GENETICS OF MERLE PATTERNING IN THE DOMESTIC DOG AND GENE TRANSCRIPT PROFILING AND IMMUNOBIOLOGY OF DERMATOMYOSITIS IN THE SHETLAND SHEEPDOG

A Dissertation

by

JACQUELYN MARIE BELL WAHL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2008

Major Subject: Veterinary Microbiology

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Approved by:

Chair of Committee,	Keith E. Murphy
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ABSTRACT

Genetics of Merle Patterning in the Domestic Dog and Gene Transcript Profiling and Immunobiology of Dermatomyositis in the Shetland Sheepdog. (May 2008)

Jacquelyn Marie Bell Wahl, B.A., Baylor University

Chair of Advisory Committee: Dr. Keith E. Murphy

Since its domestication, the dog has served in many roles, from protector, guide, hunter, and best friend, to model organism. Every role in which the dog serves is important; however, this work highlights the importance of the dog as a model organism for study of human hereditary diseases. Roughly half of the 450 hereditary diseases found in the dog have clinical presentations similar to those found in the human. Included in these are auditory-pigmentation conditions and skin diseases for which the dog is a working model.

Described herein are studies of the merle coat pattern and dermatomyositis. Through research on these topics, important information can be obtained that can be used to help both the dog and the human. Merle is a pattern of coloring observed in the coat of the domestic dog and is characterized by patches of diluted pigment. Dogs heterozygous or homozygous for the merle locus exhibit a wide range of auditory and ophthalmologic abnormalities. Linkage disequilibrium was identified for a microsatellite marker with the merle phenotype in the Shetland Sheepdog. This region of the human genome contains *SILV*, a gene important in mammalian pigmentation. Therefore, this

gene was evaluated as a candidate for merle patterning. A short interspersed element insertion at the boundary of intron 10/exon 11 was found, and this insertion segregates with the merle phenotype in multiple breeds. These data show that *SILV* is responsible for merle patterning and is associated with impaired function of the auditory and ophthalmologic systems.

Dermatomyositis (DM) is an inflammatory disease of the skin and muscle that occurs most often in the rough collie and Shetland Sheepdog. Gene transcript profiles were generated for affected and normal skin using a canine-specific oligonucleotide array. Two-hundred and eight-five gene transcripts, many of which are involved in immune function, were found to be differentially regulated in these tissues. Also reported are western blot, immunohistochemistry, and immunofluorescence analyses. While our work suggests that canine DM is a disease that may be immune mediated, it did not detect the production of specific disease-associated autoantibodies.

DEDICATION

For my mom

For Jab and the rest of my menagerie

ACKNOWLEDGEMENTS

I would like to first thank my advisor, Dr. Keith Murphy, for allowing me to join the Canine Genetics Laboratory at Texas A&M University. His passion for canine genetics research has served as an inspiration to me.

I would like to thank my committee members, Dr. Womack, Dr. Kier, and Dr. Rees, for their guidance and support throughout the course of my graduate career. I would especially like to thank Dr. Rees for helping me conquer my fear of public speaking and always having an ear to listen to my problems.

The Collie Health Foundation and The American Shetland Sheepdog Association made this research possible. I would also like to thank the breeders and owners, especially Sherry Lindsey, Beverly Wymola, Don Abney, Kerry Kirtley, Susan Schroeder, and Sharon Vanderlip, for generously providing samples from affected and unaffected dogs. In addition, I would like to thank Dr. George Strain for BAER testing the dogs in the study.

I would also like to thank the members of the Canine Genetics Laboratory for their years of guidance and support. In particular, I would like to thank Dr. Leigh Anne Clark for her friendship, guidance, and help with all of my research. She has served an invaluable role in my graduate education. I would especially like to thank four very important Murphy lab survivors, Drs. Stephanie Herbst (my Huckleberry), Ashley Hamlett, Jessica Moody, and Michelle Boggs, for their unwavering friendship, encouragement, love, and support. Without them I would have lost my sanity a long time

ago. We not only worked together, but we laughed together, cried together, partied together, stood by each other, stood up for each other, and in the end built life-long friendships. I cannot thank you all enough. A mention of Stephanie would not be complete without also mentioning her family. Her husband Todd and two, beautiful little girls Madeleine and Anna, also had a huge influence in my life. Todd and the girls would sometimes stop by the lab to say hi and just a five minute visit from them would be enough to put a smile on my face the rest of the day. I would also like to thank Matt Danziger. His support and encouragement has meant the world to me. He believed in me when I did not believe in myself and has helped push me through the harder times.

Finally, I would like to thank my family. I would like to thank my brothers for their encouragement and support throughout my graduate career. They have always been there to help me unwind and re-focus. I would like to thank my grandparents for their everlasting love and kind words not only throughout my graduate career, but throughout my lifetime. I would also like to thank my dad, without his support I would not be able to be where I am today. Last but not least, I would like thank my best friend, my mom. My mom has been the most influential person in my life. She has always supported my goals and has encouraged me never to be defeated. Her endless love and support has been the most important thing in my graduate success.

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CHAPTER I

INTRODUCTION

The Dog as a Model

The domestic dog (*Canis familiaris*) has served as a focal point of scientific curiosity for ages. Originating in East Asia, domestic dogs diverged exclusively from the gray wolf approximately 40,000-15,000 years ago, according to mtDNA evidence (Vila *et al.*, 1997; Leonard *et al.*, 2002; Savolainen *et al.*, 2002). Alternatively, archeological evidence suggests a later domestication event, around 14,000-9,000 years ago (Leonard *et al.*, 2002; Savolainen *et al.*, 2002). To date, there are over 1000 regional varieties and recognized breeds of dog world-wide, the majority of which were created in the past 250 years (Ostrander and Giniger, 1997; Neff and Rine, 2006). This rapid breed differentiation was accomplished using selective inbreeding practices, often from a very small number of founders (Ostrander and Kruglyak, 2000). The desire to breed dogs with specific traits led to a species that is unrivaled among mammals in diversity. Dog breeds differ in size, coat color and texture, craniofacial structure, body conformation, and behavior.

Interbreeding among dogs of the same breed is necessary in order to maintain breed characteristics. This inbreeding, coupled with an already limited genetic pool, leads to a greater expression of recessively inherited traits and diseases in the dog than observed in other species. Forty-six percent of the identified genetic diseases occur

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This dissertation follows the style of Gene.

predominately in one or few breeds (Ostrander *et al.*, 2000). Interestingly, the reproductive isolation of dog breeds has created relatively closed gene pools that can be studied as isolated populations (Patterson, 2000; Parker *et al.*, 2004).

To date, there are more than 450 different hereditary diseases identified in the dog, 58% of which have clinical presentations similar to their human counterparts (Ostrander and Giniger, 1997). Of these 450 diseases, greater than 70% are autosomal recessive, X-linked, or complex traits. Diseases that are inherited in an autosomal dominant fashion are not as common because they can easily be selected against by removal of affected dogs from breeding programs (Ostrander *et al.*, 2000). The dog has more naturally occurring hereditary diseases than any other species other than human (Crook and Hill, 1998; Patterson, 2000; Sargan, 2004). In addition, many diseases are influenced by environmental factors, making the dog an ideal model for human diseases because they often share our same living space, exercise habits, and even food. Coupled with the fact that there is a higher sequence similarity between orthologous genes of the human and dog than is found between the human and mouse, this makes the dog an ideal model system in which to study human hereditary diseases (Kirkness *et al.*, 2003).

The importance of the dog as a model for study of diseases was clearly indicated when canine genome was selected as the fourth mammalian genome to be sequenced. The first sequence was a 1.5 X sequence of a male Standard Poodle in 2002 (Kirkness *et al.*, 2003). This sequence provided 77% coverage of the genome and was assembled by a private company, Celera Genomics (Kirkness *et al.*, 2003). In 2004, the National Human Genome Research Institute (NHGRI) completed a 7.8X sequence of a female Boxer.

This provides greater than 95% coverage (Lindblad-Toh *et al.*, 2005). This sequence is publicly available and, since its release, has propelled the field of canine genetics research into a new era. With the published DNA sequences of both the dog and the human, the canine model can now be more effectively used to study both canine and human diseases.

Canine Coat Color Genetics

The field of canine coat color genetics has been studied for a number of years, and is particularly intriguing because phenotypes are easily measured. Studies of the inheritance of coat color began in 1977 with the Labrador Retriever (Templeton *et al.*, 1977). Labradors were of interest because they exhibit three basic coat colors: black, chocolate, and yellow. Previous studies identified the dominant *B* allele at the *B* locus as responsible for black coloring (Little, 1957). The yellow coat color is determined by the recessive *e* allele at the *E* locus and is epistatic to the *B* locus in the homozygous condition. The inheritance of the brown coat color, however, remained unknown (Little, 1957). The recessive *b* allele at the *B* locus was identified by Templeton *et al.* as responsible for the chocolate coat color (1977). In addition, it was determined that the hypostatic *B* alleles have some expression in *e/e* dogs, as well, and are responsible for iris, nose, and lip pigmentation (Templeton *et al.*, 1977). The conclusions made by Little and Templeton, which were based solely on the phenotypes of the offspring produced from planned breedings. Today, with the availability of the published canine sequence, coat color genetics in the dog can be more thoroughly evaluated at the molecular level. As a result, such data support and refute various conclusions of Little and Templeton.

There are four main loci for basic coat colors that have been discovered to date: *melanocortin 1 receptor(MC1R)*, t*yrosinase related protein 1(TYRP1)*, *agouti signal peptide(ASIP)*, and *β-defensin 103* (*CBD103*) (Candille *et al.*, 2007; Schmutz and Berryere, 2007).

MC1R is the gene referred to by Little as the *E* locus. A loss-of-function mutation in *MC1R* results in the clear red coat color in dogs (Everts *et al.*, 2000), the allele referred to by Little as *e,* while the wild type allele was called *E* (Little, 1957). A single nucleotide substitution in *MC1R* results in an allele producing a melanistic mask and is referred to as allele E^M. The mask, observed in dogs with fawn or brindle coloring, is the result of a single copy of this allele. Dogs that have solid black, brown, or blue coloring do not have a distinguishable mask even though they may carry the E^M allele (Schmutz and Berryere, 2007).

