Comparison and Identification of Autoimmune Antigens in Seropositive and Seronegative Myasthenia Gravis

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Abstract

Myasthenia gravis (MG) was the first autoimmune disorder of the Peripheral Nervous System to be characterized. This disease is generally caused by autoantibodies that bind to the nicotinic acetylcholine receptors (AChR) at the post-synaptic muscle membrane. However, about 15% of patients do not have detectable levels of this antibody and are diagnosed to have seronegative myasthenia gravis (SNMG). Previous research in this lab has focused on autoimmune rippling muscle disease (ARMD), which has been reported to appear prior to the onset of (MG). Autoantigens have been characterized through the immunoscreening of a human skeletal muscle cDNA expression library using sera from patients with seropositive myasthenia gravis (SPMG) and (SNMG). SDS-PAGE analysis and Western blot studies have been carried out to better understand these target proteins. The patient's IgG and IgM autoantibodies were used to identify three unique muscle proteins. One of the three muscle proteins was titin-isoform (N2A). Titin is a large well known protein that is found in skeletal muscle. The protein extends from the Z line to the M line of the sarcomere. Titin provides passive tension to the muscle and acts as a template for normal muscle formation. Previous research performed in the lab has shown that the ARMD patients' sera also bound to titin-isoform (N2A). The Western blots of both the ARMD and the SPMG sera showed a unique doublet at about 123kDa. and 140kDa. The significance of this study is that the SPMG titin sequence fell in the Main Immunogenic Region (MIR) of the muscle protein. This region is believed to play a possible role in myasthenia gravis. Future studies are aimed at 2DE proteomic comparative analysis to help understand the connection between ARMD and SPMG

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Introduction

Myasthenia gravis (MG) was the first autoimmune disorder of the peripheral nervous system to be characterized. MG is the most understood autoimmune disorder and one of the few conditions in which the effector mechanisms are thought to be entirely antibody-mediated (Lang and Vincent 2002). The disease is associated with the clinical symptoms of muscle weakness and fatigability (Sharshar, Lacroix-Desmazes et al. 1998). A hallmark of this disease is the presence of autoimmune antibodies specifically directed against the α-subunit of the nicotinic acetylcholine receptors (AChR) at the post-synaptic muscle membrane (Brostoff, Scadding et al. 1991).

MG is an autoimmune disease that affects the strength of a normal muscle contraction. The controlled movement of a muscle contraction is modeled after the Hanson and Huxley sliding filament model proposed in 1954. The sliding filament mechanism for muscle contraction is an intricate well coordinated sequence of events that leads to a controlled contraction. Initiation of a muscle contraction starts with the propagation of an action potential from a motor neuron. The depolarization of the axon acts to release the neurotransmitter acetylcholine (ACh) from the neuron into the synaptic cleft. The ACh binds specifically to the α-subunit of the AChR located on the surface of the skeletal muscle. The binding initiates a Na^{+} influx, a change in polarity, and a depolarization of the skeletal muscle, along the surface of the cell into the T-tubule (Vander. A, Sherman. J et al. 2001). The depolarization causes a sequential cascade of ion channels, Ca^{+2} channels, to open starting with the dihydropyridine receptor (DHPR), ryanodine (RyR), and ending with the release of Ca^{+2} from the sarcoplasmic reticulum

(SR). The Ca^{+2} ions that are released from the SR bind to the Ca^{+2} regulated troponin C molecules. The binding causes a conformational change which exposes binding sites for the myosin cross bridges on the actin filament. The cross bridges are free to form between the myosin and actin filaments which enables the sliding muscle contraction (Vander. A, Sherman. J et al. 2001).

Studies have shown that MG is caused by the direct binding of autoantibodies to the α-subunit of the AChR (Strauss and Kemp 1967). The autoantibodies bind to the AChR, which are clustered in the synaptic cleft, and inhibit the amount of ACh that can bind to propagate the muscle contraction. Studies have shown this through the use of a snake toxin, α-bungarotoxin (BUTX), which is a specific ligand for the α-subunit of the AChR (Brostoff, Scadding et al. 1991). The study shows that the binding of this subunit blocks the receptor/neurotransmitter interaction and hinders muscle contraction (Brostoff, Scadding et al. 1991). Myasthenia gravis can typically affect any skeletal muscle. Although it is still unclear how this autoimmune response originates, it has been hypothesized that some kind of trauma may have occurred to the skeletal muscle which will present antigen to autoantibodies.

MG is a complicated disease to treat and study because it has a number of subgroups that present the same clinical symptoms ([1]Romi, Skeie et al. 2000). Each subgroup varies in severity. The first subgroup is ocular MG, with the clinical symptoms being ocular weakness. In 10-15% of patients, the weakness is restricted to the ocular muscles resulting in ptosis (droopy eyelids) and diplopia (double vision), these patients are termed ocular myasthenics (Lang and Vincent 2002). The constant rapid contractions

of the ocular muscles that control the eye appear to fatigue the quickest which results in the ptosis and diplopia.

The second group of patients is termed full blown myasthenics. Studies demonstrate that these patients experience the muscle weakness and fatigable which affects most of their large muscle groups and even the extremities (Vincent 2001). The last group is classified as late-onset MG (over the age of 50) (Romi, Gilhus et al. 2002).

The late on-onset MG is usually accompanied with a thymic tumor, know as a thymoma. This tumor creates a paraneoplastic syndrome which results in medical complications due to a released substance, autoantibodies in this case, that affect the function of normal tissue rather than the tumor itself affecting the tissue (Romi, Gilhus et al. 2002). The thymus is a small organ located in the upper chest in humans which produces lymphocytes cells early in development and coordinates immunological functions in the body. At puberty, the thymus is affected by steroids resulting in atrophy (Brostoff, Scadding et al. 1991). 15% of MG patients are diagnosed with a thymoma consisting of neoplastic epithelial cells and immature T cells. A thymectomy, surgical removal of the thymus, is a procedure that was introduced as a treatment for MG in the 1950s (Romi, Gilhus et al. 2002). Although no randomized controlled studies exist, thymectomies are still carried out in MG patients and there is clinical support for post-thymectomy improvement in early onset MG (Romi, Gilhus et al. 2002).

Diagnosis of MG is based on specific clinical tests that asses ACh activity. The tests examine clinical patterns such as a positive edrophonium Tensilon test, a short acting acetylcholinesterase inhibitor. Acetylcholinesterase is the chemical that clears ACh from the synaptic cleft. The test inhibits the Acetylcholinesterase to extend the time ACh

has in the cleft. This will indicate if the ACh is in low titer or having trouble binding to the AChR ([1]Romi, Skeie et al. 2000). The other test is a neurophysiological investigation with decrement at repetitive stimulation and increased jitter using single fiber electromyogram (EMG). This investigation uses the single fiber EMG to measure the electrical activity of the muscles and asses the strength of the action potentials. This is done by measuring the jitter, the variability in time between activation of the motor nerve and the generation of the action potentials ([1]Romi, Skeie et al. 2000).

The roles these autoantibodies play in the disease are still not fully understood. It has been found that 80% of MG patients have detectable levels of AChR antibodies, these patients are termed seropositive myasthenia gravis (SPMG); however, about 15% of patients do not have detectable levels of this antibody and are diagnosed to have seronegative myasthenia gravis (SNMG) (Vincent, Bowen et al. 2003). SPMG may appear to be more prevalent, due to the fact that it is the easiest to detect.

In the first comprehensive study of autoantibodies in patients with MG, Vincent found that AChR antibodies were undetectable in about 15% of patients and postulated that they might have antibodies to another protein (Vincent, Bowen et al. 2003). This idea, and the lack of detectable AChR antibodies, helped define the patient's disease as SNMG. In SNMG, the symptoms of generalized MG are present but a specific autoantibody could not be detected. Vincent did discover that over time low levels of AChR autoantibodies became detectable in some SNMG patients. This data suggests that the autoantibodies were present all along, but at an undetectable low titer (Vincent and Newsom-Davis 1985).

There are three hypotheses regarding the mechanism for SNMG and the related autoantibodies associated with it. All three hypotheses believe that the disease is associated with autoantibodies binding to specific autoantigens. The difference between the three hypotheses involves the isotype of the autoantibodies and the autoantigen they affect. When dealing with the topic of autoimmunity, one must take in to consideration the "Witebsky's postulates". Witebsky was a pioneer in autoimmune diseases, first publishing his work on autoimmune thyroiditis in 1956 (Masllorens 2000). Witebsky laid down a framework of criteria, similar to Koch's postulates for infectious diseases, which makes it possible to state, on an objective basis, that a disease is in fact autoimmune driven. The postulates stated that in order to establish that a disease is autoimmune; one must demonstrate the existence of autoantibodies or autoreactive cells, identify that an autoantigen is involved, and induce a similar autoimmune response in some experimental animal (Masllorens 2000).

The first hypothesis describes a mechanism that is mediated by some IgG autoantibody that targets an unknown antigen on the surface of the skeletal muscle (Mier and Havard 1985). This mechanism is somewhat similar to the SPMG mechanism that inhibits muscle contraction and control by inhibiting neurotransmission. This hypothesis is based on experimental evidence detecting IgG autoantibodies in SNMG patients. Researchers were led to believe this due to the patients' response to plasmaphorisis (plasma exchange), passive transmission of the disease from the mother to a newborn, and the infection of most laboratory animals which all suggest involvement of autoantibodies in the blood, mainly of the IgG isotype (Mier and Havard 1985).

The second hypothesis for a mechanism of SNMG suggests the autoantibody is a non-IgG antibody, possibly thought to be of the IgM isotype. Studies have shown that the plasma from SNMG patients reduce AChR function in the human AChR-expressing TE671 rhabdomyosarcoma cell line (Li, Forester et al. 1996). The laboratory showed that SNMG sera had non-IgG fractions that inhibited the function of the AChR but did not block/inhibit the binding of the α-subunit of the AChR (Li, Forester et al. 1996). Li and colleagues propose that the autoantibodies act indirectly via phosphorylation of the AChR. They have shown that substances such as the β_2-adrenergic agonist, salbutamol, cholera toxin, and calcitonin-gene-related-peptide (CGRP) increase intracellular cAMP via binding of specific cell-surface receptors which reduce function of the AChR in the TE671 cell line. These results imply that the non-IgG, perhaps IgM, autoantibody acts on a cell-surface receptor and triggers intracellular cAMP or other second messengers which leads to the desensitization or damage of the post synaptic membrane (Li, Forester et al. 1996).

The third hypothesis is that SNMG onset is due in part to the IgG anti-muscle-specific receptor tyrosine kinase (MuSK) and its role in the agrin/MuSK pathway (Liyanage, Hoch et al. 2002). MuSK is muscle specific and localized to the neuromuscular junction (Vincent, Bowen et al. 2003). The MuSK protein is a single transmembrane polypeptide containing an endodomain with the 12 subdomains characteristically significant of a functional tyrosine kinase (Liyanage, Hoch et al. 2002). Scuderi and colleagues have identified IgG MuSK antibodies in SNMG patients. In the study, proteins from the TE671 cell line were immunoprecipated and subjected to Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE), transferred

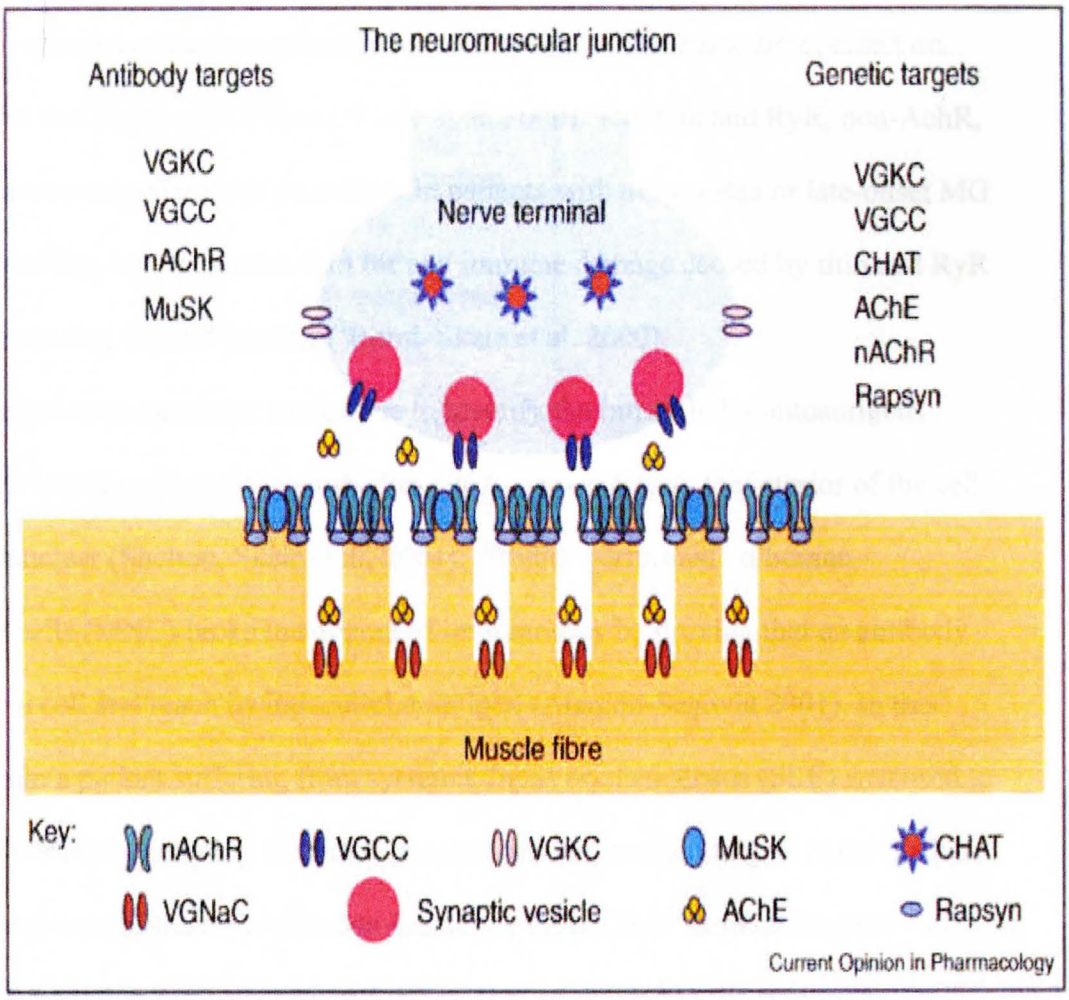
to nitrocellulose membranes, and exposed to the SNMG patients' sera (Scuderi, Marino et al. 2002). Immunoreactivity was expressed to a 110 kDa membrane protein from the TE671 cell line, which is not recognized by SPMG sera. This protein was later confirmed to be the MuSK protein using a specific anti-muscle specific kinase (MuSK) antiserum (ABGENT catalog # AP7664a). (Scuderi, Marino et al. 2002).

