a ligand expressed on a cell. However, during labeling of cells for flow cytometry, the fusion protein is first bound to the expressed ligand on the cell followed by binding of anti-IgG Fc PE to the IgG portion of the fusion protein. This protocol works well and the linkage is resistant to centrifugation up to 200 g. The linkage is also stable several hours after initial incubations without fixing. In theory, the linkage of ligand to fusion protein to anti-IgG Fc coated plate should work in the panning plate environment; however, the strength of the linkage may not survive even the gentlest washing. While initial attempts only used one wash, two washes proved more productive in removing unbound cells while still preserving any possible cells bound to the panning plates.

Finally, B16 cells may be deficient in the necessary enzymes that provide for crucial glycosylations. This form of post translational modification is required for NKp44 to recognize its ligand. Since the B16 cell line is derived from mice, it should provide for the same glycosylations as human cells. However, since the cell line is also derived from melanomas, it may lack the proper glycosylations machinery due to its transformed state.

Going forward, this will not be the last attempt at resolving the ligand for NKp44. First, a return to cDNA construction is necessary to create a library with less empty vector. Such a library will be better in assessing the success of panning attempts as well as provide stronger odds for resolving the ligand. In addition, a high quality cDNA library will also enhance the power of cell sorting techniques. Second, future panning attempts will continue to receive two washes. This appears to be most beneficial over one or three washes. Panning plates will also be produced containing more anti-IgG Fc coated to the bottom. A measure of this coating may also be attempted by fluorescently tagging the anti-IgG Fc and analyzing the plates under a fluorescent microscope. Finally, additional mammalian cell lines will be used for mammalian expression cloning attempts in case the B16 cell line does contain some malfunction in glycosylation machinery. Resolving the ligand for NKp44 will require full optimization of panning plate conditions, a new DB cDNA library free of contaminating empty vectors, as well as a mammalian expression system that provides for proper glycosylation events.

REFERENCES

1. Cantoni, C. et. al. (1999). NKp44, A Triggering Receptor Involved in Tumor Cell Lysis by Active Human Natural Killer Cells, Is a Novel Member of the Immunoglobulin Superfamily. *J. Exp. Med.* 189 (5): 787-795.

2. Vitale, M. et. al. (1998). NKp44, a Novel Triggering Surface Molecule Specifically Expressed by Activated Natural Killer Cells, Is Involved in Non-Major Histocompatibility Complex-restricted Tumor Cell Lysis. *J. Exp. Med.* 187 (12): 2065-2072.

3. Cantoni, C. et. al. (2003). The Three-Dimensional Structure of the Human NK Cell Receptor NKp44 a Triggering Partner in Natural Cytotoxicity. *Structure.* 11: 725-734.

4. Poggi, A. et. al. (2005). Tumor-Induced Apoptosis of Human IL-2 Activated NK Cells: Role of Natural Cytotoxicity Receptors. *Journal of Immunology.* 174: 2653-2660.

5. Campbell, K. et. al. (2004). NKp44 Triggers NK Cell Activation Through DAP12 Association that is not Influenced by a Putative Cytoplasmic Inhibitory Sequence. *Journal of Immunology.* 172: 899-906.

6. Fuchs, A. et. al. (2005). Paradoxic Inhibition of Human Natural Inteferon-Producing Cells by the Activating Receptor NKp44. *Blood.* 106 (6): 2076-2082.

7. Arnon, T. et. al. (2001). Recognition of Viral Hemagglutinins by NKp44 but not by NKp30. *Eur. J. Immunol.* 31: 2680-2689.

8. Arnon, T. et. al. (2004). The Mechanisms Controlling the Recognition of Tumor- and Virus-Infected cells by NKp46. *Blood.* 103 (2): 664-672.

9. Bloushtain, N. et. al. (2004). Membrane-Associated Heparan Sulfate Proteoglycans are Involved in the Recognition of Cellular Targets by NKp30 and NKp46. *Journal of Immunology.* 173: 2392-2401.

10. Hershkovitz, O. et. al. (2007). Characterization of the Recognition of Tumor Cells by the Natural Cytotoxicity Receptor, NKp44. *Biochemistry.* 46: 7426-7436.

11. Toyabe, S. et. al. (2004). Decreased DAP 12 Expression in Natural Killer Lymphocytes from Patients with Systemic Lupus Erythematosus is Associated with Increased Transcript Mutations. *Journal of Autoimmunity.* 23: 371-378.

12. De Maria, A. et. al. (2003). The Impaired NK Cell Cytolytic Function in Viremic HIV-1 Infection is Associated with a Reduced Surface Expression of Natural Cytoxicity Receptors. *Eur. J. Immunol.* 33: 2410-2418.

13. Costello, R. et. al. (2002). Defective Expression and Function of Natural Killer Cell-Triggering Receptors in Patients with Acute Myeloid Leukemia. *Blood.* 99: 3361-3367.