The gene responsible for the brown coat color is *TYRP1,* referred to by Little as the *B* locus. The recessive allele of this gene results in brown coloring, while the dominant allele produces solid black (Schmutz *et al.*, 2002). To date, three different alleles of *TYRP1*have been detected. Allele b^s contains a premature stop codon in exon 5, allele b^c contains a base pair substitution in exon 2 that changes a serine residue to a cysteine residue, and allele b^d has a deleted proline residue in exon 5 (Schmutz *et al.*, 2002). Any combination of two of these alleles will result in a brown coat color in the dog (Schmutz and Berryere, 2007).

The coat color phenotype of a dog is the result of interaction between alleles of *TYRP1* and *MC1R* (Schmutz *et al.*, 2002). A dog with a yellow, cream, or red coat color

but the nose, eye rims, and pads of feet which have keratinized epidermis are either black or brown or a dilution of these has the genotype of *e/e* (Schmutz and Berryere, 2007). This mechanism of gene interaction was hypothesized by Templeton and associates years ago but is still not fully understood (Templeton *et al.*, 1977). All dogs that are either solid or spotted brown, black, or grey have at least one *E* or E^M allele and inherit the coat color in a dominant fashion. Additionally, these dogs also possess at least one K^B allele (Kerns *et al.*, 2007; Candille *et al.*, 2007). For most dog breeds, in order to express the eumelanin (brown, black , or grey) coat color the dog must carry one *E* or E^M allele at the *MC1R* locus and one dominant allele at the *CBD103* locus (Candille *et al.*, 2007; Schmutz and Berryere, 2007).

ASIP is another gene that determines coloring in the dog. *ASIP* possesses four alleles, which exhibit a hierarchy: $a^y > a^w > a^t > a$ (Schmutz and Berryere, 2007). The dominant allele of the *ASIP* alleles, a^y, results in the fawn or sable coat color found in breeds such as the Shetland Sheepdog, Collie, and Great Dane (Berryere *et al.*, 2005). Allele a^w is the wild type allele and results in a banding pattern of the hair, consisting of a band of eumelanin (brown, black, or grey), followed by a band of phaeomelanin (red, yellow, or cream), and another band of eumelanin from the base of the hair to the tip (Schmutz and Berryere, 2007). The a^t allele produces a coloring pattern of black with tan areas around the eyes and on the muzzle, chest and legs, seen commonly in Doberman pinschers and Rottweilers. The recessive allele, *a*, results in a black coat color in dogs which are from primarily herding decent, such as the Puli and Schipperke and is the sole

allele responsible for of black coloring in the German Shepherd Dog and the Shetland Sheepdog(Kerns *et al.*, 2004; Berryere *et al.*, 2005; Schmutz and Berryere, 2007).

CBD103, formerly known as the *K* locus, is the fourth main color gene in the dog. This gene is responsible for causing the dominant black coat color. As mentioned above, in most cases (with the exception of the recessive a allele), in order for a dog to have a eumelanin coat color it must carry one *E* or E^M allele at the *MC1R* locus and one dominant allele at the *CBD103* locus. The *CBD103* locus has three alleles which exhibit a dominance hierarchy: K^B (black) > k^{br} (brindle)> k^y (yellow) (Candille *et al.*, 2007). A single copy of k^{br} in the presence of k^y produces the brindle phenotype, which consists of alternating stripes of phaeomelanin and eumelanin of various shades (Kerns *et al.*, 2007). Brindle, as for many coat colors, is variable between dog breeds, with some breeds having very thin bands of phaeomelanin and thus appearing black and others having very thin bands of eumelanin (Schmutz and Berryere, 2007). Furthermore, depending on the genotype of the dog at the *ASIP* locus, dogs with k^y/k^y could have fawn coloring, eumelanin and tan, or wolf sable coloring (Schmutz and Berryere, 2007). Dogs with an a^y allele have the brindle patterning over their entire body whereas dogs possessing the a^t/a^t genotype have brindle patterning only along the ventral surfaces of the body (Berryere *et al.*, 2005).

The four genes, *MC1R*, *TYRP1*, *ASIP*, and *CBD103*, are responsible for the main coat colors observed in most domestic dogs; however, there are also coat patterns of the dog, such as harlequin, ticking, and white spotting. The causative genes for harlequin and ticking remain unknown (Schmutz and Berryere, 2007). The exact mutation for

white spotting is unknown, but the phenotype was mapped to an associated region of less than 1 Mb containing only one gene: *microphthalmia-associated transcription factor* (*MITF*). *MITF* is an ideal candidate locus for white spotting (Karlsson *et al.*, 2007). The causative mutation for another modification, the merle phenotype, has been determined (Clark *et al.*, 2006) and will be discussed in detail in chapter II.

Coat color genetics is an important area of research because it demonstrates the complexity of inheritance of what appears to be a simple trait and may also help identify deleterious alleles that produce phenotypes which are associated with some coat colors, such as deafness, skin anomalies, and vision abnormalities (Schmutz *et al.*, 1998; Clark *et al.*, 2006; Von Bomhard *et al.*, 2006). Studies of the inheritance of coat color in the domestic dog have identified several genes responsible for specific coat colors and patterns. These genes, in addition to others, contribute to the diverse level of phenotypic variation found in the domestic dog, making it unique among all mammals.

Dermatomyositis

Dermatomyositis (DM) is a devastating dermatologic condition that most commonly affects the skin and/or muscles of the collie and Shetland Sheepdog (Haupt *et al.*, 1985; Hargis and Mundell, 1992

; Scott *et al.*, 2000). The DM phenotype is extremely variable, with some affected dogs experiencing mild, transient symptoms, and others developing a life threatening form of the disease. Clinical findings consistent with DM include the development of skin lesions, which include hair loss, redness, scaling and crusting on the face, ears, legs, and tail tip. Lesions in individual dogs vary, and may affect multiple

areas, and dogs afflicted with DM often develop disfiguring scarring and secondary skin infections. In addition, cases of DM with muscle involvement are characterized by muscle atrophy, megaesophagus, aspiration pneumonia, and difficulty eating or walking, causing some dogs to walk with an abnormal high stepping gait. For those dogs with severe DM, the quality of life becomes a concern, often necessitating euthanasia(Hargis and Mundell, 1992).

In the collie, the mode of inheritance of DM is thought to be autosomal dominant with incomplete penetrance (Haupt *et al.*, 1985; Scott *et al.*, 2000). The age of onset for DM varies widely, with some affected dogs showing clinical signs of DM as early as eight weeks of age, while others are several years old before clinical symptoms are detectable. The inability to diagnose DM prior to the development of clinical symptoms has made control of the disease difficult.

Identification of the gene(s) causative for DM would allow for the development of a DNA-based test to identify dogs that carry the mutation responsible for DM. Such a test would allow early detection of affected dogs, in addition to dogs that have mild, undetectable clinical symptoms. This knowledge would benefit affected dogs, allowing early treatment and possibly improving their prognoses, and also help breeders make informed decisions in their breeding programs and eliminate DM in their lines.

CHAPTER II

RETROTRANSPOSON INSERTION IN *SILV* IS RESPONSIBLE FOR MERLE PATTERNING IN THE DOMESTIC DOG*

Overview

Merle is a pattern of coloring observed in the coat of the domestic dog and is characterized by patches of diluted pigment. This trait is inherited in an autosomal, incompletely dominant fashion. Dogs heterozygous or homozygous for the merle locus exhibit a wide range of auditory and ophthalmologic abnormalities, which are similar to those observed for the human auditory–pigmentation disorder Waardenburg syndrome. Mutations in at least five genes have been identified as causative for Waardenburg syndrome; however, the genetic bases for all cases have not been determined. Linkage disequilibrium was identified for a microsatellite marker with the merle phenotype in the Shetland Sheepdog. The marker is located in a region of CFA10 that exhibits conservation of synteny with HSA12q13. This region of the human genome contains *SILV*, a gene important in mammalian pigmentation. Therefore, this gene was evaluated as a candidate for merle patterning. A short interspersed element insertion at the boundary of intron 10/exon 11 was found, and this insertion segregates with the merle phenotype in multiple breeds. Another finding was deletions within the oligo(dA)-rich

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*Reprinted with permission from "Retrotransposon insertion in *SILV* is responsible for merle patterning of the domestic dog" by Clark, L.A., Wahl, J.M., Rees, C.A., and Murphy, K.E., 2006. *Proc Natl Acad Sci USA,* 103, 1376-81, Copyright 2006 by Copyright National Academy of Sciences, U.S.A.

tail of the short interspersed element. Such deletions permit normal pigmentation. These data show that *SILV* is responsible for merle patterning and is associated with impaired function of the auditory and ophthalmologic systems. Although the mutant phenotype of *SILV* in the human is unknown, these results make it an intriguing candidate gene for human auditory–pigmentation disorders.

Introduction

Merle is a coat pattern in the domestic dog characterized by patches of diluted pigment intermingled with normal melanin. It is a standard coloration for several breeds recognized by the American Kennel Club, including the Shetland Sheepdog, Australian Shepherd, Cardigan Welsh Corgi, and Dachshund. The merle phenotype in the Dachshund is known as dapple. Although merle is not an acceptable color in the Great Dane, the desirable harlequin pattern results from the interaction of the merle locus (*M*) and a separate harlequin locus (*H*) (O'Sullivan and Robinson, 1988). In addition, many breeds (e.g., Catahoula Leopard Dog, Bergamasco Sheepdog, and Pyrenean Shepherd) accepted by other kennel clubs present with merle patterning.

Merle is inherited in an autosomal, incompletely dominant fashion (Mitchell, 1935). Although rare, a dog that does not present with the overt merle phenotype may possess the merle genotype and subsequently produce merle offspring. Such a dog is termed a cryptic merle. The mechanism for this phenomenon is unknown. Dogs homozygous for merle (*MM*) are known as double merles and are predominantly white (Fig. 1C).