Early in neuromuscular junction development agrin, a heparin sulfate proteoglycan, is released from the motor neuron and becomes embedded in the basal lamina of the muscle fiber. Agrin activates MuSK which dimerises and autophosphorylates. This reaction phosphorylates a cytoskeleton protein rapsyn, which helps cluster AChR together on the surface of the skeletal muscle (Vincent, Bowen et al. 2003). The MuSK protein maintains a high concentration of AChR in the synaptic cleft, which allows the ACh maximum exposure to the AChR before being degraded by acetylcholinesterase (Liyanage, Hoch et al. 2002). The agrin/MuSK pathway is believed to cause a complement-mediated reaction that disassembles the AChR and thus destroys the motor endplate causing defective neuromuscular transmission (McConville and Vincent 2002). Figure # 1 summarizes the interactions that are thought to be involved.

Figure # 1

Localization of possible proteins at the neuromuscular junction that may interact with autoimmune antibodies: This figure displays the voltage-gated channels (Ca+2/KC), along with the MuSK and Rapsyn targets. Note that the Rapsyn clusters the AChR tightly within the synaptic cleft.

Figure 1



(McConville and Vincent 2002)

Cases have been reported where autoantibodies to intracellular striated muscle proteins have been characterized with MG (Lubke, Freiburg et al. 1998). Autoantibodies to the sarcomeric muscle protein titin and the ryanodine receptor (RyR) of the skeletal muscle have been detected in sera from MG patients (Skeie, Lunde et al. 1998). The RyR is a Ca^{+2} channel of the sarcoplasmic reticulum (SR) which has a direct effect on muscle control and contraction ([2]Romi, Skeie et al. 2000). The titin and RyR, non-AchR, autoantibodies appear to be more prominent in patients with a thymoma or late-onset MG (after the age of 50), but the mechanism for any immune damage caused by titin and RyR autoantibody binding are still unclear ([2]Romi, Skeie et al. 2000).

There has been extensive work done to identify the intracellular autoantigens present in MG but the pathway the antibodies use to gain access to the interior of the cell still remains unclear (Shelton, Skeie et al. 2001). Studies performed on human mononuclear cells (MNC) broke the dogma of immunology by proving that an antibody can penetrate a cell and reach its intracellular antigen (Alarcon-Segovia 2001). In this study, sera from a patient suffering from systemic lupus erythematosus (SLE) was used to show the antibodies, penetrating the MNC, reached the autoantigen targets in the nucleus and caused apoptosis of the cells (Alarcon-Segovia 2001). This "Broken Dogma" provides the foundation for further investigations that could lead to the proposed mechanisms of penetration that the titin and RyR autoantibodies are exhibiting.

Myasthenia gravis has been clinically diagnosed to be associated with another autoimmune disease termed autoimmune rippling muscle disease (ARMD) (Ansevin and Agamanolis 1996). ARMD is an autoimmune form of a genetic disorder termed rippling muscle disease (RMD). RMD was first described in 1975 by Torbergsen as an autosomal

dominant genetic disorder affecting the larger skeletal muscle groups (Torbergsen 1975). RMD is characterized by wave like contractions that ripple and roll through the skeletal muscle initiated by physical stretch or percussion of the muscle group (Ricker, Moxley et al. 1989). Ricker's further studies demonstrated that there was no action potential propagating the muscle contractions, thus terming the ripples "electrically silent". It has been hypothesized that the silent muscle contractions display an independent depolarization caused not from the neuron but perhaps an ion channel, possibly Ca^{+2} dependent (Burns, Bretag et al. 1994).

ARMD was first characterized by Dr. Ansevin in a patient who presented the rippling muscle contractions that were stretch and percussion activated but genetically, through the patient's family history, displayed no signs of RMD or any other neuromuscular diseases (Ansevin and Agamanolis 1996). This patient later presented the symptoms of MG. Along with other patients, once immunosuppressant treatment was started to help with the MG the rippling muscles stopped. ARMD is now considered autoimmune rippling muscle disease associated with myasthenia gravis (Walker, Watkins et al. 1999).

Studies have shown that sera from ARMD patients posses autoantibodies that immunoreacted to a very large, > 400 kDa suggestive of a titin-like, protein from whole muscle (Walker, Watkins et al. 1999). Further studies indicated immunoreactivity to autoantigens, from a whole muscle sub-cellular fraction, that co-localized with the ryanodine receptor (RyR), dihydropyrdine receptor and other proteins associated with the muscle triad (Watkins 2004). These results suggest that that the high molecular weight protein may play a role in the mechanosensitive characteristics of the rippling muscles,

these results also suggest the RyR and other Ca^{+2} channels may play a role in the independent Ca^{+2} hypothesis.

The specific aim of this study was to identify and characterize the unique autoantigens targeted by the autoantibodies of the IgG and IgM isotype. The study also compares the unique set of autoantigens between the SPMG and the SNMG forms of the disease. There has been a connection associated with ARMD and MG, stated above. Drs. Ansevin and Agamanolis describe patients with ARMD associated with MG and how they related (Ansevin and Agamanolis 1996). This study was designed to help determine and compare the biological similarities between ARMD and MG.

Specific Aims and Scope

1) Identify unique autoantigens associated with SPMG and SNMG through biochemical and genomic techniques.
2) Characterize the isotypes of the autoantibodies associated with the disease, IgG/IgM.
3) Compare the autoantigens from SPMG and SNMG with ARMD autoantigens to better define the biological similarities and correlation these two autoimmune diseases share.

Materials and Methods

This study was designed to identify the autoantigens in human skeletal muscle. Auotantibodies from SPMG and SNMG patients's sera were used as probes for screening a cDNA expression library. IgG and IgM secondary antibodies were used to characterize the antibody isotypes. Immunoreactive clones were sequenced and analyzed through proteomics and genomic techniques. Throughout this study specific notations will be used to designate patients, disease, and antibody isotypes. Table # 1 reports patients' sera used and the notations for antibody recognition that were used throughout this study. A summary of patients' clinical descriptions are displayed in Table # 2, along with the empirical data of antibody isotyping.

Patient's Serum

The sera from patients diagnosed with seronegative myasthenia gravis (SNMG) and seropositive MG with a thymoma (SPMG) were used in this study as probes in screening the expression library. The serum was used as the primary antibody to exhibit specific antigen/antibody binding to the translated fusion proteins from the cDNA fragments of the human skeletal muscle. The seropositive serum was obtained through the office of Dr. Carl F. Ansevin, M.D. in collaboration with Humility of Mary Health Systems. The seronegative serum was obtained through Dr. Shani Vaturi, M.D. All sera and medical records were obtained through recorded consent forms in accordance with the affiliated institutions or doctors.

Table # 1: Patient summary

Patient	Notation for IgG (+)	Notation for IgM (+)
MG10	MG10-1	MG10-2
SNMG15	SNMG15-1	SNMG15-2

Table # 2: Patient information summary

Patient	Anti-ACh	Thymoma	IgG	IgM
MG10	+	+	+	+
SNMG15	-	-	-	+

The SPMG serum was collected in red capped tubes. The red cap denotes that there were no chemicals or preservatives added to the whole blood. The serum was centrifuged to separate the serum and pellet the red blood cells. The serum was preserved in 100μL aliquots at -80°C. The SNMG serum was collected in red and gray capped tubes. The gray color represents the presence of a silicon gel, a serum separator/clotting activator to help in the division of the blood cells and the serum. The tubes were refrigerated, -4°C, for 20 minutes, to induce clotting, and then centrifuged. Again, the serum was maintained as stated above.

Screening of the Stratagene® Human Skeletal Muscle Copy DNA (cDNA) Expression Library

The Lambda Zap® II expression library was purchased from Stratagene®, Inc. This premade amplified expression library contained roughly 3 million human cDNA segments engineered into the Lambda Zap expression vector and packaged into a pBluescript bacteriophage. The library came with the host strains of XL1-Blue MRF′ and SOLR™, as well as f1 helper phages ExAssist™ interference-resistant and VCSM13 interference-resistant helper phage. All materials were maintained at -80°C.

The library was used to identify immunoreactive skeletal muscle autoantigens that displayed immunoreactivity when presented to autoantibodies from patients' sera with seronegative and seropositive MG. The cDNA segments were inserted into the vector at the endonuclease *Eco*RI site within the vector. This site was under the control of an inducible lacZ promoter which was used to drive expression of the fusion protein. The screening of the library started with growing an overnight culture of the XL-Blue in LB

broth (Appendix 2). The cells were then washed in TBS, pelleted, and resuspended in 10mL of $MgSO_4$ (Appendix 2) to an optical density$_{600}$ (O.D.) of 0.5 (cells/mL) (O.D. was performed on a Beckman DU®-50 Series Spectrophotmeter). The host strain of *E.coli* (XL-Blue) cells were then infected with the diluted phage (phage diluted to 1mLe-4 in SM buffer) (Appendix 2). The two were incubated for 15 minutes, to allow for enough infection to produce approximately 50,000 plaque forming units (pfu). At which point, the solution was added to NZY top agar and plated onto 150mm NZY agar plates (Appendix 2) to form bacterial lawns. The plates were incubated for 6-8 hours at 37°C.

BioBond nitrocellulose membranes/filters (Sigma) were saturated for 2 hours in a 2% solution of Isopropyl-β-D-thiogalactopyranoside (IPTG) and TBS (Appendix 2). The saturated membranes were placed onto the plates and incubated for 1hour. The membranes were then lifted off the plates and washed three successive times in TBS-T (Appendix 2) for 5 minutes each and dried overnight. The membranes were rehydrated in TBS and soaked in 3% blocking buffer containing 3% non-fat dry milk, 0.2% Tween-20 in 100mL TBS (Appendix 2) for 2 hours. This blocking step ensured that all unbound portions of the membrane would be covered with milk protein to cut down on any false positives. The membranes were then placed into a 1% blocking buffer solution (Appendix 2) which contained 200µL of the patient's serum and the membranes were placed on an orbital shaker to incubate for 4-6 hours.

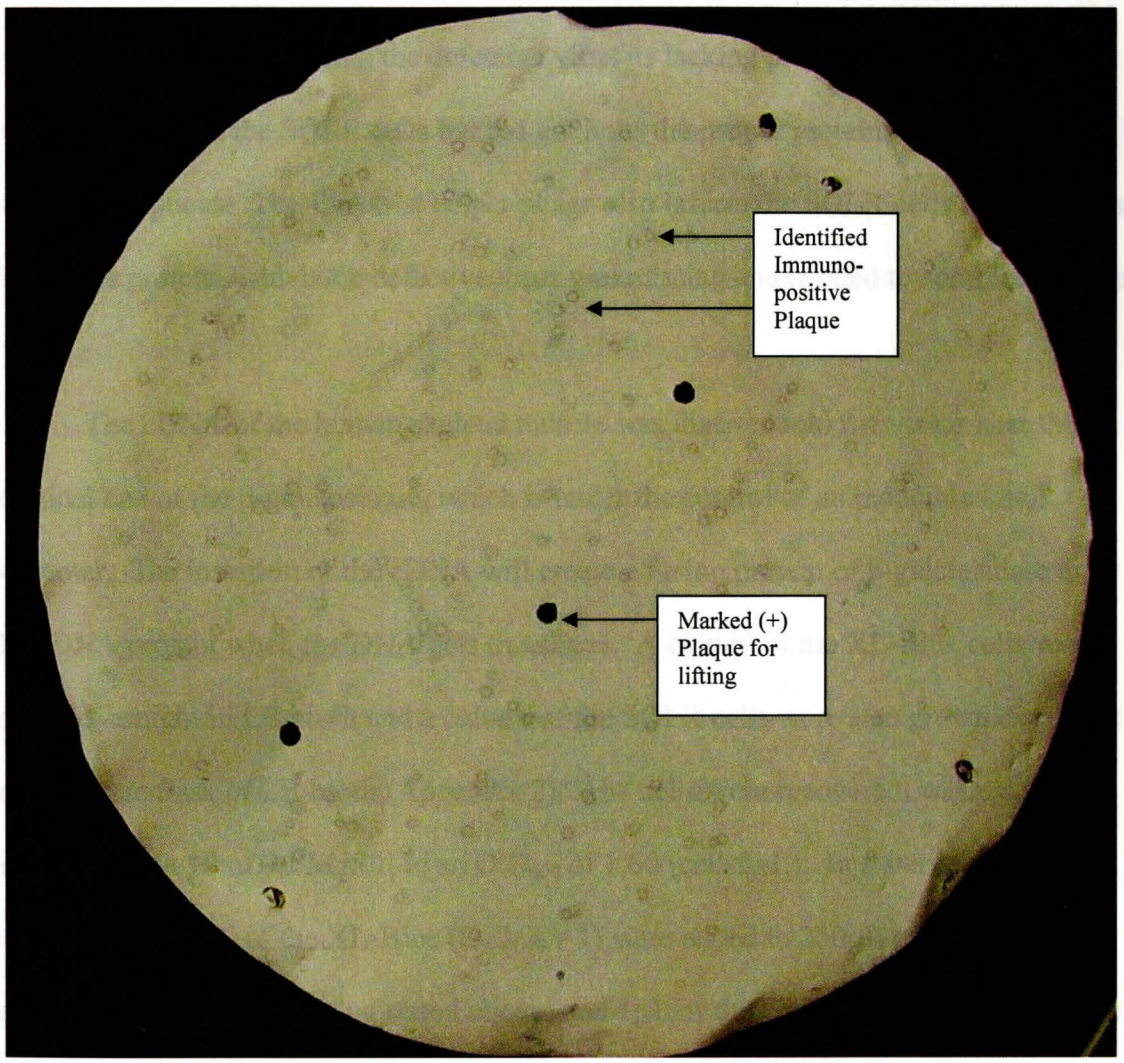
The membranes were recovered and put through another set of 3 washes of TBS-T to remove any unbound antibodies. Again, the membranes were put into a 1% blocking solution and 40µL of the secondary antibody of choice was added. The two secondary antibodies used in this study were IgG and IgM specific, both purchased from

Sigma, Inc. (Appendix 2). The two secondary antibodies were Goat anti-Human conjugated to horseradish peroxidase (HRP) and marked specific for its antibody isotype (IgG/IgM). The secondary antibody was chosen to help in the visualization of the primary antibody by chromogenic detection. The contents were again placed on the orbital shaker for 2 hours.