14. Chisolm, S. et. al. (2005). Recognition of Vaccinia Virus-Infected Cells by Human Natural Killer Cells Depends on Natural Cytotoxicity Receptors. *Journal of Virology.* 80 (5): 2225-2233.

15. Sivori, S. et. al. (2000). Involvement of Natural Cytotoxicity Receptors in Human Natural Killer Cell-Mediated Lysis of Nueroblastoma and Glioblastoma Cell Lines. *Journal of Neuroimmunology.* 107: 220-225.

16. Sivori, S. et. al. (2000). Triggering Receptors Involved in Natural Killer Cell-Mediated Cytotoxicity Against Choriocarcinoma Cell Lines. *Human Immunology.* 61: 1055-1058.

17. Von Lilienfeld-Toal, M. et. al. (2006). Activated γδ T cells Express the Natural Cytotoxicity Receptor Natural Killer p44 and Show Cytotoxic Activity Against Myeloma Cells. *Clinical and Experimental Immunology.* 144: 528-533.

18. Vieillard, V. et. al. (2005). NK Cytotoxicity Against CD4+ T Cells During HIV-1 Infection: A gp41 Peptide Induces the Expression of an NKp44 Ligand. *PNAS.* 102 (31): 10981-10986.

19. Tumova, S. et. al. (1999). Heparan Sulfate Proteoglycans on the Cell Surface: Versatile Coordinators of Cellular Functions. *IJBCB.* 32: 269-288.

20. Mavillo, D. et. al. (2003). Natural Killer Cells in HIV-1 Infection: Dichotomous Effects of Viremia on Inhibitory and Activating Receptors and their Functional Correlates. *PNAS.* 100 (25): 15011-15016.

21. Bottino, C. et. al. (2005). Cellular Ligands of Activating NK Receptors. *Trends in Immunology.* 26 (4): 221-226.

22. Lanier, L. L. (2003). Natural Killer Cell Receptor Signaling. *Current Opinion in Immunology.* 15:308-314.

23. Moretta L, Moretta A (2004). Unraveling Natural Killer Cell Function: Triggering and Inhibitory Human NK Receptors. *EMBO Journal.* 23 (2): 255-259.

24. van der Voort, R. et. al. (2000). Regulation of Cytokine Signaling by B cell Antigen Receptor and CD40-controlled Expression of Heparan Sulfate Proteoglycans. *J. Exp. Med.* 192 (8): 1115-1124.

25. Bennett, K. et. al. (1995). CD44 Isoforms Containing Exon V3 are Responsible for the Presentation of Heparin-binding Growth Factor. *J. Cell Bio.* 128 (4): 687-698.

26. Forster-Horvath, C. et. al. (2001). Constitutive Intracellular Expression and Activation-induced Cell Surface Up-regulation of CD44v3 in Human T Lymphocytes. *Eur. J. Immunol.* 31: 600-608.

27. Woerner, S. et. al. (1995). Expression of CD44 Splice Variants in Normal, Dysplastic, and Neoplastic Cervical Epithelium. *Clinical Cancer Research.* 1: 1125-1132.

28. Esin, S. et. al. (2008). Direct Binding of Human NK Cell Natural Cytotoxicity Receptor NKp44 to the Surfaces of Mycobacteria and Other Bacteria. *Infection and Immunity.* 76 (4): 1719-1727.

29. Newman K, Riley E. (2007). Whatever Turns You On: Accessory-Cell-Dependent Activation of NK Cells by Pathogens. *Nat. Rev. Immun.* 7: 279-291.

30. Garg, A. et. al. (2006). Vimentin Expressed on *Mycobacterium tuberculosis*-Infected Human Monocytes is Involved in Binding to the NKp46 Receptor. *Journal of Immunology* 177: 6192-6198.

31. Hallet W, Murphy W. (2006). Positive and Negative Regulation of Natural Killer Cells: Therapeutic Implications. *Seminars in Cancer Biology.* 16: 367-382.

32. www.lymphoma.org

33. Hunt K, Reichard K. (2008). Diffuse Large B-Cell Lymphoma. *Arch Pathol Lab Med.* 132: 118-124.

34. Invitrogen fast track 2.0 mRNA kit manual

35. Seed B, Aruffo A. (1987). Molecular Cloning of the CD2 Antigen, the T-cell Erythrocyte receptor, by a Rapid Immunoselection Procedure. *Proc. Nat. Acad. Sci. USA.* 84: 3365-3369.

36. Zannis-Hadjopoulos, Maria. et. al. (2007). 14-3-3 Cruciform-binding Proteins as Regulators of Eukaryotic DNA Replication. *Trends in Biochemical Sci.* 33 (1): 44-50.

37. Babady, Ngolela Esther. et. al. (2007). Cryptic Proteolytic Activity of Dihydrolipoamide Dehydrogenase. *PNAS*. 104 (15): 6158-6163.

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