Dogs having *Mm* and *MM* genotypes typically have blue eyes and often exhibit a wide range of auditory and ophthalmologic abnormalities (Sorsby and Davey, 1954). Reetz *et al.* (1977) studied the auditory capacity of Dachshunds and found that 54.6% of *MM* and 36.8% of *Mm* dogs had auditory dysfunction, ranging from mild to severe deafness. All control dogs (*mm*) in the study had normal hearing. Klinckmann *et al.* (1987; 1987) conducted ophthalmologic studies with three groups of Dachshunds (*MM, Mm*, and *mm*) and found that merles and double merles had significantly greater frequencies of ocular abnormalities, including increased intraocular pressure and ametropic eyes. Microphthalmia and colobomas are well described in merle and double merle Dachshunds and Australian Shepherds (Sorsby and Davey, 1954; Gelatt and McGill, 1973; Dausch *et al.*, 1977). In all breeds, the double merle genotype can be sublethal and is associated with multiple abnormalities of the skeletal, cardiac, and reproductive systems (Sorsby and Davey, 1954; Little, 1957; Sponenberg and Bowling, 1985). For these reasons, merle-to-merle breedings are strongly discouraged (Little, 1957).

Interestingly, many of the abnormalities associated with merle dogs are remarkably similar to those observed in Waardenburg syndrome (WS) (Schaible and Brumbaugh, 1976). WS is an autosomal dominant auditory–pigmentation disorder in humans (1 per 40,000 live births) that accounts for 2% of all cases of congenital deafness (Nayak and Isaacson, 2003). Several genes have been implicated in the four clinical varieties of WS: Mutations in *PAX3* cause WS type 1 and type 3 (Baldwin *et al.*, 1992; Tassabehji *et al.*, 1992) and mutations in *SOX10, EDNRB,*or *EDNR3* cause WS

type 4 (Puffenberger *et al.*, 1994; Pingault *et al.*, 1998; McCallion and Chakravarti, 2001). Mutations in *MITF* cause WS type 2; however, the genetic basis for 85% of type 2 cases remains unidentified (Tassabehji *et al.*, 1994; Choi *et al.*, 2004).

To identify a chromosomal region segregating with merle, we carried out a whole-genome scan for the Shetland Sheepdog by using the multiplexed Minimal Screening Set 2 (Guyon *et al.*, 2003; Clark *et al.*, 2004). Linkage disequilibrium (LD) for merle was identified with a microsatellite marker in a region of CFA10 that exhibits conservation of synteny with HSA12q13. This region of HSA12 harbors the *SILV* gene.

SILV (also known as *Pmel17*; gp100) is a pigment gene best known as the *Silver* locus responsible for a recessive trait in an inbred strain of black mice in which the hair color dilutes with age (Dunn and Thigpen, 1930; Kwon *et al.*, 1995). Although this gene is known to have a central role in pigmentation, the precise function of *SILV* remains controversial (Theos *et al.*, 2005). Multiple studies have provided data to suggest that *SILV* is involved in the biogenesis of premelanosomes (Kobayashi *et al.*, 1994; Zhou *et al.*, 1994; Berson *et al.*, 2001). Significant expression of the gene is almost exclusive to the skin and eye, providing further evidence to support a role in pigmentation (Theos *et al.*, 2005). Although mutations in *SILV* have not been implicated in disease, the aforementioned LD data and the role of *SILV* in pigmentation made it a candidate gene for merle.

Characterization of *SILV* in merle and nonmerle Shetland Sheepdogs revealed a short interspersed element (SINE) insertion at the intron 10/exon 11 boundary. The SINE segregates with the merle phenotype in multiple breeds and is absent from dogs

representing breeds that do not have merle patterning. All examined harlequin Great Danes harbored the insertion in either a heterozygous or homozygous state. Described herein is LD with merle and characterization of a SINE insertion in *SILV* that is responsible for merle patterning in the dog.

Results

LD with the Merle Phenotype

Genotype data for 279 Minimal Screening Set 2 markers were generated for 9 merle and 32 nonmerle Shetland Sheepdogs. Only one marker had an allele that appeared to be more common in the merle population. For this marker, FH2537, a statistically significant *P* value (7.2×10^{-6}) was obtained. To validate this result, genotype data were generated for the aforementioned marker by using additional Shetland Sheepdogs: 7 merle, 2 double merle, and 11 nonmerle. These genotypes were combined with the original data set and used to recalculate the *P* value, which increased in significance (3.0×10^{-8}).

Candidate Gene Selection

The above result allowed narrowing of the search to those genes that are (*i*) important in pigmentation and (*ii*) also proximal to microsatellite marker FH2537 on CFA10. *SILV* encodes a melanosomal protein important in pigmentation (Sturm *et al.*, 2001) and maps to HSA12q13–q14, which exhibits conservation of synteny with CFA10 (Kwon *et al.*, 1991). A single base insertion in *SILV* causes the silver phenotype in the mouse (Kwon *et al.*, 1995), and polymorphisms in this gene are associated with the dominant white, dun, and smoky plumage color variants in chickens (Kerje *et al.*, 2004).

The dilute coloration of Charolais cattle has also recently been attributed to a mutation in *SILV* (Theos *et al.*, 2005). Furthermore, *SILV* expression is dependent on *MITF* (Baxter and Pavan, 2003), which is causative for some cases of WS type 2 (Tassabehji *et al.*, 1994).

SINE Insertion

PCR was carried out by using genomic DNA from two nonmerle, one blue merle, and one double merle Shetland Sheepdog to obtain amplicons from each exon of *SILV*. Amplification of exon 11 yielded two products: (*i*) the expected 206-bp product and (*ii*) a larger product (slightly smaller than 500 bp). These amplicons segregated with the merle phenotype among the aforementioned dogs: The nonmerle dogs were homozygous for the 206-bp product; the blue merle was heterozygous for the products; and the double merle was homozygous for the larger product (Figure 1).

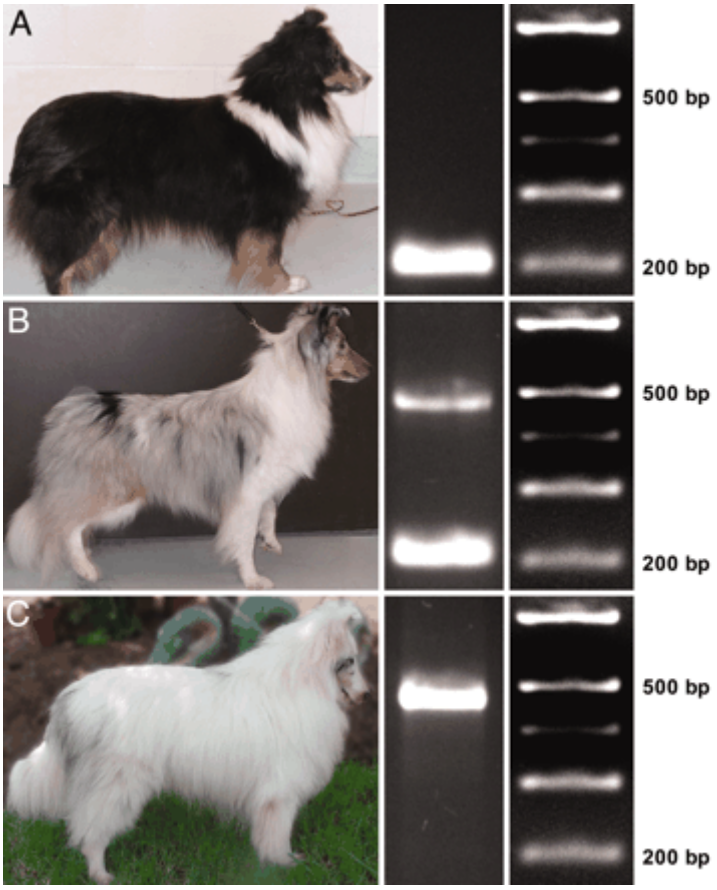

Figure 1. SINE insertion in *SILV* segregates with merle phenotype. (*A*) Tricolored (black, sable, and white), nonmerle Shetland Sheepdog (*mm*). (*B*) Blue merle Shetland Sheepdog (*Mm*). (*C*) Double merle Shetland Sheepdog (*MM*). (*Left*) Phenotypes. (*Center*) Exon 11 PCR products. (*Right*) Length markers.

Sequence analysis of exon 11 products revealed an insertion of a tRNA-derived SINE, highly similar to the unique canine SINEs described by Minnick *et al.* (1992). The insertion occurs at the boundary of intron 10 and exon 11 and is flanked by a 15-bp target site duplication (Figure 2). The SINE insertion is in reverse orientation, with the 5′ end closer to exon 11. Sequence analysis of all coding regions of the gene did not reveal any other mutations that may disrupt the function of *SILV*.

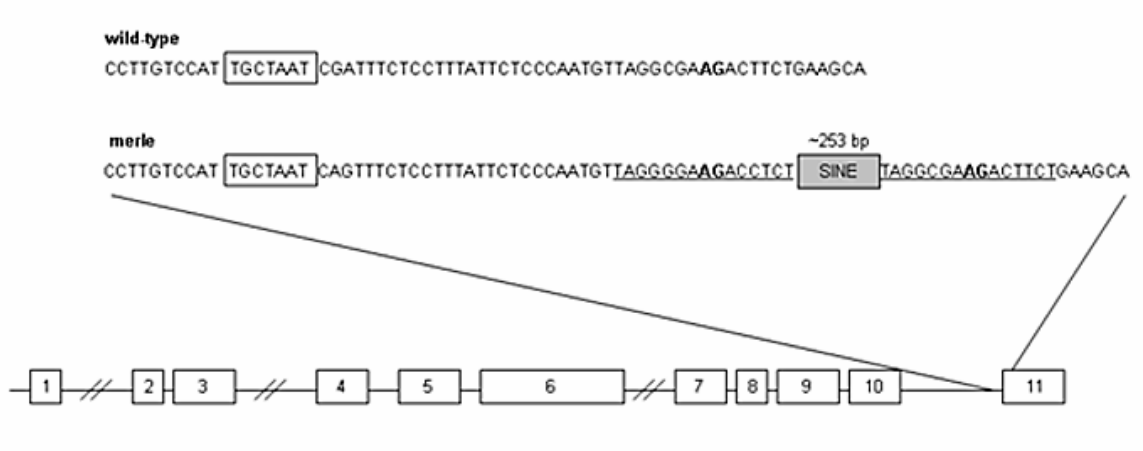

Figure 2. Structure of wild-type canine *SILV* and sequence of the SINE insertion site in merle dogs. The putative lariat branch point sequence is boxed. Splicing acceptors are indicated by bold type. In merle dogs, the splicing acceptor is located in the 15-bp duplicated sequence (underlined) that flanks the SINE insertion. The average insertion size (not including the duplicated sequence) for the merle dogs analyzed herein is 253 bp.