The recovered membranes were washed again in 3 successive washes with TBS (to get the Tween-20 out). The membranes were then subjected to a chemiluminiscent substrate detection solution (Appendix 2). The solution was made in two parts and mixed separately. The first portion was made of 60mL of methanol (MeOH) and 0.180g of 4-chloronaphthol. The second part was made by mixing 300mL of cold TBS and 180μL of 30% Hydrogen Peroxide (H_2O_2) (Appendix 2). The two parts were then mixed in a glass dissection bowl at which time the membranes were set in and incubated for 45 minutes. After the incubation period, small black immunoreactive doughnut-like plaques appeared. This was the indication of an immunoreactive clone (shown in figure # 2). The membranes were aligned back onto the plates and a 50μL plug of the agar (containing the immunoreactive cDNA) was isolated and maintained in 960μL of SM buffer and 40μL of chloroform (Appendix 2), the plug was vortexed and stored at -4°C. To establish a pure clonal colony the phage was plated again on smaller plates (100mm) (this re-screening process was done a total of three rounds). This helped to insure that the final clone used was from a single isolated clonal colony.

Figure # 2

Identified Immunoreactive Positive (+) Plaques on a nitrocellulose Membrane



In Vivo Excision of the pBluescript SK(-) Phagemid from the Zap II Vector

The use of the ExAssist helper phage with the SOLR strain helped ensure efficient excision of the pBluescript phagemid from the Lambda Zap II vector and eliminate possible co-infection. The helper phage is a virus that helps a separate defective virus reproduces by providing the defective virus its lacking proteins. The pBluescript phagemid infected the SOLR cells but did not have the proper proteins necessary to reproduce/replicate. The ExAssist helper phage also infects the SOLR cells and provides the proper proteins which the defective virus was missing and needed to complete its life cycle.

The cDNA of the human skeletal muscle was inserted into the vector near the N-terminal end of the β-galctosidase, which is under the control of an inducible LacZ promoter. The insertion of the cDNA will create a fusion protein of β-galctosidase and the cDNA protein when the DNA gets translated. A culture of the XL-Blue cells were grown overnight in LB broth and a culture of the SOLR cells were also grown overnight in a separate flask of LB broth (Appendix 2). The cells were recovered, washed, and resuspended in 10mL of $MgS0_4$ to an $O.D_{600}$ of 1.00 (cells/mL). In a sterile 15mL conical tube 200μL of the XL-Blue ($O.D_{600} = 1$) were added to 250μL of the phage (selected clone from agar plug stated above) and 1μL of the ExAssist helper phage. The tube was placed in the incubator and was left for 15 minutes. This solution was then added to 3mL of LB broth and placed in a shaker and incubated at 37°C for 3 hours. The tube was recovered and placed in a 70°C water bath for 20 minutes. The solution was centrifuged in a Beckman GPR centrifuge (Cat # 349702 (GH-3.7 rotor)) at 1,000 rpm for 15 minutes and the supernatant was saved and maintained at -4°C.

Plating and Selecting of Excised cells

The cells were plated at two different concentrations (1:10 and 1:100) (μg/μl). The plating of two different concentrations allowed for better selection of one isolated plaque. The first concentration used 100μL of the phage solution and the second concentration used 10μL of the same phage solution stated above. The selected amount of phage was then mixed with 200μL of the SOLR cells prepared above. The two incubated for 15 minutes. 200μL of the incubated solution was then plated onto LB media plates that had ampicillin (1:1000) added to them (LB+Amp) (Appendix 2) and incubated overnight. The ampicillin plates were used as a selectable marker for the excised cells. Only the cells that were excised properly will contain the plasmid (with an amp-resistant gene) that will allow for the *E.coli* to be ampicillin resistant and propagate.

Restriction Enzyme Mapping of the cloned phagemids/clones

DNA agarose gel electrophoresis was the first step in the characterization of the cDNA insert within the clone. A Miniprep Plasmid DNA Purification Kit (Cyclo-Prep™) was purchased from AMRESCO®. This kit was used to isolate the cDNA insert and prepare the DNA for agarose gel electrophoresis. Once the cells were prepared for the restriction digest, the restriction enzyme *Eco*RI was used, again this was the best choice since the cDNA was inserted using this enzyme. 20μL of the DNA solution was mixed with 2.5μL of H buffer, 1μL of sterile ddH_2O, and 1.5μL of *Eco*RI (Appendix 2). This solution was placed into a 37°C water bath for 1 hour. After an hour, 6μL of a stop buffer (Appendix 2) was added to stop further digestion.

DNA Agarose Gel Electrophoresis

DNA agrose gel electrophoresis was performed to verify that there was an insert and characterize the size of the inserted cDNA. A 1% agarose gel was made with TAE (1X) (Appendix 2). 3.5μL of the standard lambda ladder (λ-ladder) (of known b.p.sizes) was added to the first well of all gels. 20μL of the uncut DNA sample was added to a well as a control, while 20μL of the cut DNA sample was added to analyze the insert's size. The power supply was set for 100 volts and the gel was left to run for 1 hour. The gels were then stained with 1:10,000 dilution of ethidium bromide in ddH_2O for 45 minutes. Gels were destained in ddH_2O for 20 minutes and subjected to an ultra violet (UV) light source where a picture was taken. The clone was analyzed by measuring the bands and comparing them to known sizes of the λ-ladder. The remaining clones were maintained in a 30% glycerol stock solution containing filtered sterilized glycerol and the clones in LB+Amp broth (Appendix 2) and stored at -80°C.

PCR and Automated Sequence analysis

Polymerase Chain Reaction (PCR) (Mullis and Faloona 1987) was performed to amplify the cDNA insert of interest for sequencing. The pBluescript vector came with suitable primers for the insert in question. The two primers used were an M13 Forward primer which was utilized to sequence the upstream portion of the cDNA insert and a Reverse primer that started at the β-galactosidase coding region. A Beckman-Coulter DCTS-Quick Start kit (Appendix 2) was used during the PCR reaction to label each nucleotide in the DNA sequence with the necessary deoxynucleotides (dNTPs) and

dideoxynucleotides (ddNTPs). This technique was based on Sanger's dideoxynucleotide chain termination DNA sequencing (Sanger, Nicklen et al. 1977). The ddNTPs are tagged with a dye that fluoresces when exposed to a laser. This fluorescein energy donor dye gets excited when it is passed through the laser. The excited DNA emits light at a specific wavelength that the computer analyzes and can then discriminates between the four different nucleotides to give an accurate sequence (Palladino 2002). The actual sequencing was performed by a Beckman-Coulter CEQ 2000XL automated DNA Sequencer and used dye terminator cycle sequencing (Beckman-Coulter, Inc.).

Bioinformatics: Sequence Analysis

Bioinformatics are methods for analyzing sequencing data using computers. The DNA sequences were analyzed using different software freely available on the World Wide Web. These websites are maintained by the NIH and are accessible through the www.pubmed.org link. All sequences must be formatted in a FASTA (Fast Alignment Search Tools Anything) format (Pearson and Lipman 1988).

>Sequence title

ATTCGATCGTACGTACGTTCGACACCAG

The program BioEdit was used to characterize the sequence. BioEdit located EcoRI sites and performed vector screens to detect any unwanted vector sequence. Screened sequences were then analyzed through the automated search tool BLAST (Basic Local Alignment Search Tool) (Altschul, Madden et al. 1997). BLAST was used to compare sequences against all the known (pub-med) sequences contained in a specific database, such as GenBank (also accessible through pub-med).

This study used BLASTx to compare the cloned DNA (FASTA formatted) sequence, obtained from the sequencer, against known protein sequences in the GenBank database. The database search reports a number of different possible proteins and scores them sequentially from greatest identity to the least. This scoring is done by using a bit score. Bit is a contraction for binary digit, the basic unit of information in the number system used by computers (Claverie and Notredame 2003). The bit score refers to the quality of the sequence over the entire length. This takes into account the number of gaps or break in the matching sequences, % identity and positives (letter to letter matches), and size/length of the sequences, the shorter sequences the more likely it is to be random thus a lower bit score would be given. There is another means of statistical scoring. Along with a bit score, an E-value is also given. This is an expectation value. This value statistically tells you the number of times you could expect this identity to happen randomly by chance. In this case, It is understood that the smaller the E-value and higher the bit score the stronger the identity (Claverie and Notredame 2003). Bit scores > 100 and E-values < 10^{-10} are universally required before any biological significance is accepted (Baxevanis and Francis-Ouellette 2001).

The biological significant sequences (in FASTA format) were analyzed in Java Script DNA translator (vesion1.1). This software translated the DNA sequence into an amino acid sequence (in the same open reading frame (ORF) of reference) as the BLASTx alignment. The peptide sequence was then pasted into Microsoft Word and highlighting tools were used to match identities and positives. Identities are exact matches of amino acid residues while positives are amino acids that don't match exactly

but are chemically similar and may not change the function of the peptide so they are still significant (Shown in figure # 7).

The program CLUSTALX was used to make multiple sequence alignments. Again, FASTA format was used to upload the pMG10-1 peptide sequence and the complete peptide sequence of the muscle protein titin isoform N2A (GenBank Accession # NP_596869.1). The two protein sequences were aligned to display the overlapping region. (Shown in figure # 9).

SDS-PAGE Protein/Immunoblot (Western Blot) analysis of the fusion protein

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli 1970) and Western blot (Towbin, Staehelin et al. 1979) techniques were used to separate the proteins based on size and identify the proteins (translated from the cDNA) that immunoreact with autoantibodies from the patient's sera.

First, an overnight culture was grown from the glycerol stock in LB+amp media (Appendix 2). 1mL of that culture was added to 100mL of fresh LB+amp broth and the cells were grown for 4 hours. 100µL of IPTG (Appendix 2) was added to induce the LacZ promoter and in turn start transcription of the fusion protein. After 1 hour, 50mL of the culture were pelleted for 10 minutes in a Beckman GPR centrifuge (as previously stated) set at 1,500 rpm and 4°C. The cells were recovered and washed in 10mL of TBS and centrifuged again under the same conditions.

The supernatant was discarded and the cells were resuspended in 5mL of TBS and subjected to point sonification for 1min at which point 500µL of 10% Triton was added and the cells were sonicated for and additional 30 seconds. The point sonification should

have lysed the cells and the triton was added to break up any inclusion bodies and help solubolize the proteins. All steps were done on ice to inhibit protease activity. The sonicated solution was again centrifuged for 20 minutes. 1mL of the supernatant was added to 330μL of 4X SDS sample buffer (Appendix 2) to denature the proteins and leave a uniform negative charge on the peptides. 50μL of 2-β-mercaptoethanol was added and the solution was boiled for 2 minutes.

The proteins were then separated by using a SDS-PAGE mini-slab apparatus (Matsudiara and Burgess 1978). The gel consists of two distinct portions. The first is a 5% stacking gel (Appendix 2), which compresses and stacks the proteins as they pass through. The larger bottom section is referred to as the resolving gel. The resolving gel is a 10% acrylamide gel that serves to separate and give a better resolution to the protein bands. A 5μL pre-stained Kaleidoscope standard (Bio-RAD, Inc.) (Appendix 2) was added in lane one of all the gels while 10μL of the prepared sample (as stated above) was added to other lanes. The 10μL load supplied approximately 1-5μg of protein to each lane. The gel was submerged and the tank was filled with SDS buffer solution to help in the conductivity. The gel was run at 0.25amps per a gel and ran for approximately 1 hour. The gels were stained in Coomassie Brilliant Blue (Appendix 2) to visualize the proteins. Destain was added for 30 minutes to wash excess coomassie off the gel. Finally the gel was soaked in ddH_2O for 1 hour.

The immunoblotting was performed to identify which protein(s) display immunoreactivity to the autoantibodies in the patient's sera. Proteins were prepared as stated above and the SDS-PAGE was carried out as stated previously, up to the point of running the gel in the electric field. The gel was then stacked onto a PVDF membrane

and positioned into the Western blotting chamber for two hours to ensure protein transfer. The PVDF was then blocked in a 3% blocking buffer (Appendix 2) for 1 hour. The membrane was then washed 3 times in TBS-T. The membranes were incubating in a 1% blocking solution while exposed to the patient's serum, as the primary antibody, at a concentration of 1:500 for 1 hour. The membranes were again washed 3 times in TBS-T and secondary antibody, fab specific goat anti-human/horseradish per-oxidase (Sigma, Inc.) (Appendix 2) was added in a concentration of 1:3000. The membranes soaked for 1 hour. The PVDF membranes were then washed 3 times in TBS and secondary antibody binding was detected by chemiluminescent substrate (Pierce Chemical, Inc.) (Appendix 2) the membrane was then exposed to x-ray film by autoradiography and developed (Appendix 2).

Two Dimensional (2DE) gel electrophoresis and immunoblotting

The analysis of the fusion protein in a 2DE gel is far more specific due to the separation of the peptides not on size alone but also by the native charge/isoelectric focusing (IEF). This gives a more precise characterization of the immunoreactive protein. In a SDS-PAGE gel previously described, there may be many proteins of the same molecular weight in one band. The 2DE gel displays single isolated peptides on weight and pH.

An overnight culture of the glycerol stock was grown in LB+Amp broth and 1mL of that was transferred to100mL of fresh media. The culture was divided into two separate cultures, an induced and non-induced. The induced culture had IPTG added to increase protein production via the LacZ promoter, while IPTG was not added to the non-

induced culture. The cells that were induced had 100μL of IPTG added (Appendix 2). The cells were incubated for an additional 1 hour. The recovered cells were pelleted and washed (as stated above). The pellet was then resuspended in 5mL of a rehydration buffer solution (Appendix 2) and subjected to point sonification (on ice) for 3 minutes. The solution was centrifuged (Beckman GPR) at 3,000 rpm for 20 minutes and the supernatant was kept. A modified Bradford Assay was performed (Bradford 1976) (as described by Bio-RAD) to measure the amount of isolated protein (μg/μL). The approximate amount of loaded protein needed for each strip was 170μg of protein/120μL of sample. 120μL of the calculated sample was passively rehydrated overnight. The saturated IPG strip was then added to the Isoelectric focusing power supply. This isoelectric focusing machine (Bio-RAD, Inc.) creates an electric field that separates the proteins on the immobilized strips by pH. The proteins were focused for 20,000 volt hours at 50μamps per a strip. The strip was soaked in two equilibration buffers (equilibration buffers I and II) (Bio-RAD, Inc.) and then soaked in SDS-PAGE buffer for 2 minutes before running the second dimension (Appendix 2).