DNA was available from 50 of the 61 Shetland Sheepdogs used in the linkage analysis. These 50 dogs were analyzed by gel electrophoresis for the insertion. The insert was present in the heterozygous state in 12 merles and in the homozygous state in 2 double merles. Thirty-one nonmerle dogs did not harbor the insertion and four nonmerle dogs were heterozygous for a smaller insertion. Sequence analysis of this smaller insertion from two Shetland Sheepdogs revealed a deletion within the oligo(dA)-rich tail of the SINE. This smaller insertion was also present in a nonmerle that is suspected to be cryptic because it was sired by a double merle; however, no test breedings have been conducted to date to conclusively classify the dog as cryptic.

To determine whether the *SILV* mutation causing merle patterning in the Shetland Sheepdog population was breed specific, merle and nonmerle dogs representing six other breeds (Collie, Border Collie, Australian Shepherd, Cardigan Welsh Corgi, Dachshund, and Great Dane) were analyzed for the insertion. Merle dogs from all six breeds were heterozygous and one double merle Great Dane was homozygous for the insertion (Figure 3). Sequence analyses of one representative merle dog from each of the aforementioned breeds showed that they have the same SINE insertion in *SILV* (Figure 4).

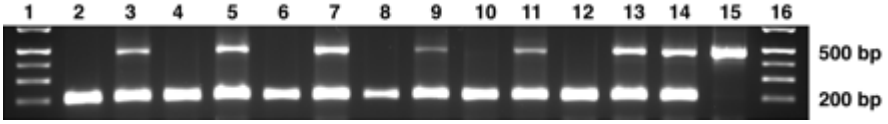

Figure 3. Mutation analysis of *SILV* and its segregation in six breeds. PCR on genomic DNA from a sable/white Collie (lane 2), blue merle Collie (lane 3), black/white Border Collie (lane 4), blue merle Border Collie (lane 5), red Australian Shepherd (lane 6), blue merle Australian Shepherd (lane 7), brindle Cardigan Welsh Corgi (lane 8), blue merle Cardigan Welsh Corgi (lane 9), black/tan Dachshund (lane 10), red dapple Dachshund (lane 11), fawn Great Dane (lane 12), blue merle Great Dane (lane 13), and harlequin Great Danes (lanes 14 and 15).

Genotypes of 12 harlequin Great Danes were also analyzed by gel electrophoresis: 9 were heterozygous and 3 were homozygous for the insertion. Additionally, seven Great Danes (six harlequin and one black) were heterozygous for a smaller insertion, and sequence analysis from one of these dogs showed a deletion within the oligo(dA)-rich tail, as was observed in the Shetland Sheepdogs (Figure 4).

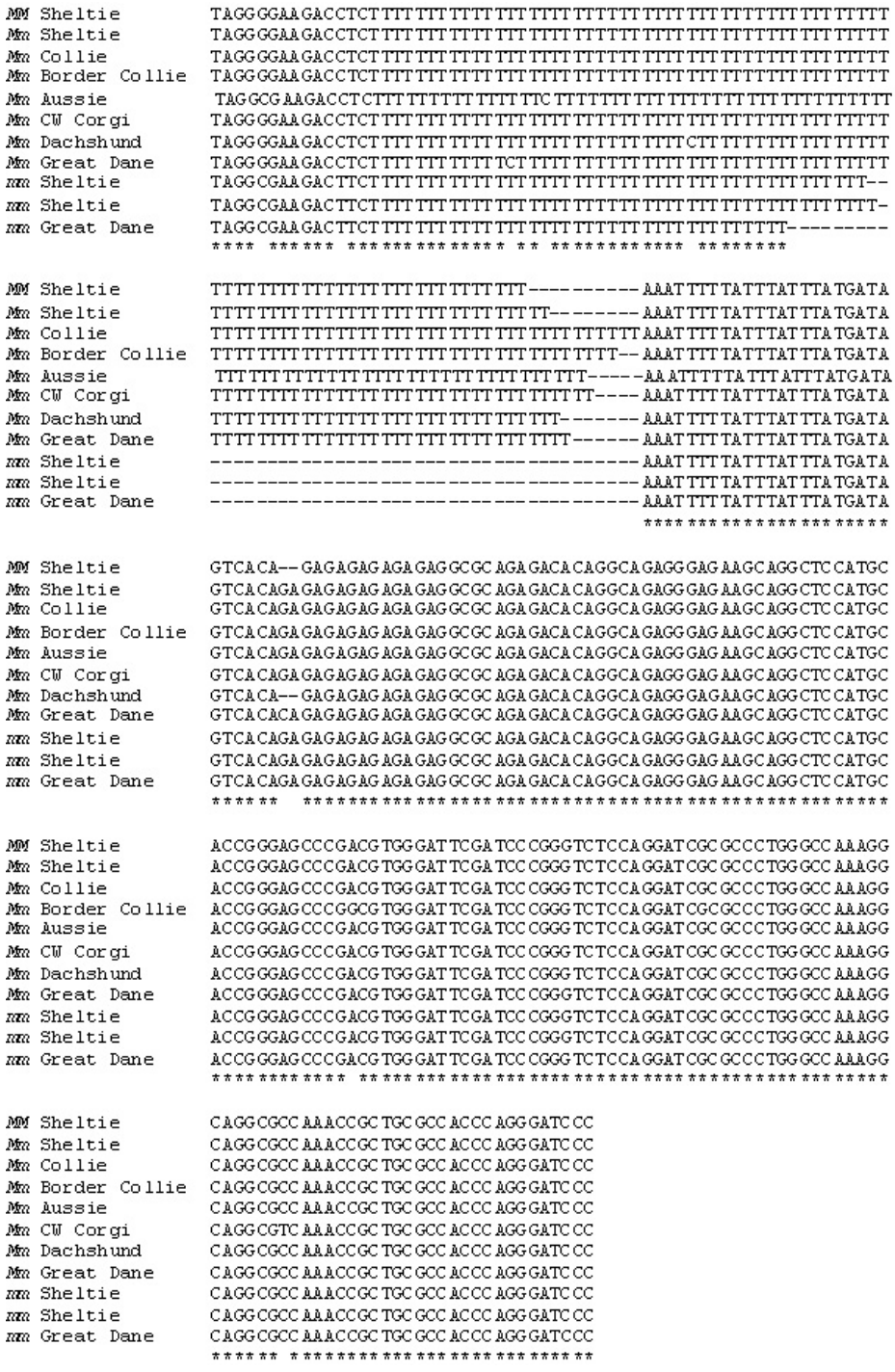

Figure 4. Sequence alignment of the SINE insertion in eight merle dogs from seven breeds (Shetland Sheepdog "Sheltie," Collie, Border Collie, Australian Shepherd "Aussie," Cardigan Welsh Corgi, Dachshund, and Great Dane) and three nonmerle dogs from two breeds (Shetland Sheepdog and Great Dane) with the smaller insertion.

Gel electrophoresis analysis showed that the SINE insertion also segregated with the merle phenotype in dogs from five additional breeds (American Pit Bull Terrier, Catahoula Leopard Dog, Chihuahua, Miniature Poodle, and Pyrenean Shepherd). Analysis of the intron10/exon 11 segment from 29 dogs representing 26 breeds that do not have merle patterning revealed that they do not have the insertion.

Discussion

Melanocytes are pigment-producing cells present in many tissues, including the epidermis, hair follicle, inner ear, and choroid of the eye (Steingrimsson *et al.*, 2004). Melanocyte cell populations differentiate from unpigmented melanoblasts released from the neural crest during embryogenesis (Steingrimsson *et al.*, 2004). The complex process in which melanoblasts migrate and differentiate into melanocytes is not fully understood; however, the study of pigmentary anomalies may accelerate identification of genes important for normal development (McCallion and Chakravarti, 2001).

The merle phenotype of the dog is a pattern of pigmentation associated with a wide range of developmental defects. A whole genome scan for merle by using 41 Shetland Sheepdogs showed LD with FH2537 on CFA10. No genes previously implicated in WS (*PAX3, MITF, SOX10, EDNRB*, and *EDN3*) map to this region. However, another gene important in pigmentation, *SILV*, is located ≈0.2 Mb from this marker. The *SILV* protein appears to be necessary for the formation of the fibril matrix upon which melanin intermediates are deposited late in melanosome maturation (Theos *et al.*, 2005). Other studies have shown that *SILV* may also participate in melanin biosynthesis by accelerating the conversion of 5,6-dihydroxyindole-2-carboxylic acid to

melanin (Chakraborty *et al.*, 1996; Lee *et al.*, 1996). The mutant phenotype of *SILV* in the human is unknown (Sturm *et al.*, 2001).

A SINE, structurally similar to a class of canine SINEs described by Minnick *et al.* (1992), was identified in *SILV* for all merle dogs analyzed. This SINE shows high sequence similarity (95–97%) with canine SINEs previously identified in the canine D2 dopamine receptor gene (Jeoung *et al.*, 2000), the dystrophin gene (Fletcher *et al.*, 2001), and the *PTPLA* gene, implicated in centronuclear myopathy (Pele *et al.*, 2005). These SINEs are tRNA-derived and highly abundant in the dog, representing 7% of the genome (Kirkness *et al.*, 2003).

It was previously hypothesized that the merle locus contains a transposable element (Whitney and Lamoreux, 1982). This theory is based, in part, on the finding that matings of homozygous merle dogs have produced nonmerle offspring (Whitney and Lamoreux, 1982; Sponenberg, 1984). Subsequent breedings with these offspring produced only nonmerle puppies, providing evidence for a stable germinal reversion (Sponenberg, 1984). It is estimated that the reversion rate in the germ line is 3–4% (Sponenberg, 1984).

In the human, *Alu* insertions causing disease are usually found in coding exons or near the boundaries of exons and introns (Druker and Whitelaw, 2004). These insertions result in disease, presumably by affecting splicing (Druker and Whitelaw, 2004; Pele *et al.*, 2005). Similarly, SINE insertions at these locations have been reported in the dog. A SINE insertion within exon 2 of the *PTPLA* gene causes aberrant splicing patterns associated with centronuclear myopathy in the Labrador Retriever (Pele *et al.*, 2005).

Lin *et al.* (1999) identified a SINE insertion in the 5′-flanking intronic region of exon 4 of *HCRTR2* that causes narcolepsy. The insertion displaces a putative lariat branch point sequence necessary for proper splicing (Lin *et al.*, 1999). The SINE insertion reported herein also occurs at an intron/exon boundary and may displace the putative lariat branch point sequence (Figure 2). This change in sequence of the gene is further complicated by the fact that in humans and presumably in other mammals as well, *SILV* has alternative splicing patterns (Bailin *et al.*, 1996; Nichols *et al.*, 2003). Therefore, cDNA transcripts should be analyzed to determine splicing patterns in nonmerle and merle dogs and the effect of the insertion on the encoded protein.

Fifty Shetland Sheepdogs were analyzed for the insertion, which segregated perfectly with the merle phenotype. The mutation also segregated with merle among dogs representing the Collie, Border Collie, Australian Shepherd, Cardigan Welsh Corgi, Dachshund, and Great Dane breeds, and sequence analysis confirmed that they have the same insertion. Aside from a single point mutation in two breeds each, the sequence of the SINE is identical. This finding suggests that the SINE insertion is identical by descent, and that the breeds analyzed in this study share a common ancestor. The occurrence of merle in many breeds and the fact that the first breeds to diverge from the working Sheepdog population in the 1800s have merle patterning (Collie, Old English Sheepdog, and Shetland Sheepdog), suggest that the founding mutation may predate the divergence of breeds (Neff *et al.*, 2004).

Harlequin is a popular coat pattern in the Great Dane and is characterized by black patches on a white background. Studies of the inheritance of harlequin support the

hypothesis that it is the result of two genes: *M* and the dominant gene *H* (Sponenberg, 1985). A deficiency of white dogs (*MM*) from harlequin to harlequin matings provides evidence to suggest that the *H+ MM* genotype has reduced viability (O'Sullivan and Robinson, 1988). The identification of harlequin Great Danes homozygous for the SINE insertion in the present study demonstrates that harlequin dogs may be either *H+ Mm* or *H+ MM*. These data suggest that the *H* gene is dominant to *M* and that white Great Danes have the ++ *MM* genotype. This misclassification of phenotype could account for the deficiency of white dogs observed in the aforementioned harlequin studies (Sponenberg, 1985; O'Sullivan and Robinson, 1988). Further studies are necessary to confirm this hypothesis.

Polymorphisms in the 3′ end of the SINE insertion were identified in four nonmerle Shetland Sheepdogs from two families and in seven Great Danes (six harlequin and one black) from one kindred. These Shetland Sheepdogs were heterozygous for the allele in LD with the merle phenotype, suggesting that the deletion may have occurred as a secondary mutation. The 3′ end of retroelements is typically polymorphic because of the presence of an oligo(dA)-rich tail, which is subject to strand slippage during replication and unequal crossing-over (Roy-Engel *et al.*, 2002). Roy-Engel *et al.* (2002) report an association between longer oligo(dA)-rich tails and diseased loci. In general, the oligo(dA)-rich tails decay gradually, with older insertions having shorter tails, although they can also shorten significantly in a single generation (Roy-Engel *et al.*, 2002). Abdelhak *et al.* (1997) report that the oligo(dA)-rich tail of an *Alu* insertion into the *eya1* locus shortened from A_{97} to A_{31} in one generation.

The present work identifies variable oligo(dA)-rich tail lengths associated with the SINE insertion: An $A_{91–101}$ segment present in merle dogs from seven breeds and an $A_{54–65}$ segment present in the aforementioned nonmerle Shetland Sheepdogs and Great Danes. No oligo(dA)-rich tail lengths intermediate to these were found. These data suggest that the truncation may have occurred in a single generation and could represent a reversion of the merle mutation (Sponenberg, 1984). Additionally, expansion of the oligo(dA)-rich tail in the germ line of a nonmerle dog having the smaller insertion may result in merle offspring and may be the mechanism behind the cryptic merle phenotype. Although the data presented herein suggest that the oligo(dA)-rich tail length determines phenotype (i.e., merle, nonmerle), they are not sufficient to determine the precise threshold for this phenomenon.

Here we have described a mutation in *SILV* that results in a disease phenotype and presented evidence to suggest a critical role for *SILV* in normal mammalian development. Phenotypic similarities between merling in the dog and WS in the human suggest that *SILV* may be involved in human auditory–pigmentation disorders. This work also enables genetic testing to identify dogs that carry the SINE insertion and may produce merle offspring. A genetic test for the merle locus can help responsible breeders of merle dogs prevent undesirable double merle progeny by allowing them to (*i*) distinguish merle from nonmerle in light-colored dogs that show little contrast between areas of dilution and full pigmentation, (*ii*) classify harlequin Great Danes as single or double merle, and (*iii*) identify cryptic merles. We have submitted a provisional patent application (Murphy *et al.*, 2005) for the identification of the mutation causing merle.

Materials and Methods

Sample Collection

DNA samples were obtained during previous studies conducted in the Canine Genetics Laboratory at Texas A&M University and through contributions from participating owners and breeders. Whole blood or buccal cells were collected from all dogs and genomic DNA was isolated by using the Puregene DNA Isolation kit (Gentra Systems).

Genotyping

Fluorescently labeled primers were synthesized and multiplex PCR was performed for Minimal Screening Set 2 markers as described by Clark *et al.* (2004). PCR products were resolved with an internal size standard (GeneScan 500 LIZ, Applied Biosystems) by using an ABI 3730xl DNA Analyzer (Applied Biosystems). Genotypes were determined by using genemapper 3.5 software (Applied Biosystems).

Linkage Analysis

Analyses for LD were carried out for all genotyped Minimal Screening Set 2 markers by using 41 Shetland Sheepdogs. For each marker, the allele more often associated with the merle dogs was identified, and all other alleles were combined into a second independent class. Fisher's exact probability test for 2 × 2 tables was used to evaluate allelic frequencies between the merle and nonmerle dogs. By convention, a *P* value of <0.0001 provides evidence for LD. For one marker with evidence of LD, an additional 20 Shetland Sheepdogs were genotyped and the *P* value was recalculated.

Sequencing

Primers were designed to amplify the complete exon and partial flanking intronic sequences for the 11 exons of *SILV* (see Table 1) by using the Boxer and the human intron/exon boundaries reported in Bailin *et al.* (1996). Concentrations for an 8.45-µl PCR volume were 0.09 units/µl *Taq* DNA polymerase with 1.2× buffer B (Fisher Scientific), 3.55 mM $MgCl_2$, 1.2× MasterAmp PCR Enhancer (Epicentre Technologies, Madison, WI), 0.59 mM total dNTPs, 5.9 ng/µl DNA, 0.47 µM each forward and reverse primer, and 2.8 µl of water. All exons were amplified with a single stepdown thermal cycling program: 5 min at 95°C followed by 5 cycles of 30 sec at 95°C, 15 sec at 58°C, and 10 sec at 72°C, and an additional 30 cycles of 20 sec at 95°C, 15 sec at 56°C, and 10 sec at 72°C, with a final extension of 5 min at 72°C.

Table 1. Primer sequences for the 11 exons of canine *SILV*

Exon	Forward	Reverse
1	GTAGCGGGATGTCCAGGG	GAGAAAAATCAGAGCAGGTGTG
2and3	ATGGTGCTGTCCCCTGA	ATCTGAGCCCTTGGAATAA
4	GGTTTGAGGGTGACTCTGTGT	GGGCAGTGAAGATTTAGGGAA
5	TTCCCTATGCTCAGTTCTTCC	GCTTTGCCCCTTCCCA
6a	GGTGTGCCTGTGAAAGAAG	CAAGCGTAGTGCCTGTGAC
6b	GCAGATGACGACCACGG	GTCCCACCTCAATGAACCT
7	GCCTCTTCAATCCTCTCC	CAAGGTATGCTTTCACTGG
8	GAAGCAGCCTTACGGTTTT	CGGAGTTCTCAGGACAATCA
9	CCATTGCCCTGACCTAAGC	AGCCTGTCCAACGCCTG
10	TGGCGGGGAGCAGACA	AAGAATGAGCAGTGGCAAGAG
11	CAGTTTCTCCTTTATTCTCCCA	CCTCGGCAAATCACAGCA

PCR products were analyzed by electrophoresis on a 3% agarose gel. The Gel Extract kit was used to purify amplicons (Qiagen, Valencia, CA). Products were ligated into pCR4.0-TOPO (Invitrogen) and transformed into chemically competent *Escherichia coli* TOP-10 cells (Invitrogen). Two clones for each dog were selected for sequencing. Nucleotide sequencing was performed by using the Big Dye Terminator version 1.1 Cycle Sequencing kit (Applied Biosystems) and an ABI 3130 Genetic Analyzer (Applied Biosystems). Sequences were aligned by using Clustal W (www.ebi.ac.uk/clustalw).

CHAPTER III

CANINE SINES AND THEIR EFFECTS ON PHENOTYPES OF THE DOMESTIC DOG

Overview

Short interspersed elements (SINEs) are mobile elements that contribute to genomic diversity through the addition of genetic material. Recent genomic analyses have vastly augmented our knowledge of both human- and canine-specific SINEs. SINEC_Cf is a major SINE of the canid family that has undergone recent expansion and is thought to be present in half of all genes. To date, only three phenotypes of the domestic dog have been attributed to a SINE. One of these is merle, a coat pattern characterized by patches of full color on a diluted background and associated with ocular and auditory anomalies. A SINEC_Cf in the *SILV* gene causes merle patterning by altering the cDNA transcript and has unique characteristics that are likely responsible for the random nature of the phenotype.

Short Interspersed Elements

Short interspersed elements (SINEs) are non-autonomous transposons of approximately 100 to 400 bp. SINEs have an internal promoter and are transcribed by RNA polymerase III but rely on long interspersed elements (LINEs) for reverse transcription and integration into the genome (Dewannieux *et al.*, 2003). A typical SINE is characterized by three features: the head, which contains the promoter necessary for initiation of transcription; the body, which is similar at the 3' end to LINE RNA and is necessary for reverse transcription; and the tail, which is usually A- or AT- rich but can

vary in sequence and length and may be involved in transcription termination, RNA delivery, and/or RNA stability (Kramerov and Vassetzky, 2005).