The second dimension is similar to the first procedure described above for SDS-PAGE. The only difference is the thickness of the gel. The 2DE gel is a 1.00mm thick gel that does not have a stacking gel. The strip is loaded directly on top of the resolving gel and overlaid with agarose. The running and Western blot techniques are all the same as stated above. Sypro Ruby Red (Appendix 2) was used to stain the gel overnight. A Chemidoc XRS (Bio-RAD model # 170-8070) digital camera was used to enlarge and photograph the stained gels. PD Quest 2DE Analysis software analyzed the induced and non-induced protein constellation (map). The software matches similar proteins spots that

appear on both gels (induced/non-induced). The software also measures the intensity of the spots for concentration of protein within the gel. Through computer analysis and Western blot studies, the inducible immunoreactive protein was identified (Shown in Figure # 15).

Results

cDNA library screening and identification of autoantigens

The results of this study characterized the three skeletal muscle autoantigen targets that were discovered and analyzed their possible association in MG. Two different patients are characterized in this study, the seropositive myasthenic (MG10) and the seronegative myasthenic (SNMG15) (Shown in table # 1). Each patient's sera contained autoantibodies that were targeted for IgG or IgM autoantigens (Shown in table # 2). The study identified 3 immunoreactive clones/plasmids; pMG10-1 (**IgG**), pMG10-2 (**IgM**), and pSNMG15-2 (**IgM**). Table # 3 summarizes the results from the immunoreactive clones isolated in this study.

DNA restriction digests and characterization of cDNA inserts

DNA restriction digest produces cDNA inserts of various base pair (b.p.) sizes. The size of each insert was calculated using the Blastx sequence and the 2960 b.p. of the known pBluescript vector. Clone pSNMG15-2 displays an insert with a size calculated to be about 3000 b.p. long (shown in lane 4, figure # 3). Clone pMG10-2 has an insert calculated to be 1000 b.p. long (shown in lane 4, figure # 4). Clone pMG10-1 produces an insert of 2000 b.p. long (shown in lane 2, figure # 5). The Clone pSNMG15-1 does not appear to posses an insert thus no sequence or any further data was obtained or analyzed. All figures display a standard (STD) lane which contains a lambda (λ) ladder digestion for estimation of base pair (b.p,) sizes.

Table # 3: Summary of peptides from the immunoreactive clones

Immunoreactive Plasmid Name	Encoded peptides	E-Value	Bit Score	% Identity
pSNMG15-1	No immunoreactivity	No Sequence	No sequence	No sequence
pSNMG15-2	aldolase A	e-117	423	96 %
pMG10-1	titin Isoform N2A	3e-44	158	67 %
pMG10-2	enolase-3,beta	9e-82	288	87 %

Figure # 3

***Eco*RI restriction digest of plasmid pSNMG15-2:**

Lane two is the uncut plasmid. Lane four is the *Eco*RI digest of the plasmid with an insert size calculated to be 3000 base pairs long and a pBluescript vector is expected to have a length of 2960 base pairs.

Figure # 3

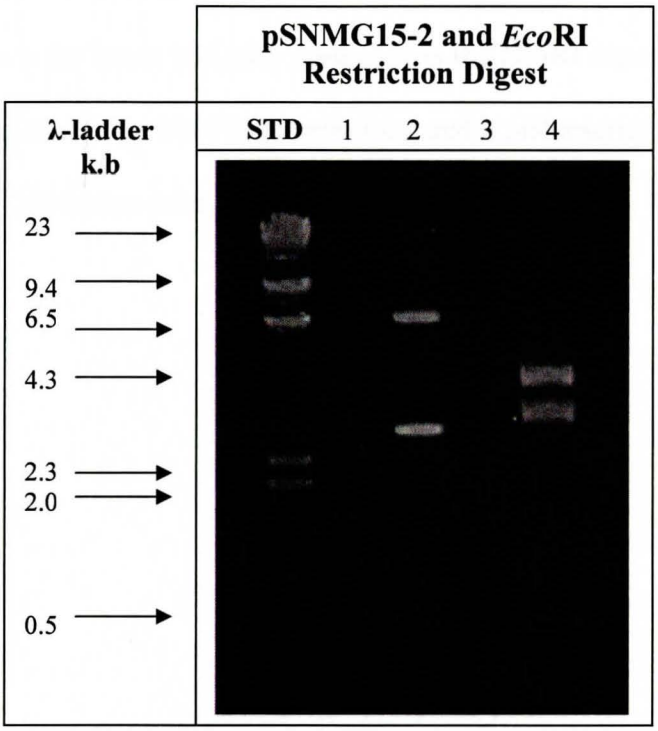

pSNMG15-2 and *Eco*RI
Restriction Digest
λ-ladder
k.b
STD
1
2
3
4
23
9.4
6.5
4.3
2.3
2.0
0.5

Figure # 4

***Eco*RI restriction digest of plasmid pMG10-2:**

Lane two is the uncut plasmid. Lane four is the *Eco*RI digest of the plasmid with an insert size calculated to be 1014 base pairs long and a pBluescript vector expected to have a length of 2960 base pairs.

Figure # 4

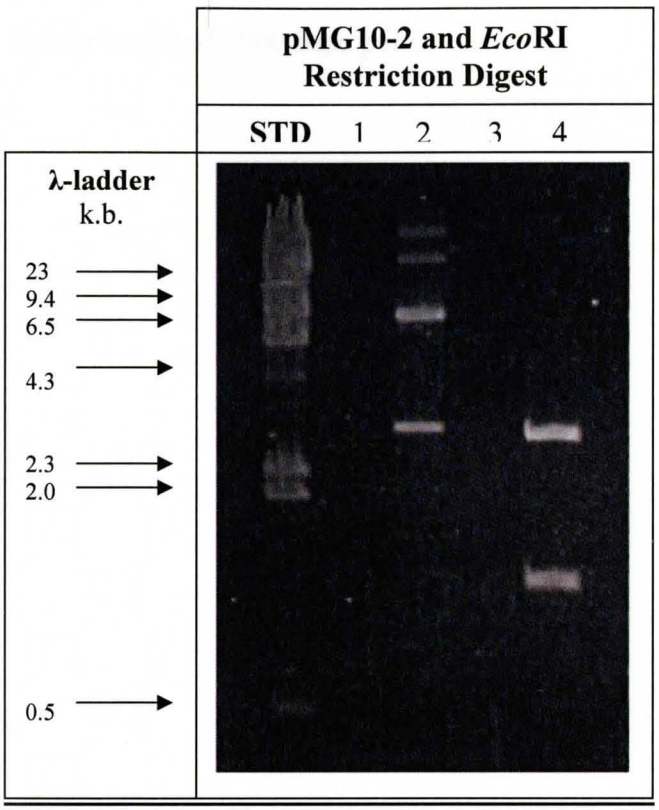
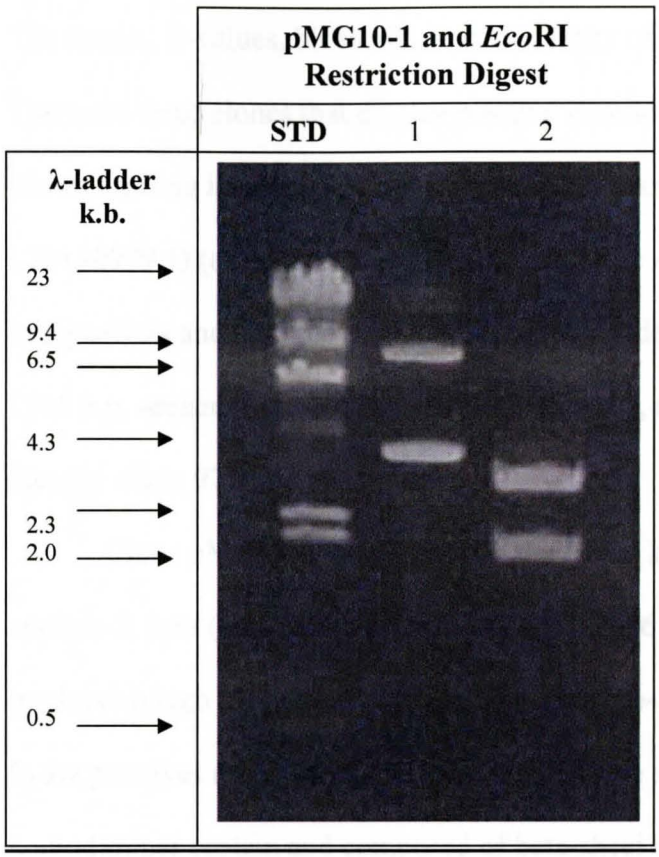

pMG10-2 and EcoRI
Restriction Digest
STD 1 2 3 4
λ-ladder
k.b.
23
9.4
6.5
4.3
2.3
2.0
0.5

Figure # 5

***Eco*RI restriction digest of plasmid pMG10-1:**

Lanes one is the uncut plasmid. Lane two is the *Eco*RI digest of the plasmid with an insert size calculated to be 2000 base pairs long and a pBluescript vector expected to have a length of 2960 base pairs.

Figure # 5


pMG10-1 and *Eco*RI Restriction Digest
STD
1
2
λ-ladder k.b.
23
9.4
6.5
4.3
2.3
2.0
0.5

Automated DNA sequencing identification

The automated DNA sequencing along with the NCBI BLAST and SWISS-PROT database searches shows high score pairing of the positive clones with known proteins. The results, E-values, bit scores, and % identity of these searches are shown in table # 3. There are three clones that display positive immunoreactivity. The clone pSNMG15-2 shows a strong identity with the *Homo sapiens* protein aldolase A (Gen Bank Accession # CAA30979.1) (alignment in figure # 6). Aldolase A is a homotrimeric enzyme involved in glycolysis and the breakdown of glucose (Vander. A, Sherman. J et al. 2001). The 1165 b.p. sequence produces an E-value of e-117, a bit score of 423, and displays 96 % identity while 97% are positives.

Clone pMG10-2 identifies with the *Homo sapiens* protein muscle specific enolase-3, beta (Gen Bank Accession # NP_001967.1). This 1014 b.p. sequence also produces a high bit score of 288, an E-value of 9e-82, and displays 87% identity while 88 % are positives (alignment in figure # 7). Muscle specific enolase-3, beta is a homodimmer protein and composed of beta-sheet subunits and is also involved in the glycolysis pathway (Dolken and Leisner 1975).

Immunoreactive clone pMG10-1 shows sequence identity with *Homo sapiens* muscle protein titin isoform N2A (Gen Bank Accession # NP_596869.1). The 873 b.p. sequence has a bit score of 158, an E-Value of 3e-44, and displays 67% identity while 72 % are positives (alignment in figure # 8). This sequence is similar to other target autoantigen sequences from autoimmune rippling muscles associated with MG (ARMD) patients (Watkins 2004). The computer based alignment program ClustalX was used to align the clone's query to the complete mapped titin sequence (GenBank Accession #

NP_596869.1) (Bang, Centner et al. 2001). This alignment can be seen in figure # 9 which shows the strong alignment between the two starting around amino acid # 14,170. The importance of this amino acid sequence is that the end of it falls inside the Main Immunogenic Region (MIR) of the titin protein (shown in figure # 10) and is consistent with the other literature on titin isoform N2A and MG (Gautel, Lakey et al. 1993).

Figure # 6

Plasmid pSNMG15-2 amino acid sequence shows identity with the protein aldolase A: A 1165 b.p. sequence (truncated here) produced by the reverse primer with the amino acid translation in the -1 reading frame. Identity to aldolase A (Gen. Bank Accession # CAA30979.1) indicates a bit score of 423 and an E-value of e-117. The BLASTx alignment produces 210/217 identities (96%) while 212/217 (97%) are positives. The identities are indicated in red and the positives are shown in yellow.