Our knowledge of SINEs has increased greatly in recent years, largely due to the assembly of whole genome sequences. Analyses of the human sequence revealed that SINEs comprise 13% of the genome (Lander *et al.*, 2001). In the human, the dominant SINE is the *Alu* element, which is also the only *active* SINE family in the genome (Lander *et al.*, 2001). The promoter region of *Alu* elements is derived from 7SL RNA sequences. This is different from most SINEs in that the majority of SINEs have promoters derived from tRNA sequences (Ullu and Tschudi, 1984; Lander *et al.*, 2001). It is estimated that 75% of human genes contain *Alu* elements, but many of these were inserted prior to divergence and thus are fixed in the population (Wang and Kirkness, 2005).

In 1992, Minnick *et al.* described a family of SINEs specific to canids. These SINEs, which are tRNA-Lys derived, are characterized by a (TC) repeat and an oligo-dA rich tail (Minnick *et al.*, 1992). A subfamily of these SINEs is SINEC_Cf. It is estimated that there are 170,000 SINEC_Cf elements in the genome and that half of all genes contain a SINEC_Cf insertion (Wang and Kirkness, 2005). Analyses of the first available canine genome sequence, which provides 1.5X coverage (a Standard Poodle was used), suggest that SINEC_Cf elements have experienced a large expansion in recent history and that this subfamily is highly conserved, exhibiting only 4.8% divergence from the consensus sequence (Kirkness *et al.*, 2003). When this sequence was compared to the 7.5X coverage sequence (from a Boxer), 10,000 insertion sites

were found to be bimorphic (differing by the presence or absence of a SINE), indicating that there are a substantial number of bimorphic sites present in the entire canine population (Lindblad-Toh *et al.*, 2005).

The first published report of a disease-causing retrotransposon in the dog came in 1999 when Lin *et al.* discovered that narcolepsy in the Doberman Pinscher results from a 226 bp SINEC_Cf insertion in intron 3 of *Hcrtr2*. The SINE, located 35 bp upstream of the exon 4 splice acceptor, causes skipping of exon 4. Exon skipping as a result of an intronic SINE is also reported in the human, causing autoimmune lymphoproliferative syndrome (Tighe *et al.*, 2002) and hemophilia A (Ganguly *et al.*, 2003). In both organisms, this phenomenon presumably results from displacement of the lariat branch point sequence.

A second canine disorder was recently attributed to a retrotransposon. Pele *et al.* (2005) showed that exon 2 of *PTPLA* is disrupted by a 236 bp SINEC_Cf element in Labrador retrievers having autosomal recessive centronuclear myopathy. The result of the insertion is variable; seven different cDNA transcripts are identified. Exon-skipping and exonization (the incorporation of SINE sequence into mRNA) are observed in the mutant transcripts. Interestingly, one of the seven transcripts is wild-type, indicating that the SINE is spliced out.

Merle Patterning

While the most devastating impact of SINE insertions is disease such as the two mentioned above, SINEs may also have a dramatic effect on physical appearance of dogs, without necessarily causing overt clinical disease. An example of this is a

SINEC_Cf insertion in the gene *SILV* that causes merle patterning in several breeds of dog (Clark *et al.*, 2006). In a single dose, the mutation causes dilution of the base fur color, which is determined by a separate locus, and can also cause blue eye color. The predominant characteristic of merling is random patches of full color distributed in various sizes and patterns across the coat. In some breeds (e.g., Australian Shepherd Dog, Catahoula Leopard Dog) this coat pattern is extremely popular and, according to breed standard, is the preferred phenotype.

Unfortunately, merle is problematic when two mutant copies of *SILV* are inherited (dogs may be referred to as double merles or double dilutes). Double merles have very little pigmentation and are afflicted with a variety of auditory and ocular defects (Figure 5). The most common abnormality in double merles is deafness. One study concluded that 54.6% of double merle and 36.8% of single merle dogs have mild to severe deafness (Reetz *et al.*, 1977). Also, merle dogs exhibit greater frequencies of ocular abnormalities than do non-merle dogs. These include increased ocular pressure, ametropic eyes, microphthalmia, and colobomas (Sorsby and Davey, 1954; Gelatt and McGill, 1973; Dausch *et al.*, 1977; Klinckmann *et al.*, 1987; Klinckmann and Wegner, 1987). Skeletal defects and sterility have also been reported in double merle dogs (Treu *et al.*, 1976; Sponenberg and Bowling, 1985)

Figure 5. Three red (e/e) Australian Shepherd dogs. From left to right: a homozygous merle, a non-merle, and a heterozygous merle.

A linkage disequilibrium approach using 32 non-merle and 9 merle Shetland Sheepdogs was used to identify the merle locus (Clark *et al.*, 2006). *SILV* (also known as *Pmel17*; gp100) was selected as a candidate gene for its location and its role in pigmentation of the mouse and chicken (Kwon *et al.*, 1991; Kerje *et al.*, 2004). Although *SILV* is clearly critical for pigmentation, its precise function remains controversial (Theos *et al.*, 2005). Studies suggest that the *SILV* protein is necessary for the formation of the fibril matrix upon which melanin intermediates are deposited (Theos *et al.*, 2005).

Sequencing of *SILV* revealed a SINEC_Cf insertion at the intron 10/exon 11 boundary in merle dogs. The SINE is flanked by a 15 bp target duplication site that includes the exon 11 splice acceptor, making it impossible to determine if the insertion is

exonic or intronic. Initial sequencing of the SINE was carried out using DNA from a single dog and the total insertion size was determined to be 262 bp.

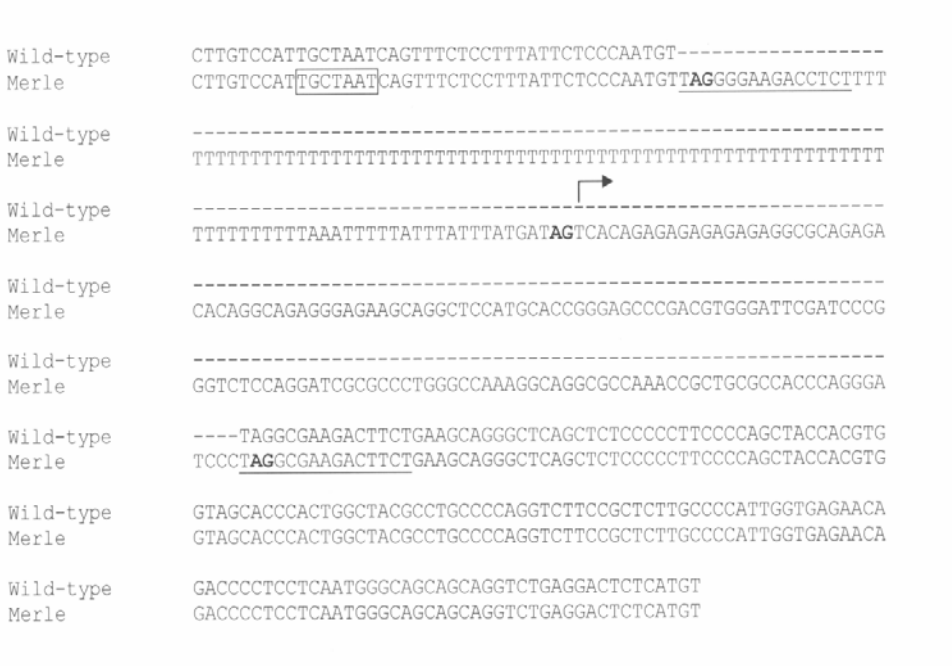

Figure 6. Genomic sequence of the 3' end of *SILV* with SINEC_CF insertion. The putative lariat branch point is boxed. The 15 bp target duplication site is underlined and contains the exon 11 3' splice site (in bold). The cryptic splice acceptor within the SINE is in bold and the arrow depicts where transcription begins in merle dogs.

cDNA transcripts from double merle dogs have a portion of the SINE incorporated between exons 10 and 11 (unpublished). This exonization is possible because the SINE is situated in reverse orientation and the reverse complement sequence of the SINEC_Cf element has an intron splice acceptor site (Figure 6) (Kirkness, 2006). Immediately following the splice acceptor is the characteristic GA tandem repeat, which is subject to strand slippage and thus is variable in length. Two alleles have been

identified to date. One allele, with seven GA repeats, maintains the reading frame. The *SILV* gene is transcribed in full with the mutant protein having a 52 amino acid insertion (Figure 7). A second allele has only six GA repeats and the insertion disrupts the reading frame. Fifty-one amino acids are incorporated after exon 10, and a premature stop codon occurs near the end of the insertion (Figure 7).

A-tails Are Important

Gel eletrophoresis analysis of the *SILV* SINE showed that the insertion size varies from dog to dog. Further sequencing revealed that the variability in the SINE is found in the poly(A) tail. A-tail length is an important factor in retrotransposition. Roy-Engel *et al.* (2002) analyzed A-tail length in *Alu* elements and found that insertions that result in disease (many are *de novo* events) have a mean length nearly twofold longer than other insertions. They also observed that overall, younger *Alu* elements have longer A-tails than older *Alu* elements (Roy-Engel *et al.*, 2002). These data suggest that the A-tail is evolutionarily unstable and subject to mutation and degradation over time. This phenomenon may exist in part because A-tails are subject to strand slippage during replication and unequal crossing over (Roy-Engel *et al.*, 2002).