Figure # 6

Translation of: pSNMG15-2

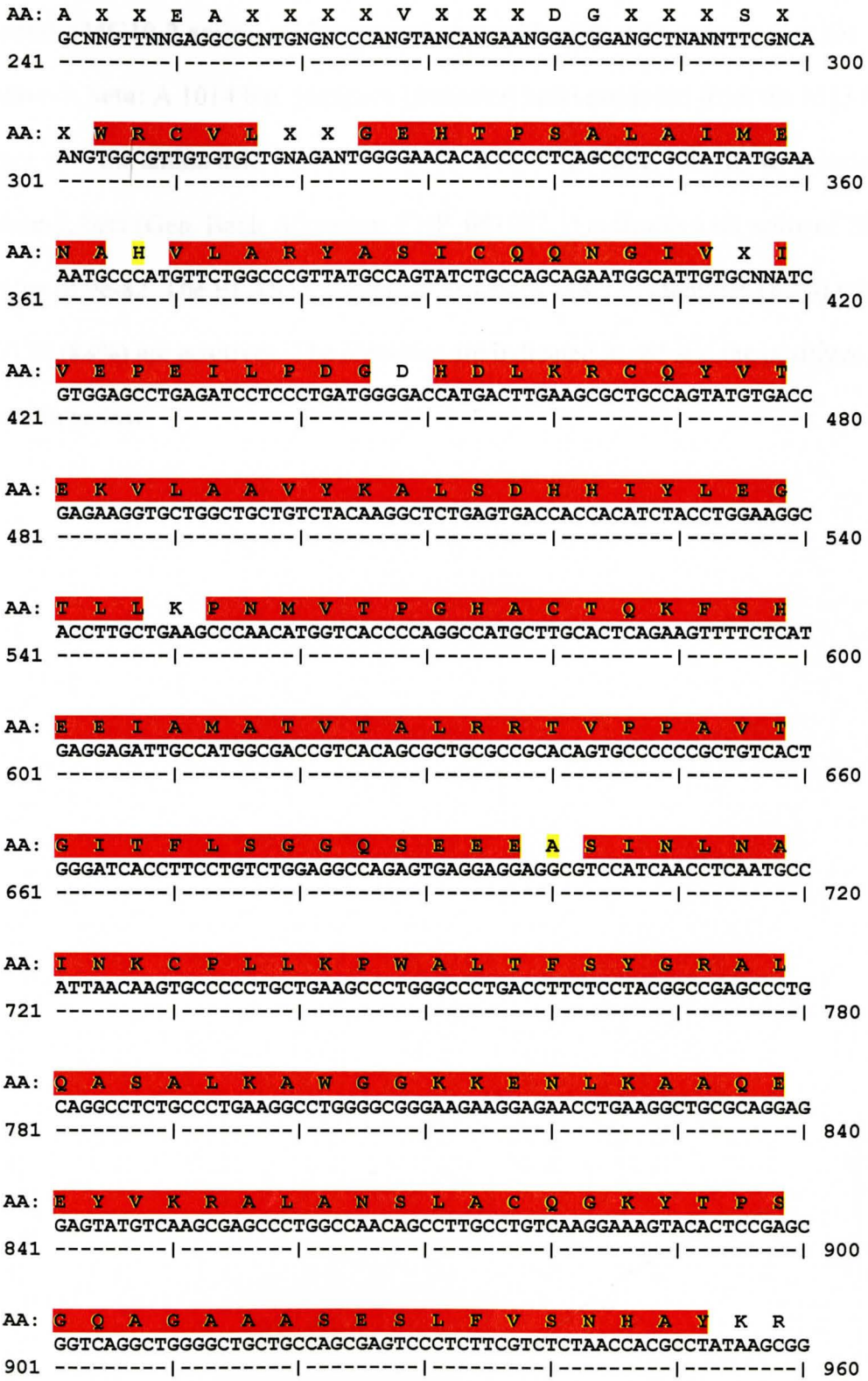


Figure # 7

Plasmid pMG10-2 amino acid sequence shows identity with muscle specific enolase-3, beta: A 1014 b.p. sequence (truncated here) produced from the M13 forward primer with the amino acid translation in the -2 reading frame. Identity to muscle specific enolase-3, beta (Gen. Bank Accession # NP_001967.1) indicates a bit score of 288 and an E-value of 9e-82. The BLASTx alignment produces 148/170 identities (87%) while 151/170 (88%) are positives. The identities are indicated in red and the positives are shown in yellow.

Figure # 7

Translation of: pMG10-2

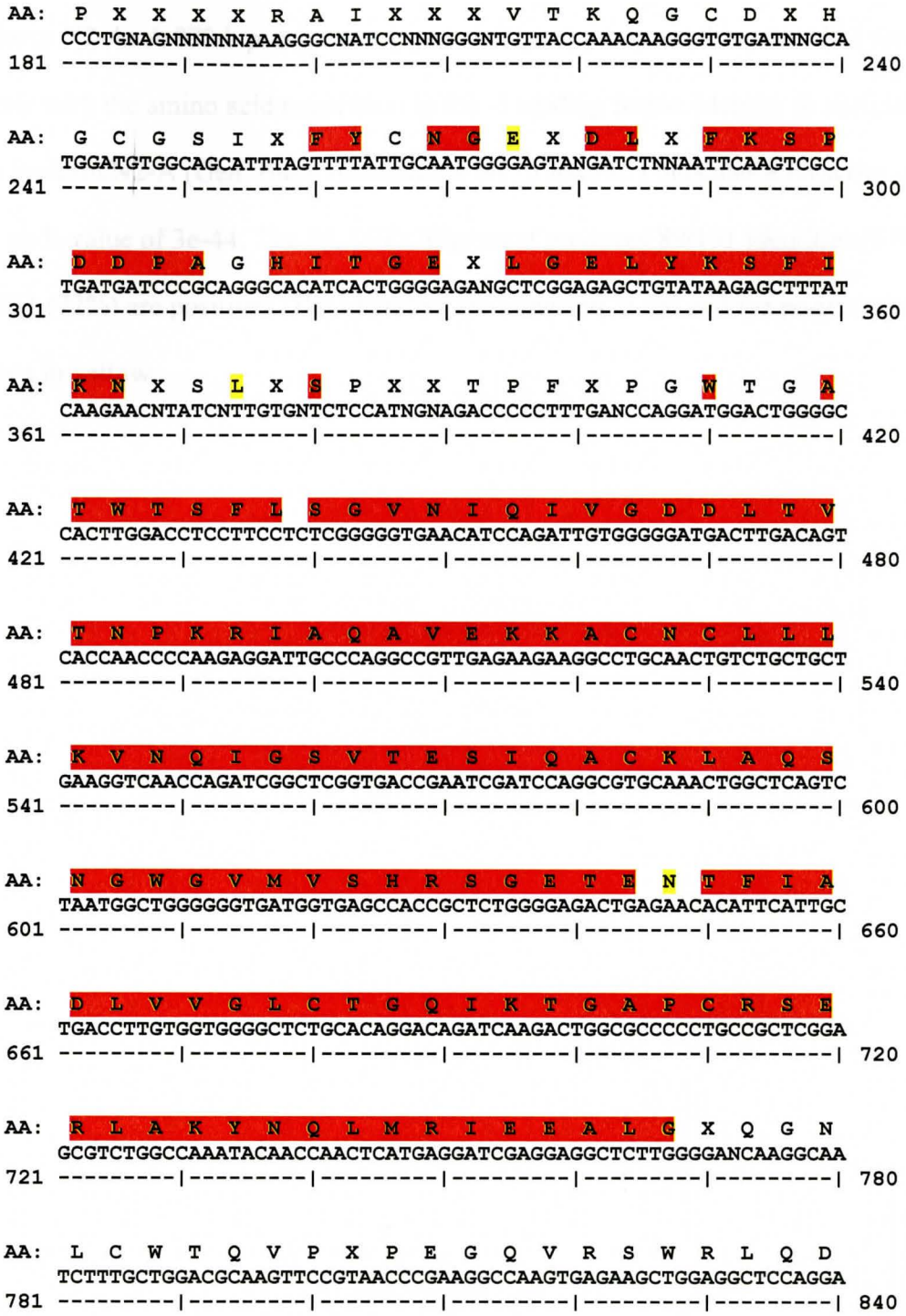


Figure # 8

Plasmid pMG10-1 amino acid sequence shows identity with skeletal muscle titin isoform N2-A: A 873 b.p. sequence (truncated here) produced from the M13 forward primer with the amino acid translation in the -3 reading frame. Identity to skeletal muscle titin isoform N2-A (Gen. Bank Accession # NP_596869.1) indicates a bit score of 158 and an E-value of 3e-44. The BLASTx alignment produces 89/131 identities (67%) while 95/131 (72%) are positives. The identities are indicated in red and the positives are shown in yellow.

Figure # 8

Translation of: pMG10-1

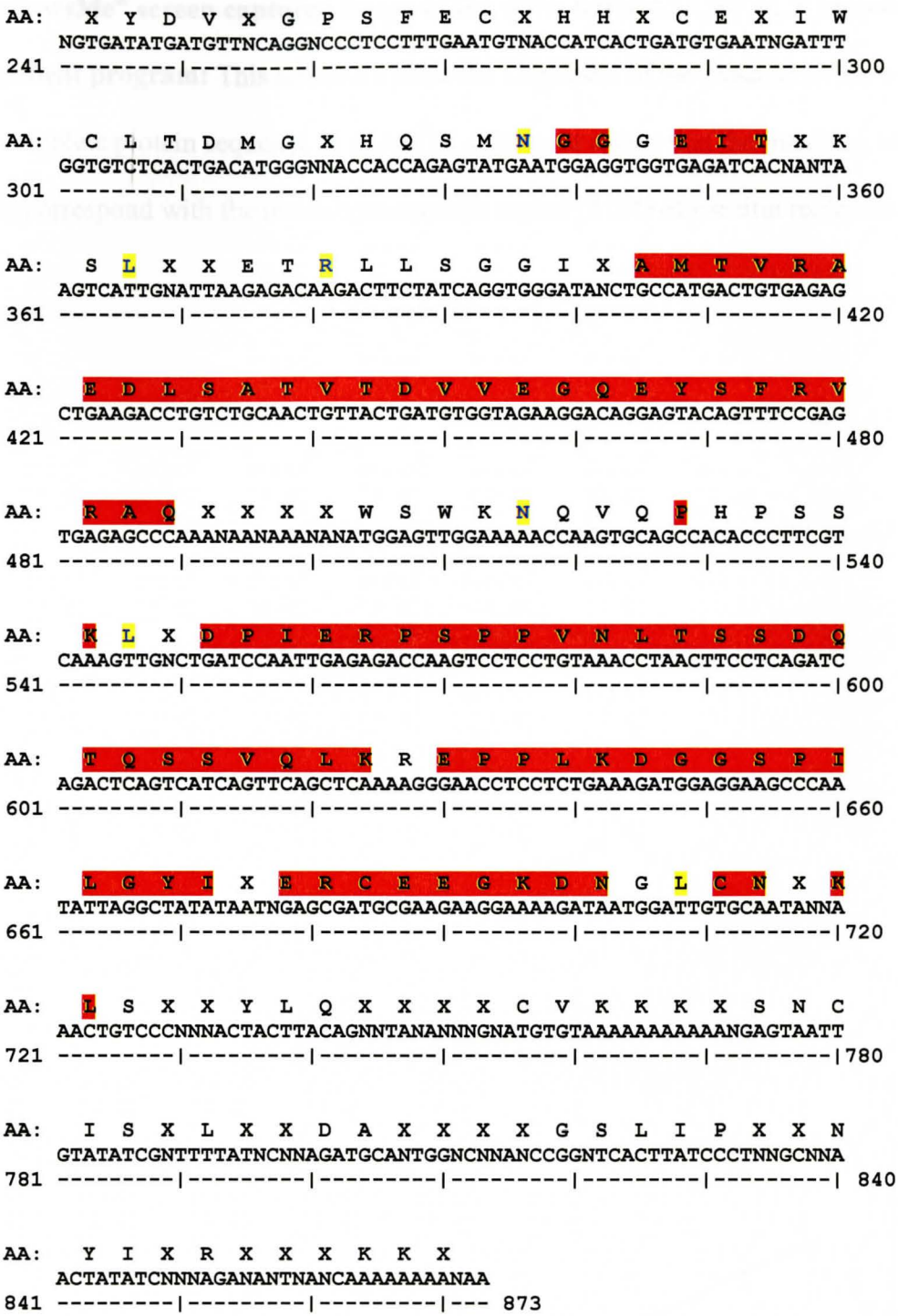


Figure # 9

WindowsMe® screen captured image of the www based CLUSTALX bioinformatics alignment program: This screen displays the alignment of the plasmid pMG10-1 and the complete protein sequence of titin. The alignment shows that the matching amino acids correspond with the main immunogenic region (MIR) of the titin molecule.

Figure # 9

Alignment of plasmid pMG10-1 and the complete titin sequence using ClustalX software