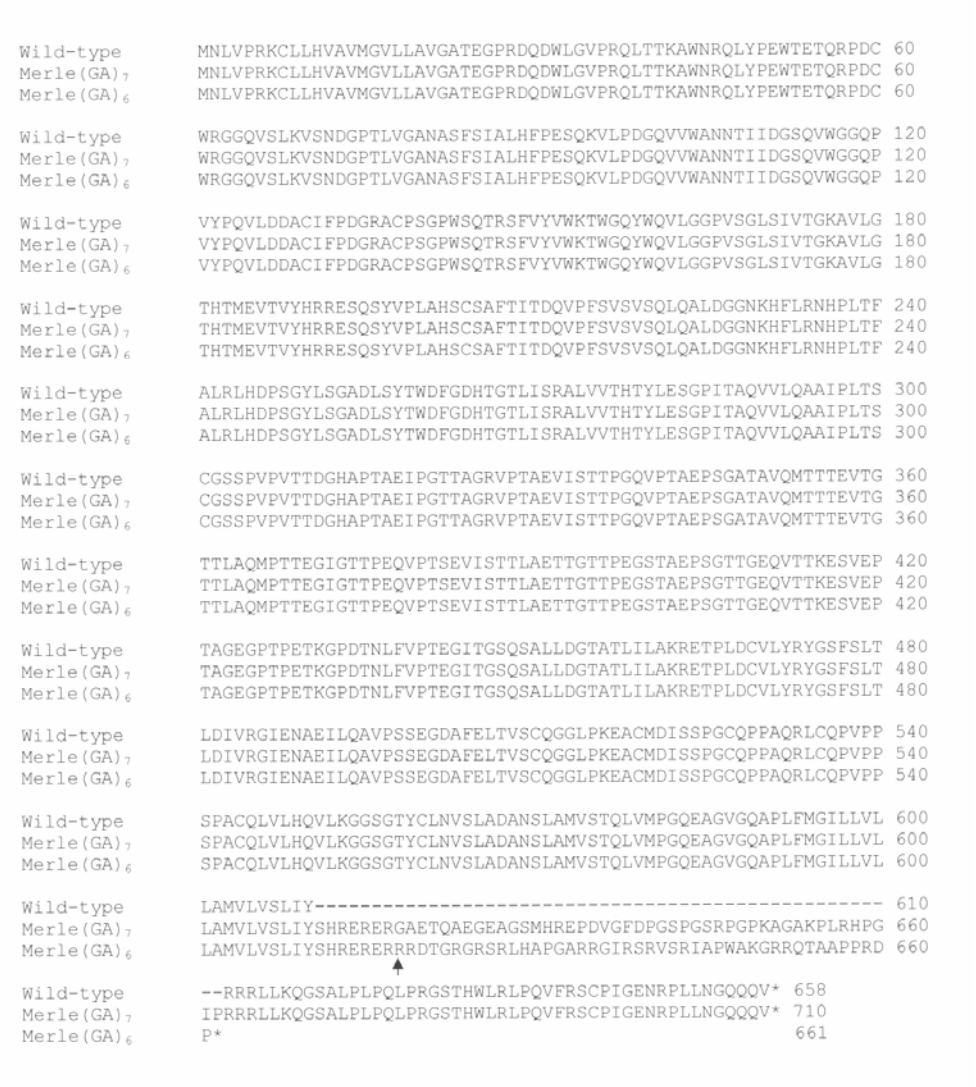

Figure 7. Wild-type and mutant SILV protein sequences. The arrow denotes where the truncated GA repeat alters the reading frame.

The SINE element that disrupts the *SILV* gene is longer than other SINEC_Cf elements that have been described in the dog (Jeoung *et al.*, 2000; Fletcher *et al.*, 2001; Pele *et al.*, 2005). The variation in length is again found in the A-tail, which we define as the region after the (TC) repeat, between the last G and the duplicated sequence. The A-

tail of the SINEs in the *PTPLA*, *dystrophin*, and *D2 dopamine receptor* genes are 64, 50, and 46 bp long, respectively (Jeoung *et al.*, 2000; Fletcher *et al.*, 2001; Pele *et al.*, 2005). In merle dogs, the tail length of the *SILV* SINE insertion ranges from 91 to 101 bp.

Along with changes in length, A-tails accumulate non-A bases that disrupt the pure A stretches. These interruptions reduce the likelihood of strand slippage, resulting in greater tail stability. The *SILV* SINE has fewer A-tail interruptions than do the *PTPLA* and *dystrophin* SINEs. Consequently, the former SINE has pure A stretches extending as long as 83 bp, while the latter SINEs have maximum pure A stretches of 14 and 15 bp, respectively. The length and purity of the A-tail of the *SILV* insertion suggest that it is a young SINEC_Cf and is subject to greater levels of instability.

A surprising find is the presence of the *SILV* SINE in dogs that do not have the merle phenotype (Clark *et al.*, 2006). In these dogs, the poly(A)-tail is shortened, ranging from 54 to 65 bp. This finding suggests that the SINE insertion is necessary but not sufficient for the merle pattern (Cordaux and Batzer, 2006). Although the threshold has not been determined, it is apparent that a long A-tail is requisite to produce the phenotype. A smaller insertion size may bring the lariat branch point to a more reasonable distance with the true splice site, encouraging proper splicing.

These data offer a possible explanation for merle patterning. During development, melanoblasts migrate from the neural crest and differentiate into the pigment-producing melanocytes (Steingrimsson *et al.*, 2004). Instability of the poly(A)-tail during this migration could result in cell populations with varying tail lengths. Melanocytes having a larger *SILV* SINE insertion would produce diluted pigment, while

those with a truncated A-tail would produce full pigment. A similar mechanism is found in pigs: black spotting results from somatic reversions of a C expansion in the *MC1R* gene (Kijas *et al.*, 2001).

The randomness of merling may also provide an explanation for the variability of the abnormalities associated with the double merle phenotype. In 2006, a hearing test known as the brainstem auditory evoked response (BAER), which detects electrical activity in the cochlea and auditory pathways in the brain (Wilson and Mills, 2005), was performed on 70 young to middle aged merles representing five breeds (unpublished). Twenty-two double merles were examined: eight were bilaterally deaf and two were unilaterally deaf. Although Reetz et. al. (1977) suggested that about one-third of heterozygous merles have deafness, only one of the 48 heterozygous merles in this study was deaf (unilateral). This dog, a Great Dane, was also piebald and consequently the deafness cannot be positively attributed to the *SILV* mutation (Strain, 1999).

Fifteen of the double merles tested were Catahoula Leopard Dogs and of these, only four were deaf. Although the sample size is small, this study suggests that only about 26% of double merle Catahoula Leopard Dogs are deaf while roughly 85% of double merles from other breeds tested (Australian Shepherd, Collie, and Shetland Sheepdog) are deaf. This finding is not entirely surprising because double merle Catahoula Leopard Dogs exhibit larger amounts of pigmentation than do other breeds. These phenotypic differences may result from a shorter average poly(A)-tail length in the breed or from modifying genes.

Conclusion

Mobile elements promote genomic diversity through the addition, and occasional deletion, of genetic material. Those mutations which occur in or near coding regions may also influence phenotypic diversity by altering gene expression and function. These changes may manifest themselves as detrimental genetic disorders or as simple physical traits. In addition, variability within phenotypes may result from instability of the causative element. This is exemplified by merle patterning in the dog in which random spotting is determined by the total size of the SINE insertion.

CHAPTER IV

ANALYSIS OF GENE TRANSCRIPT PROFILING AND IMMUNOBIOLOGY IN SHETLAND SHEEPDOGS WITH DERMATOMYOSITIS*

Overview

Dermatomyositis (DM) is a canine and human inflammatory disease of the skin and muscle that is thought to be autoimmune in nature. In the dog, DM occurs most often in the rough collie and Shetland Sheepdog. Characteristic skin lesions typically develop on the face, ears, tail, and distal extremities. The severity of lesions varies and is thought to increase with stressful stimuli. Previous studies in the collie suggest that DM is inherited in an autosomal dominant fashion with incomplete penetrance. The work presented herein concerns the different levels of gene transcripts and immunobiology of DM in the Shetland Sheepdog. Gene transcript profiles were generated for affected and normal skin using a canine-specific oligonucleotide array having 49,929 probe sets. Two-hundred and eight-five gene transcripts, many of which are involved in immune function, were found to be differentially regulated in these tissues. Also reported are western blot, immunohistochemistry, and immunofluorescence analyses which showed that staining patterns with sera from normal and affected dogs are quite similar. While our work suggests that canine DM is a disease that may be immune mediated, it did not detect the production of specific disease-associated autoantibodies.

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Introduction

In humans, dermatomyositis (DM) is an inflammatory disease of the skin and/or muscle that occurs in both children and adults. The childhood form, juvenile DM, presents much like the adult form but is accompanied by vasculitis and is classified into two types (Winkelmann, 1982). Type I exhibits rapid progression and is lethal (Banker and Victor, 1966), while Type II is chronic and less severe (Roberts and Brunsting, 1954). Canine familial DM is most similar to Type II juvenile DM (Hargis and Mundell, 1992). Canine DM is predominantly found in the Shetland Sheepdog and rough collie breeds (Haupt *et al.*, 1985; Hargis and Mundell, 1992; Scott *et al.*, 2000). In general, the Shetland Sheepdog presents primarily with skin lesions whereas the collie is more likely to have both skin lesions and muscle disease. Clinical signs in both breeds may occur as early as two to six months of age. These signs vary from mild to severe and start with small focal areas of crusting and scaling. Early signs of the disease usually begin on the face and lower extremities, often becoming more severe over time. Later stages of DM can be characterized by skin lesions that may include erythema, mottled pigmentation, and ulceration. Lesions occur primarily around the eyes, inner surfaces of pinnae, nose, lips, tail, and the distal extremities (Kunkle *et al.*, 1985; Hargis and Mundell, 1992; Scott *et al.*, 2000). One or more of these areas may be affected. Muscle lesions are rare in the Shetland Sheepdog but, when present, tend to coincide regionally with skin lesions and are more severe in the head and distal extremities. In severe cases, muscle lesions may cause difficulty in drinking and eating, reduced gag reflex, and an atypical high-stepping walk. Megaesophagus, which can be complicated by subsequent aspiration pneumonia,

may also occur. In some cases, euthanasia may be necessary due to a diminished quality of life (Hargis and Mundell, 1992; Scott *et al.*, 2000).