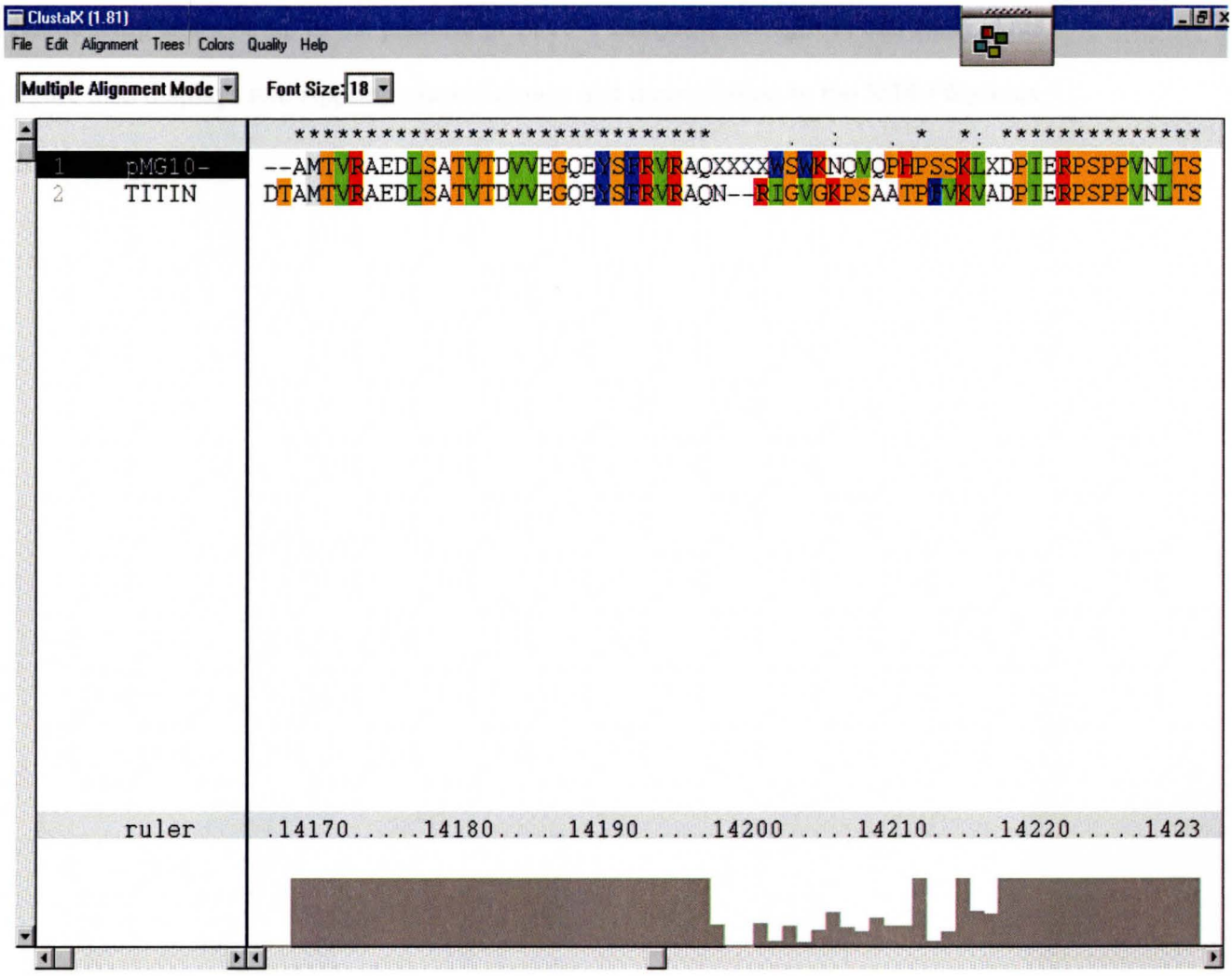
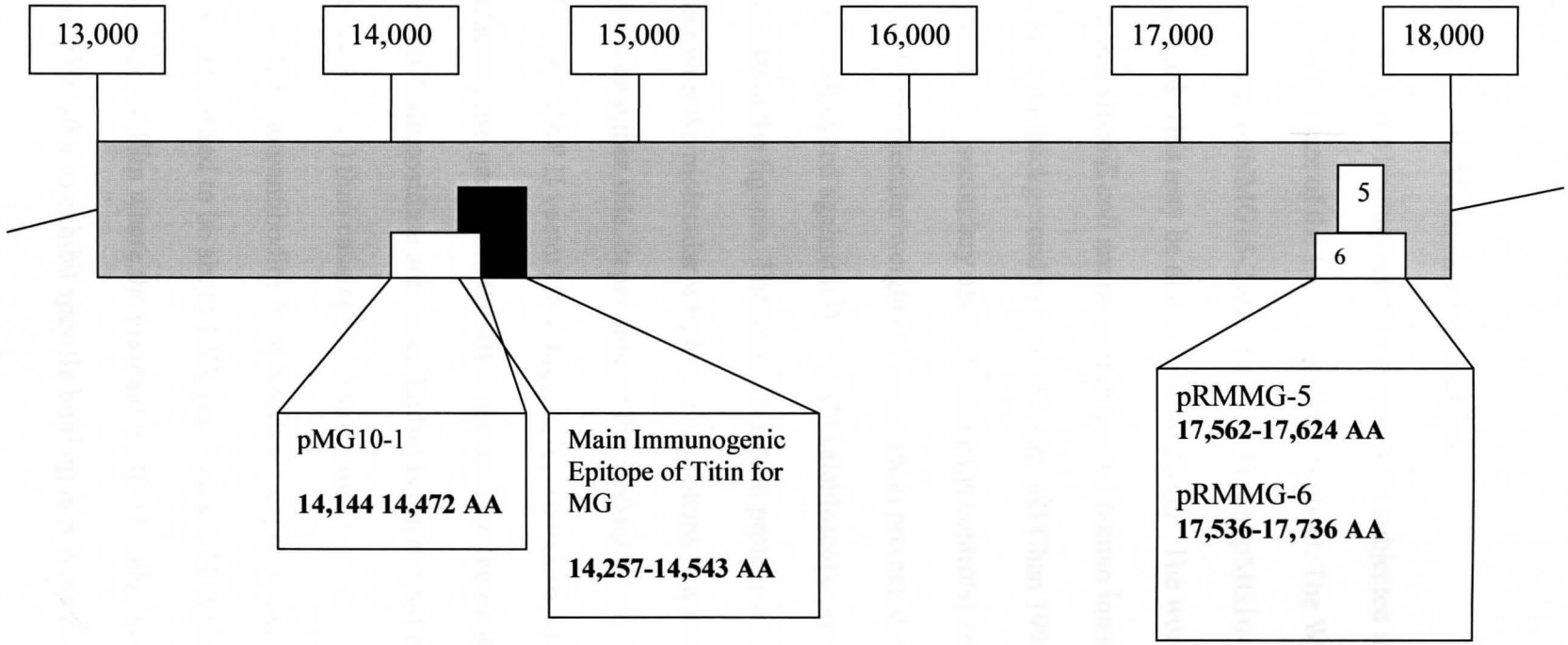


Figure # 10

Titin Isoform N2-A Map of Amino Acid 13,000 to Amino Acid 18,000: This figure depicts the (MIR) of the titin molecule (GenBank Accession # AAB28119) and the relationship and overlap of the plasmid pMG10-1 analyzed throughout this study. This figure also displays two rippling muscle clones and their relation to the MIR (Watkins 2004).

Figure # 10 **Titin Isoform N2-A Map of Amino Acid 13,000 to Amino Acid 18,000**



SDS-PAGE and Western Blot Analysis:

All three immunoreactive clones were subjected to SDS-Page and Western Blot to determine the size of the fusion proteins involved. The Western blots shown from this study, mainly pSNMG15-2 and the 2 DE blot of pMG10-1 may display some minor background. This may be due to the host *E.coli*. The work of Covini and others have shown that since *E.coli* are present in normal human intestinal flora, that they can be the cause of some background in blots (Covini and Chan 1996). The 2DE Western blots were performed with secondary antibody alone (as controls) and displayed no binding.

The molecular weights of each fusion protein, due in part of the interruption of the, was calculated against a BIO-RAD kaleidoscope pre-stained ladder (cat # 161-0324) not shown in the figures. The idea of a fusion protein should be taken into account when dealing with the molecular weights. The proteins in question have some β-Galactosidase residues, on either side, depending on the reading frame. The pBluescript phagemid (in the Lambda ZAP II vector) (Stratagene, Inc.) contains 36 amino acids of the β-Galactosidase gene from the MET sequence to the *Eco*RI site. A total of 131 amino acids of β-Galactosidase are coded for but is interrupted by the polylinker site (Stratagene, Inc.) thus causing the fusion protein.

IgM autoantibodies from SNMG15 express specific binding to a protein band that was measured to be about 100k.Da. Figure # 11 depicts the blotting membrane next to the exposed film where the immunoreactive bands line up. IgM autoantibodies from patient pMG10 also exhibit specific binding in a Western Blot. In this blot there are

actually two different bands. The more pronounce band was much smaller in size, measuring about 23kDa (shown in figure # 12). The faint band is 82kDa in size. It is hypothesized that the larger band is in fact the fusion protein and that the small fragment might have been cleaved by proteolytic enzymes displaying the 23kDa immunoreactive band. The other explanation for the smaller band is that this again could be background from *E.coli* or another protein from *E.coli* that a human has antibodies against (Covini and Chan 1996).

The IgG autoantibodies from patient MG10 also display specific binding. The analysis of this blot shows an unusual doublet at 140k.Da and 123k.Da (shown in figure # 13). This blot displays similar characteristics to ARMD blots done previously in the lab (Watkins 2004). Since clone pMG10-1 (Titin) has a number of important characteristics, 2 dimensional (2DE) gel electrophoresis was carried out, as described in the methods, to better characterize the peptide. The gel was stained and the digital photo can be seen in figure # 14. A Western blot was performed on the 2 DE gel and after analysis, the autoantibodies display binding to a specific protein(s) in the PH range of 3-8. Further analysis will need to be done to confirm if it is in fact the doublet displayed in the 1 dimension. The Western blots that show specific binding confirm that the immunoreactive binding epitope is more than likely conserved through the denaturing of the protein during SDS-PAGE (shown in figure # 15).

Figure # 11

Patient's (SNMG15-2) sera identifies proteins produced by *E.coli* cells containing the plasmids with inserts that identify protein aldolase A: This western blot showed immunoreactivity to an antigen appearing at 100kDa. The molecular weight was calculated by the matching of the blotting membrane shown on the left and comparative analysis with the BIO-RAD pre-stained ladder (not shown).

Figure # 11

Western Blot Analysis
pSNMG15-2

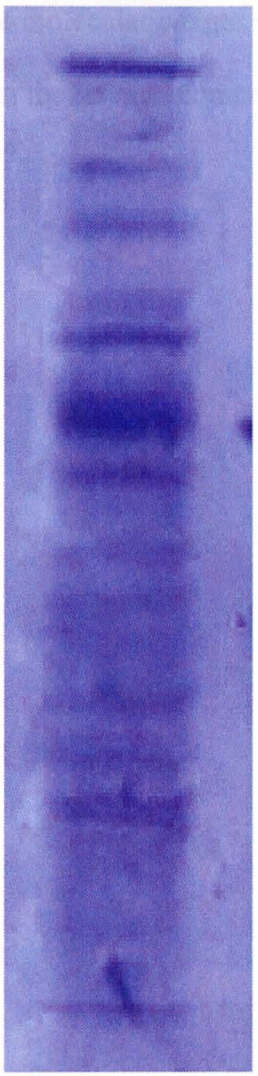
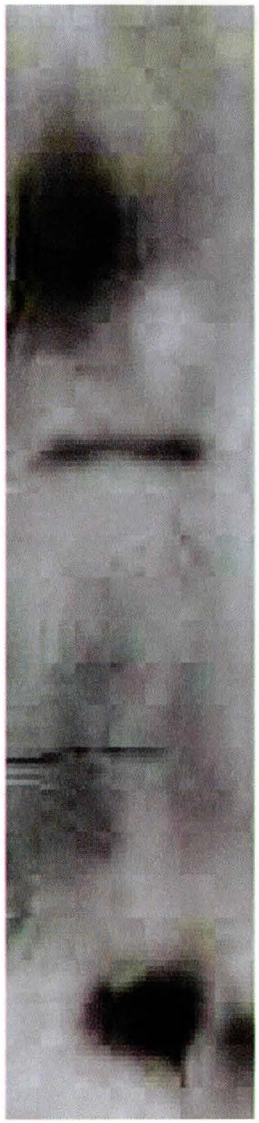
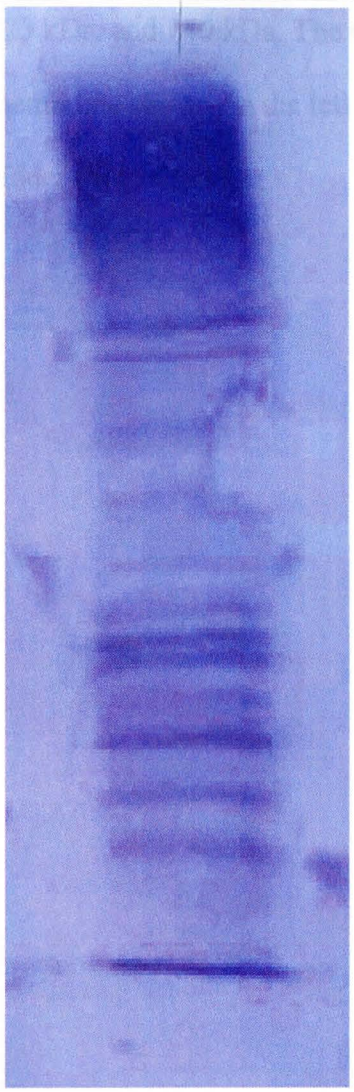
Blotting Membrane		Exposed Film	
	Protein Band ← Dye Front ←	Specific Binding → **100 k.d** Dye Front →	

Figure # 12

Patient's (MG10-2) sera identifies proteins produced by *E.coli* cells containing the plasmids with inserts that identify regions of muscle specific enolase-3, beta: This western blot showed immunoreactivity to an antigens appearing at two different weights. The lower band was calculated to be 23 kDa. The higher band expressed reactivity at 82 kDa. The molecular weight were calculated by the matching the blotting membrane shown on the left and comparative analysis with the BIO-RAD pre-stained ladder (not shown).

Figure # 12

Western Blot Analysis
pMG10-2



Protein Band ←

Protein Band ←

Dye Front ←

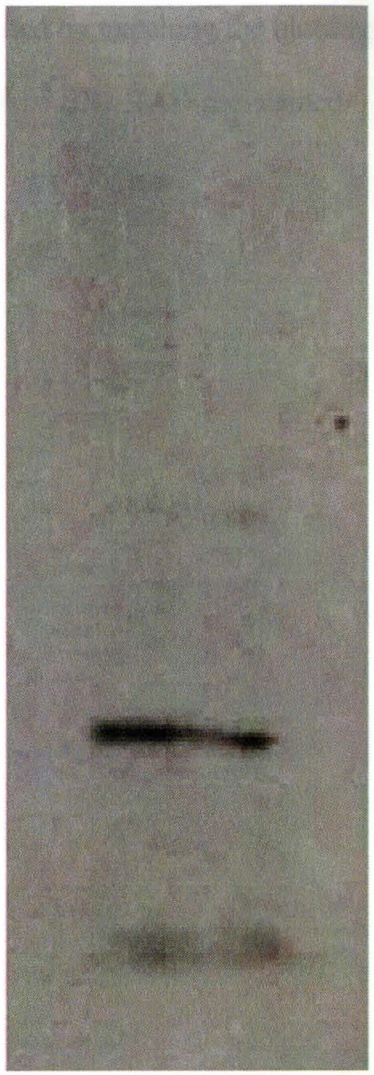
Specific Binding →

82 k.d.

Specific Binding →

23 k.d.

Dye Front →



Blotting Membrane

Exposed Film

Figure # 13

Patient's (MG10-1) sera identifies proteins produced by *E.coli* cells containing the plasmids with inserts that identify regions of skeletal muscle titin isoform N2-A: This western blot showed immunoreactivity to antigens appearing as a doublet between 123 kDa. and 140 kDa. The molecular weights were calculated by matching the blotting membrane shown on the left and comparative analysis with the BIO-RAD pre-stained ladder (not shown).

Figure # 13

Western Blot Analysis
pMG10-1

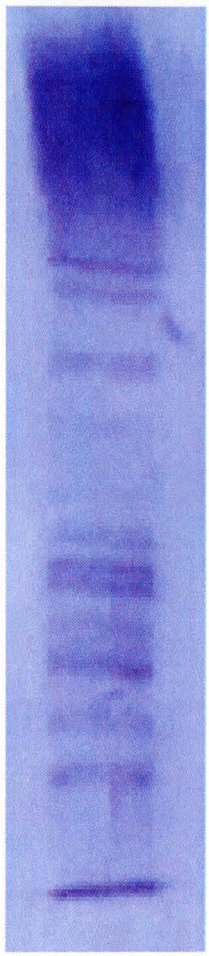
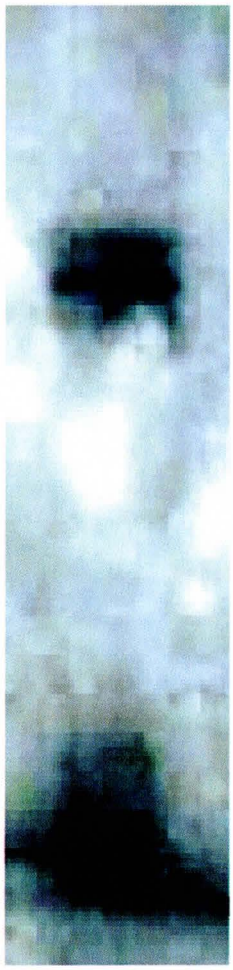
Blotting Membrane		Exposed Film	
	Protein Band ⟷	Specific Binding ⟶ **140 k.d** **123 k.d**	
	Dye Front ⟵	Dye Front ⟶	

Figure # 14

Two dimension protein gel electrophoresis of plasmid pMG10-1: the image displays the induced pMG10-1 plasmid.

Figure # 14

Two dimension protein gel electrophoresis of plasmid pMG10-1:

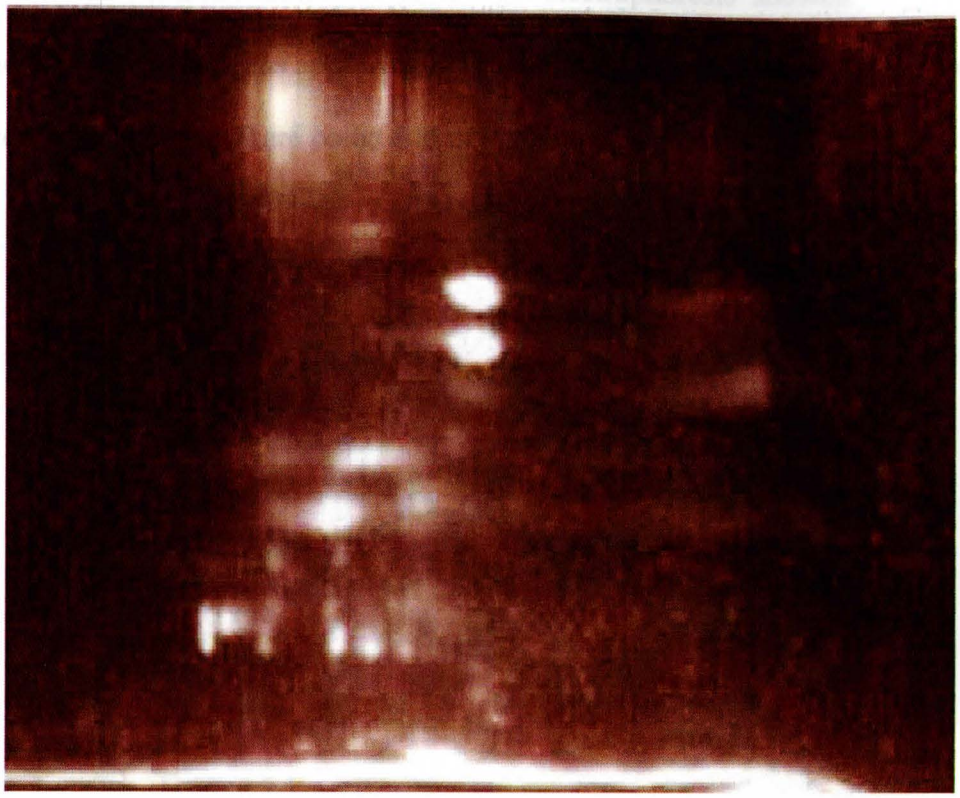


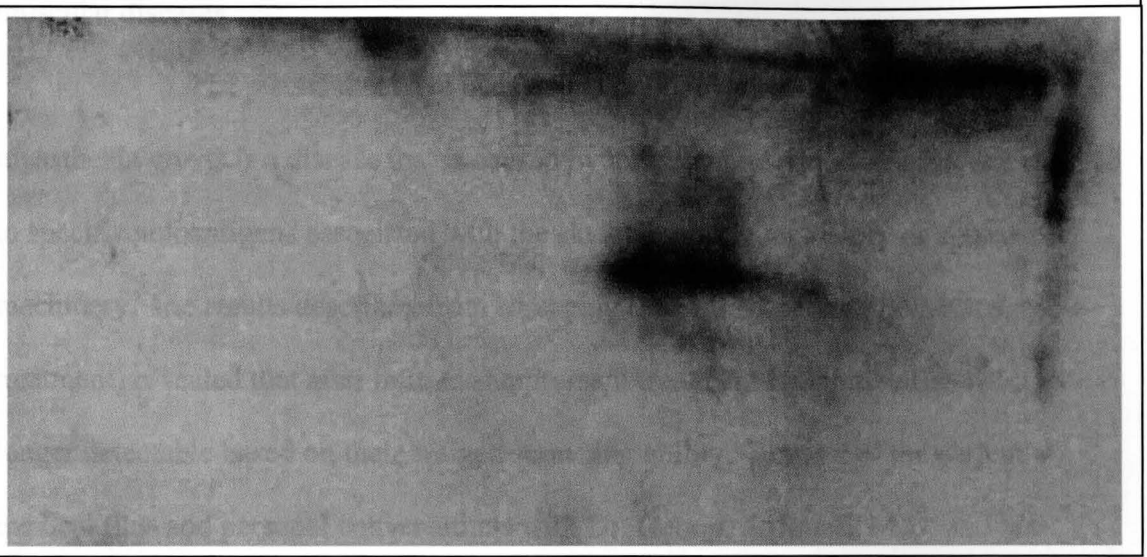
Figure # 15

Patient's (MG10-1) sera identifies proteins produced by *E.coli* cells containing the plasmids with inserts that identify regions of skeletal muscle titin isoform N2-A: This western blot of a two dimension protein gel electrophoresis showed immunoreactivity to antigens appearing in the induced plasmid. The regions of the immunoreactive antigen(s) are pointed to in the blotting membrane.

Figure # 15

Western Blot of the two dimension protein gel electrophoresis of plasmid pMG10-1

pMG10-1 Exposed Film



Discussion

The Identification and comparison of the immunoreactive autoantigens present in seronegative and seropositive myasthenia gravis are imperative for the analysis and understanding of the mechanisms behind this debilitating disease. This study helped identify possible autoantigens that may play a role in MG or similar autoimmune driven muscular diseases.

The results that have been stated previously present evidence that myasthenia gravis is a disease that is caused by autoimmune antibodies that are targeted to specific autoantigens associated with the skeletal muscle regulatory or contractile machinery. The results described from screening the SNMG patient, SNMG15, (post-treatment) revealed that after immunosuppressant treatment IgG antibodies were no longer detectable based on their antigen screening ability. Review of the patient's medical files and personal conversations with Dr. George A. Small, M.D. and his colleges affiliated with the Allegheny Neurological Associates, confirmed that there were no or very little IgG antibodies in this patient's sera.

Dr. Small, the patient's Neurologist, had prescribed two very strong drugs to help curb the myasthenia gravis. The first drug, mestinon, is an active acetyl cholinesterase inhibitor and a common medication for MG. The second drug, prednisone, is a strong immunosuppressant. Both drugs were prescribed in March 2000 at the time of the SNMG diagnosis. The sera samples were taken in January 2003. At this time, the patient was still taking 60mg of the prednisone and 120mg of the mestinon daily. It is hypothesized that after 3 years of immunosuppressant treatment that the drugs have

affected the autoimmune response in lowering the antibody titer. This would limit the amount of free IgG antibodies present in the blood. The results of this research reveal strong data and evidence that the clinical treatment for the SNMG is working by limiting the titer of the IgG antibodies present.

The muscle protein identified while screening with SNMG15-2 sera (antibody isotype IgM) was aldolase A. Aldolase A is an enzyme that is involved in the breakdown of glucose, galactose, and fructose. Aldolase is used in step 4 of glycolysis and is used to hydrolyse Fructose-1,6-Diphosphate into 3-Phosphoglyceraldehyde and Dihydroxyacetone Phosphate. Aldolase A is found in high concentration in skeletal muscle due to the constant need of ATP for energy. Aldolase is usually found in high levels in fast twitch muscle as opposed to slow twitch (Spitz and Demignon 1998).

Myasthenia gravis is a disease well know as an inflammatory autoimmune disease of the skeletal muscle (Lang, Badea et al. 1997). The inflammation of the skeletal muscle tissue leads to the damage of the cellular membrane. The inflamed tissues may present antigens that normally would not be present at the cell surface. It has been documented that an antibody can cross the cell membrane to reach an intracellular antigen (Alarcon-Segovia 2001), proving that the SNMG mechanism may be driven by a autoantibody directed to an internal antigen/protein. This idea of penetrating antibodies breaks a long believed dogma, but in fact this theory could explain the unknown IgM antibody pathway and mechanism for causing the seronegative form of the disease (Vincent, Bowen et al. 2003).

The results from screening MG10-2 produced a sequence that displays high identity with the muscle specific enolase-3,beta. Enolase-3, beta is also part of

glycolysis. This enzyme is found in step 9 and enolase converts 2-Phosphoglyceric acid to Phosphoenol Pyruvate (Vander. A, Sherman. J et al. 2001). Enolase is present as homodimers and heterodimers formed from three distinct subunits with the same molecular weight but they all differ in their biochemical and immunological properties, as well their tissue distribution (Xu, Zweier et al. 1995). Alph-enolase is found in many adult cells, while the gamma, a neuron-specific enolase, is found exclusively in neurons (for the remainder of this study I will be making reference to the muscle specific-enolase). The beta isoform of enolase is so named due to the beta-sheets that make up its subunits. β-enolase has been associated to muscle and muscle development in a number of different ways. Sato and others described that beta-enolase showed up in small amount in myoid cells that were tested from MG patients that had thymomas. These myoid cells displayed positive immunoreactivity to the beta-enolase through a immunoperoxidase technique using three different striated muscle markers (Sato and Tamaoki 1989). Aldolase A and muscle –specific enolase were both detected using the IgM immunoscreening. Interestingly enough they also share a number of physiological similarities. It has been tested that ATP production by SR-associated glycolytic enzymes(aldolase and enolase) play a role in cellular Ca^{2+} homeostatsis by driving the SR Ca^{2+} pump (Xu, Zweier et al. 1995). This research sets down a foundation that these enzymes help control Ca^{2+} release and control. The results stated here could be an example of the malfunctioning of the Ca^{2+} production and channel regulation and in turn lead to the extra Ca^{2+} needed for the rippling effect of ARMG.

Both these proteins exhibit unique properties that tie in to data already researched in the lab. Both proteins stained positive and were found to be located in the I-Band

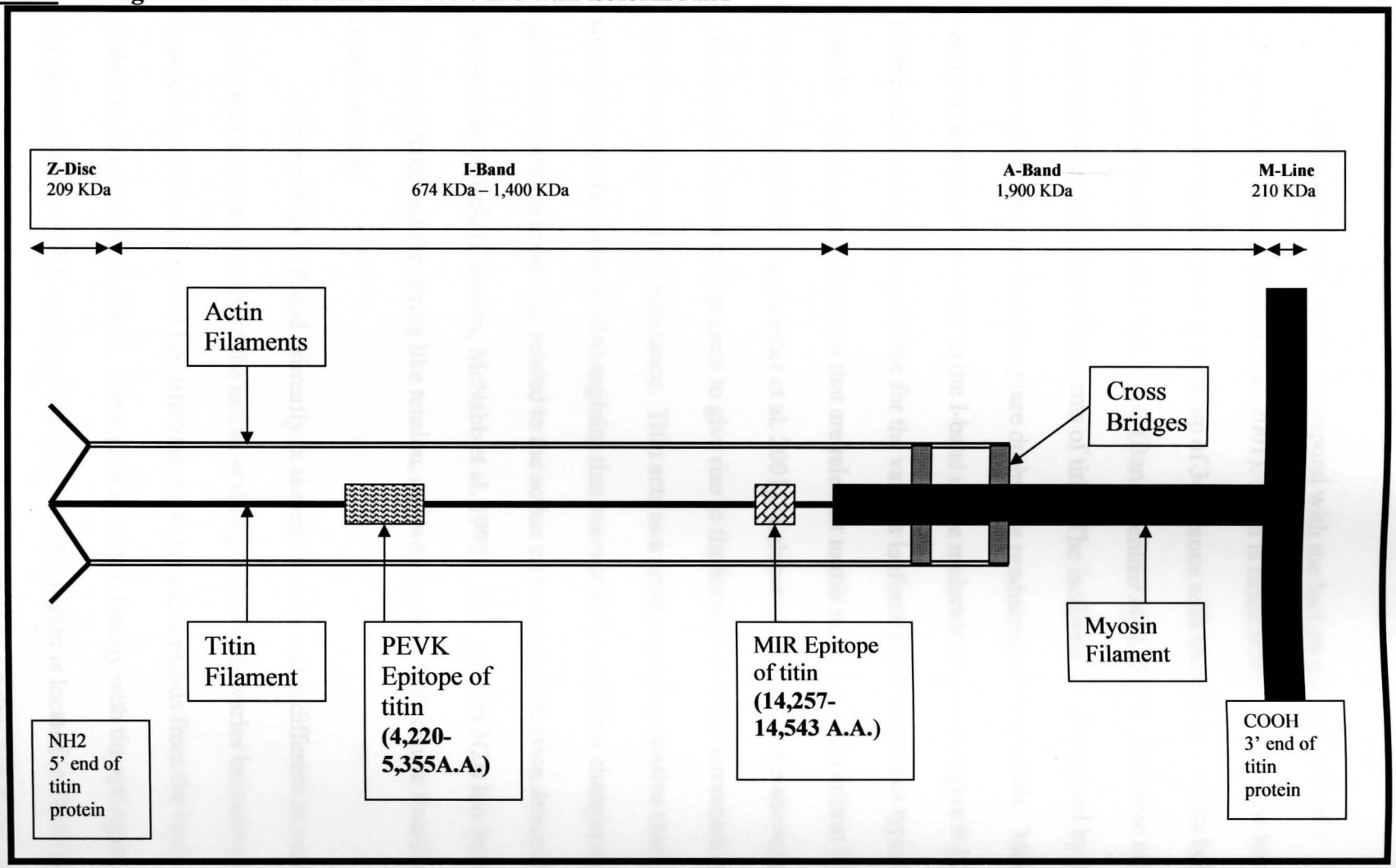
region of both cardiac and skeletal muscle. This coincides with work already done that displayed immunoreactive staining of rippling muscle patient's sera to the same I-Band region (Zelinka 2002).

Screening the library with sera from SPMG, MG10-1, revealed the most significant data of this study. The muscle protein identified was titin isoform N2A. This large muscle protein is not a new antigen associated with MG but is a significant one with MG and ARMD. Titin is the largest known protein and is the 3rd most abundant in the sarcomere (Lahmers, McNabb et al. 1995). Titin is roughly 27,000 amino acids (A.A.) long with a molecular mass of 3,000 kDa (Skeie 2000). Titin isoform N2A is found on the long arm of chromosome 2, position 2q31 (LocusLink-Locus ID # 7273). Titin extends from the Z-disk to the M-line of the sarcomere (shown in figure # 16). This centralized protein is the foundation and template for proper sarcomeric formation and muscle contraction. Titin along with enolase-beta are develop closely together and are expressed during human development as markers for myogenesis, with enolase being detected as early as three weeks in a developing embryo (Fougerousse 2001).

Figure # 16

Diagram of human skeletal muscle and titin isoform N2A: The above diagram was formulated to explain the relationship titin has with skeletal muscle. The diagram also depicts the PEVK epitope (SWISS-PROT Accession # Q812E1) and the main immunogenic region (MIR) of titin Isoform N2A (GenBank Accession # AAB28119) in spatial relation to the sarcomere.

Figure # 16 **Diagram of human skeletal muscle and titin isoform N2A**



The results stated earlier correspond with the loci on the long arm of chromosome 2 (Bang, Centner et al. 2001). Titin is made from 1 gene on the long arm of chromosome 2 and the gene is made up of 363 exons with three unique exons being discovered later and named Novex 1-3 (Bang, Centner et al. 2001). The three unique exons help form the different isoforms of titin. The isoforms are determined by the different splice variants that take place during the production of the peptide. Most of the variant amino acids are found in the I-band of the molecule (shown in figure # 16). The differential splicing is responsible for the various isofoms found in various types of muscle. The two main isoforms that are relevant to this study are titin isoform N2A and titin isoform N2B (Bang, Centner et al. 2001). Lahmers and others have shown that the Titin molecule is not only present to give rise to the formation of the sarcomere but to also give passive muscle resistance. Titin acts as a spring applying passive tension to a stretching muscle. Lahmers also explains that every muscle's isoform changes as we grow. The isoform is directly related to the action and amount of tension demanded by that specific muscle (Lahmers, McNabb et al. 1995). Titin isoform N2B has fewer elastic elements increasing the spring like tension, and why isoform N2B can be found in cardiac muscle.

Isoform N2A is found primarily in skeletal muscle and is different in each muscle. The length and composition of the amino acid sequence in titin varies because each muscle is a different size and has different physiological demands from the body (Lahmers, McNabb et al. 1995). The region involved directly with the spring property is a region called the PEVK region of the protein. This region is located in the I-band (shown in figure # 16). The PEVK is named due to the region being rich in the tandem

repeats of (P)-Pro, (E)-Glu, (V)-Val, (K)-Lys. This region's length varies from 163a.a.-2200 a.a. depending again on the isoform and the physiological demands placed on that muscle (SWISS-PROT Accession # Q812E1). The length of the region is directly related to the muscle's elasticity, the longer the muscle the more elastic the molecule (Skeie 2000). From this point on, the main focus of this study is on skeletal muscle isoform N2A.

There is an important epitope of titin named the Main Immunogenic Region (MIR) that seems to be the most relevant to this study. This region was first recognized to be affiliated with MG antibodies through the use of laboratory animals (Skeie 2000). Gautel and others have done extensive research on the immunogenic region. They mapped this epitope to amino acids 14,257-14,543 (GenBank Accession # AAB28119) (shown in figure # 16). This epitope has been named the MIR due to the specific binding of antibodies from MG patients to that region. There is empirical evidence that the titer of titin antibodies to the MIR correlate to the severity of the MG (Skeie 2000). Using the bioinformatics program ClustalX, clone pMG10-1 was aligned with the complete titin peptide sequence (shown in figure # 9). Strong alignment can be seen to start at 14,170a.a. and continue through 14,472 a.a. This comparative alignment displays an over lap of pMG10-1 into the MIR (shown in figure # 10). This is consistent with Gautel's literature describing patients with thymomas have a higher titer of antibodies to the MIR. The titer of antibodies to the MIR correlates to the severity of the disease (Gautel, Lakey et al. 1993). Sequence analysis previously done in this lab placed two ARMD clones to titin but at different regions of the molecule (Watkins 2004). Clones pRMMG5 aligned from amino acid 17,562a.a.-17,624a.a. and pRMMG6 aligned from 17,536a.a.-17,736a.a.

There is an extreme overlap between the two rippling clones but are found much farther down the titin molecule away from the MIR. The rippling muscle clones are actually showing alignment in the region of the I-band A-band interface. This is significant because this is where titin starts to interact with the heavy chain of the myosin molecule which is needed and used to controls muscle contraction (Vander. A, Sherman. J et al. 2001). The result of antibodies binding to the same molecule but at different epitopes is a phenomenon termed Epitope Spread (ES). The idea behind ES is that an antigen specific autoimmune response can spread to multiple epitopes on the same molecule/protein, this is termed intramolecular ES. The other way ES occurs is the autoimmune response can spread to epitopes on other molecules/protein, this is termed intermolecular ES (Powell and Black 2001). These secondary responses that arise later appear after the "original antigenic sin" diminishes (Steinman 1999). This could be a reasonable hypothesis to the different epitopes of titin and even the different proteins that have been identified in this study.

The relation/connection of MG and ARMD is still unclear. There is evidence present that clinically and empirically link these two autoimmune skeletal muscle diseases (Ansevin and Agamanolis 1996). This study has been a comparative study to assess the proteins present in both diseases. This study concludes that while both patients' sera recognized the same muscle protein, the antibodies recognized the molecule at very different physiological regions. This study is now progressing towards a more proteomic analysis of titin and the use of an expression vector to isolate the immunoreactive peptide for further analysis. The screening of a cardiac muscle expression library has also been looked at. There seem to be a strong correlation with

cardiac myopathies and antibodies reacting with titin. This has been shown with Western Blotting sera from pMG10-1 to cardiac muscle. The Western blot displayed specific binding to the cardiac muscle (Watkins 2004). Titin isoform N2B plays an intricate role in the tension of cardiac muscle as mentioned earlier. This is a highly regulated mechanism. The interaction of antibodies or physiological damage to the peptide would hinder the normal function which is prevalent in many cardiac myopathies (Lahmers, McNabb et al. 1995).

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Appendix 1: List of Abbreviations

Abbreviation	Term
ACh	Acetlycholine
AchR	Acetlycholine receptor
ARMD	Autoimmune rippling muscles associated with myasthenia gravis
BLAST	Basic Local Alignment Search Tool
b.p.	Base pairs
BuTX	α-bungarotoxin
CGRP	Calcitonin-gene-related peptide
DHPR	Dihydropridine receptor
EMG	electrmyogram
FASTA	Fast Alignment Search Tools Anything
HRP	Horse Radish Peroxidase
IEF	Isoelectric focusing
IPG	Immobilized pH gradient
IPTG	Isopropyl -β -D-thiogalactopyranoside
KDa	kilodalton
MG	Myasthenia gravis
MNC	Mononuclear cells
MuSK	Muscle specific Kinase
O.D.	Optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PFU	Plaque forming units
RMD	Rippling Muscle Disease
RyR	Ryanodine receptor
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SLE	Systemic lupus erthematosus
SPMG	Seropositive myasthenia gravis
SNMG	Seronegative myasthenia gravis
SR	Sarcoplasmic Reticulum

Appendix 2: Solutions/Reagents

Reagents	Purpose	Contents
	SDS-PAGE	
7.5% Acrylamide gel	Resolving gel: Separates proteins based on size	1.6g glycerol, 6.0mL running gel buffer, 10mL ddH_2O, 6.0mL 30% acrylamide gel stock, 80µL 10% ammonium persulfate, 24µL TEMED
10% Acrylamide gel	Resolving gel: Separates proteins based on size	1.6g glycerol, 6.0mL running gel buffer, 10mL ddH_2O, 8.0mL 30% acrylamide gel stock, 80µL 10% ammonium persulfate, 24µL TEMED
5% Acrylamide stacking gel	Stack/compress proteins entering resolving gel	4.5mL stacking gel buffer, 10.5mL ddH_2O, 3.0mL 30% acrylamide gel stock, 60µL 10% ammonium persulfate, 23µL TEMED
Electrode Buffer	pH buffer for running SDS-PAGE	6.05g tris, 28.84g glycine, 2.00g SDS, 2000mL of ddH_2O
Resolving gel buffer	pH buffer for resolving gel	18.17g tris, 8.20mL 3M HCl, 0.4g SDS,100mL of ddH_2O
Stacking gel buffer	pH buffer for stacking gel	6.05g tris, 1mL 2M HCl, 0.4g SDS, 100mL of ddH_2O (titrated to pH 6.8)
SDS sample buffer (4X)	Solubilize/denature proteins for analysis	50% glycerol, 200mM TRIS (pH 6.8), 20% 2-β-mercaptoethanol and 9.2% SDS
Equilibration Buffer I	Equilibrates IPG strips for the 2^{nd} dimension	6M Urea, 0.375M tris (pH 8.8), 2% SDS, 20% glycerol, 2% (w/v) DTT
Equilibration Buffer II	Equilibrates IPG strips for the 2^{nd} dimension	6M Urea, 0.375M tris (pH 8.8), 2% SDS, 20% glycerol, 2.5% (w/v) iodoacetamide
IPG strips	Used to focus peptides based on their pH	Commercially available from Bio-RAD
Coomassie Brilliant Blue	Stain Proteins	.5g Coomassie Brilliant Blue R-250, 250mL ethanol, 100mL glacial acetic acid, 650mL ddH_2O
Destain	Eliminates excess stain	350mL ethanol, 100mL glacial acetic acid, 550mL H_2O
Sypro Ruby Red	Stains 2DE gels	Commercially available from Bio-RAD

Immunoblot/Western Blot		
Goat anti-human IgG (Fab' region specific)	Secondary antibody labeled with HRP for detection of human immunoglobulin	Commercially available from Sigma
Goat anti-human IgM (Fab'region specific)	Secondary antibody labeled with HRP for detection of human immunoglobulin	Commercially available from Sigma
Chemiluminiscent substrate	Detection of HRP labeled immunoglobulin	Commercially available from Pierce
Colorimetric substrate	Detection of HRP labeled immunoglobulin	Commercially available from Sigma
PVDF membrane	Binds proteins transferred from gel by Western Blotting	Commercially available from Bio-RAD
Transfer buffer	Establishes pH and transfers proteins onto PVDF membranes	57.6g glycine, 12.1g tris, 800mL methanol, 3200mL ddH_2O
Tris buffered Saline (TBS)	Buffer/pH	20mM tris, 0.5M NaCl, (titrate to pH 7.5 with HCl)
Tris buffered Saline with Tween-20 (TBS-t)	Buffer/pH	20mMtris, 0.5M NaCl, (titrate with HCl with 0.2% Tween-20)
3% Blocking Buffer	Blocks nonspecific binding	3% dry non-fat powder milk in TBS-T
1% Bloicking Buffer	Blocks nonspecific binding	1% dry non-fat powder milk in TBS-T
Developer	Develops X-ray Film	250mL DeKtol, 500mL H_2O
Fixative	Fixes X-Ray Film	500mL Rapid Fixer, 500mL H_2O

Growth/Media		
LB/Agar	Growth media for XL-Blue MRF' and SOLR	Broth: 10g NaCl,10g tryptone, 5g yeast extract, add ddH_2O to 1L, pH 7.0 w/ NaOH, autoclaved Agar: add an additional 20g agar
LB ampicillin/agar (LB+Amp)	Growth media for XL-Blue MRF' containing pBluescript phagemid	Broth: 10g NaCl,10g tryptone, 5g yeast extract, add ddH_2O to 1L, pH 7.0 w/ NaOH, autoclaved, cooled and 10mg/mL ampicillin added Agar: add an additional 20g agar
NZY/agar	Agar for phage infection/screening	Broth:5g NaCl, 5g $MgSO_4 \cdot 7H_2O$, 10g NZ amine, 5g yeast extract, add ddH_2O to 1L, pH7.5, autoclaved Agar: added an additional 20g agar
NZY top agar	Plating agar for phage infection/screening	NZY broth with 0.7% w/v agarose
SM buffer	Phage dilutant	5.8g NaCl, 2.0g $MgSO_4 \cdot 7H_2O$, 50mL 1M tris-HCl (pH 7.5), 5.0mL 2% w/v gelatin and ddH_2O to 1L final volume
Biologicals (Included in the Stratagene Lambda Zap II Library)		
pBluescript	Phage Vector containing cDNA insert and unique restriction sites	Stratagene
ExAssist	Helper phage: helps in excision of pBluescript phagemid	Stratagene
SOLR	Excision of pBluescript phagemid	Stratagene
XL-Blue MRF'	Host strain for growth of phage library	Stratagene

cDNA chemicals/Manipulations		
Agarose gel	DNA electrophoresis	1% electrophoresis grade agarose in TAE
BioBond Membrane	Binds translated cDNA protein for antibody screening	Commercially available from Sigma
DCTS-Quick Start	PCR kit for sequencing	Beckman-Coulter commercial kit for amplifying DNA for sequencing
EcoR1	Restriction Enzyme	Commercially available from Sigma
IPTG	Promotes translation of insert cDNA (induces LacZ promoter)	Isopropyl-β-D-thiogalactopyranoside Commercially available from Sigma
Plasmid miniprep kit	Separation of plasmid DNA from *E.coli* genomic DNA	Kit Commercially available from Eppendorf
Primer M13 (Forward primer)	PCR primer	Primer sequence 5'GTAAAACGACGGCCAGT 3'
Reverse Primer	PCR primer	Primer sequence 5'GGAAACAGCTATGACCATG 3'
Stop Buffer (10x loading buffer)	Stops restriction digestion and prepares DNA for gel electrophoresis	20mL Ficoll 400, 1g SDS, 3.72g Na EDTA dehydrate pH 8.0, 0.25g bromophenol blue
TAE	Agarose gel electrophoresis running gel buffer	242g tris, 57.1mL glacial acetic acid, 37.2g Na EDTA dehydrate, ddH_2O to 1L, titrate to pH 8.5 (makes 50X)