Familial studies of DM in the collie suggest that it is inherited in an autosomal dominant fashion with incomplete penetrance (Haupt *et al.*, 1985). To date, inheritance studies in the Shetland Sheepdog have not been carried out. The undefined transmission genetics of DM combined with the inability to diagnose cases before symptoms are evident, make this a difficult disease to manage. An additional complication is that even when signs of disease are present, there is marked clinical heterogeneity among dogs, even those of the same breed. Interestingly, circulating autoantibodies in affected human patients have been found, thereby lending credence to the hypothesis that DM may be an autoimmune disease, at least in the human (Kaufmann *et al.*, 2005; Targoff *et al.*, 2006; Matsushita *et al.*, 2007). The aims of the study reported herein were to (1) generate gene transcript profiles for dogs with DM and (2) characterize the immunobiology of this disease in the Shetland Sheepdog.

Materials and Methods

Population Selection and Sample Collection

Dogs were selected to participate in the study by a veterinary dermatologist (CR) based on historical, clinical and histopathological findings consistent with a diagnosis of canine familial DM. Dogs which were selected had developed skin lesions within the first two years of life. Skin lesions were located within one or more of the typical DM areas (face, ear tips, extremities, tail tip). These skin lesions consisted of erythema, alopecia, scaling, crusting, papules, erosions and ulcers. One or more of these lesions

was present in each dog. The histopathological slides from all of the dogs included in the study were reviewed by one veterinary dermatohistopathologist (JM). The histopathological findings, as previously reported (Gross *et al.*, 1992), consisted of scattered degeneration of basal epidermal cells which was either vacuolated or necrotic along with dermal inflammation which was superficially diffuse and contained lymphocytes and histiocytes. Some biopsy samples had dermal inflammation that contained neutrophils. Follicular basal cell degeneration with follicular atrophy was consistently noted in all histologic sections. The normal control dogs that participated in the study were unaffected dogs without family histories of DM and of ages similar to the affected dogs.

Skin samples for microarray and quantitative real time PCR analyses were obtained using a 6 mm punch biopsy instrument. Samples were collected from eight Shetland Sheepdogs: four dogs with DM (one male and three females; age range: 1 to 7 years) and four normal dogs (one female and three males; age range: 0.5 to 9 years). Two biopsies were taken from each of the dogs with DM: one from a lesional area and a second from a non-lesional site (lateral thorax). A single skin sample was taken from the lateral thorax of normal dogs. Tissues were stored in RNAlater® (Ambion, Austin, TX, USA) until processed.

Whole blood was collected from the four dogs with DM, three of the normal Shetland Sheepdogs and three normal non-Shetland Sheepdogs using standard venipuncture procedure. Sera was separated from whole blood and stored at 4 ^{o}C until used for western blot analyses and immunofluorescence studies.

All procedures were approved by the Clinical Research Review Committee of Texas A&M University, College of Veterinary Medicine & Biomedical Sciences.

Microarray

The Canine 2.0 oligonucleotide microarray used in this study is manufactured by Affymetrix (http://www.affymetrix.com/products/arrays/specific/canine_2.affx ; Santa Clara, CA, USA)and contains 42,929 probe sets. RNA was isolated from skin by the method of Gauthier *et al.* (1997) and purified using RNeasy Mini columns (Qiagen, Chatsworth, CA, USA). Syntheses of first strand cDNA, second strand cDNA, and biotin-labelled cRNA were carried out with the linear RNA amplification kit MessageAmp^{TM} aRNA (Ambion). Two micrograms of total RNA were used to start the single round of amplification. Duplicate experiments were performed for all samples. Labelled cRNA was fragmented at 95^{o}C for 35 min in a solution containing 40 mM Tris-acetate (pH 8.1), 100 mM KOAc, and 30 mM MgOAc. Forty micrograms of fragmented cRNA were hybridized to each GeneChip®. The chips were washed, stained and scanned as described by Ji *et al.* (2003).

GeneChip® images were collected on the high resolution GeneChip Scanner 3000. Image data were quantified and gene expression values were calculated using Affymetrix GeneChip Operating Software. The raw data were filtered to get rid of any probesets that had a signal value below 100, and to remove any genes with less than 2-fold difference. The resulting ~4500 genes were then subjected to a paired t-test with Benjamin & Hochberg multiple testing correction (Benjamani and Hochberg, 1995) using GeneSpring software (Silicon Genetics, Redwood City, CA, USA). Significance

was identified with *P*-values <0.05. Skin samples from the eight Shetland Sheepdogs were analyzed in two separate groups: lesional skin from the four dogs with DM were compared to non-lesional skin from the same four dogs (Group 1) and, lesional skin from the four DM dogs were compared to non-lesional skin from the same dogs and normal skin from four control dogs (Group 2).

Quantitative Real Time PCR (Q-RT-PCR)

RNA was treated with DNA-*free* DNAse (Ambion, Austin, TX). The primers and probe for *KRT2A* were designed using Primer3 (Rozen and Skaletsey, 2000) and the 5′ and 3′ ends of the probe were labelled with 6-FAM and Black Hole Quencher™ (BHQ), respectively (Biosearch Technologies, Novato, CA, USA). The one-step QuantiTect Probe PCR kit (Qiagen, Inc., Valencia, CA) was used to carry out cDNA synthesis and Q-RT-PCR for *KRT2A*. Alternatively, a two-step protocol using TaqMan Gene Expression Assays (Applied Biosystems (ABI), Foster City, CA) was used to validate *ADIPOQ* and *CCL24*. cDNA synthesis was accomplished using the High Capacity cDNA Reverse Transcription Kit (ABI) and Q-RT-PCR was performed using TaqMan Universal PCR Master Mix, no AmpErase uracil DNA glycosylase (Longo *et al.*, 1990) (Applied Biosystems). Q-RT-PCR was carried out using a BioRad MyiQ Single-Color Real-Time PCR Detection System (BioRad Inc., Hercules, CA). A standard curve for primer probe sets was created and the amount of cDNA added to the reactions was within this standard curve. All reactions were performed in triplicate and quantification of gene expression was achieved by normalization against *GAPDH*.

The comparative C_t method, also known as the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_t = \Delta C_{t,sample} - \Delta C_{t,reference}$, was used to report the differential gene expression. Like for the microarray analysis, skin samples from the eight Shetland Sheepdogs were analysed in two separate groups: lesional skin from the four dogs with DM were compared to nonlesional skin from the same four dogs (Group 1), and lesional skin from the four DM dogs were compared to nonlesional skin from the same dogs and normal skin from four control dogs (Group 2).

Western Blot

The sera from ten dogs (four affected Shetland Sheepdogs, three normal Shetland Sheepdogs, and three normal non-Shetland Sheepdogs) were tested on western blots generated from gels loaded with protein from normal canine skeletal muscle, normal canine skin, rat skeletal muscle, and rat liver, as well as with two human cell lines: A431 carcinoma cells and U-373 MG glioblastoma cells, which both served as positive controls for blotting and staining protocols. Total tissue proteins were extracted and tissue samples were sonicated in 300 µL of sample buffer containing 10 mm Tris/Cl pH 6.8, 4% sodium dodecyl sulphate (SDS), 10% glycerol and 5 mm dithiothreitol. The samples were then centrifuged and the supernatant removed. Undissolved tissue was discarded after centrifugation. Protein concentrations were determined using the bicinchoninic acid assay (Smith *et al.*, 1985), and 30 µg of protein were separated by SDS gel electrophoresis and then transferred onto a nitrocellulose membrane (Towbin *et al.*, 1979). Non-specific binding was blocked with 5% non-fat milk solution. Incubations were then performed with dog sera from affected and normal dogs (diluted 1 : 250 with

phosphate buffered saline milk solution) at room temperature for two hours. After washing, membranes were incubated at room temperature for one hour with a horseradish peroxidase-conjugated secondary antibody (goat anti-dog IgG and IgM, diluted 1:1000 with PBS milk) (Kirkegaard & Perry Laboratories (KPL), Inc., Gaithersburg, Maryland, USA). Immunoreactivity was detected with 3,3',5,5'-tetramethylbenzidine membrane peroxidase substrate (Kirkegaard & Perry Laboratories).

Immunofluorescence

For immunofluorescence on tissues, 10 μm thick cryostat sections of frozen canine skeletal muscle or skin were fixed for five minutes in acetone at -20°C and air-dried at room temperature for one hour. All canine sera from affected and normal dogs were diluted in PBS at 1:100, and were used to stain the sections for 30 minutes at room temperature. This was followed by three PBS washes of five minutes each. The secondary, goat anti-dog IgG + IgM fluorescein isothiocyanate conjugate was diluted 1 : 100 in PBS, and incubated with the sections for 30 min at room temperature. This was followed by three PBS washes of 5 min each. After the last wash, cells were mounted in ProLong Antifade (Molecular Probes, Eugene, OR). Microscope observations were carried out with an Axiophot microscope (Carl Zeiss, Oberkochen, Germany) equipped with epi-illumination and specific filters for fluorescein.

Immunohistochemistry for CD3

Formalin-fixed paraffin-embedded tissues (skin from normal and affected dogs) were sectioned at 5 μm, mounted on silanized slides, deparaffinized and rehydrated. The

slides were heated in a coplin jar containing citrate buffer (pH 6) in a 97^{o}C water bath for 20 minutes to unmask the antigen. The rehydrated slides were blocked with 3% hydrogen peroxide for 15 minutes. Five percent skimmed milk was used as the protein block. Slides were incubated with the primary antibody, CD3 (DakoCytomation, Carpinteria, CA) at 1:400 for 30 minutes at room temperature. The antigen was detected using a biotin-free polymer (MACH 2, Biocare Medical, Concord, CA). DAB (3,3'-diaminobenzidine) (DakoCytomation, Carpinteria, CA) was used as the chromogen to visualize the target antigen (CD3). For each pair of slides, the primary antibody was substituted with a universal rabbit control (DakoCytomation, Carpinteria, CA). All slides were counterstained with Mayer's haematoxylin. Slides were evaluated by a veterinary pathologist.

Results

Microarray

cRNAs isolated from 12 skin samples (four samples each from lesional and non-lesional skin of dogs with DM and four from normal dogs) were used to probe the Canine 2.0 microarray. This revealed 285 genes from which transcripts were shown to be differentially expressed in normal dogs and dogs with DM. Transcripts were classified as either up-regulated (transcripts from the lesional skin exhibiting higher levels in comparison to the normal tissues), or down-regulated (transcripts from lesional skin exhibiting lower levels in comparison to the normal tissues). Two-hundred transcripts were up-regulated and 85 were down-regulated. A complete list of these can be found at http://www.cvm.tamu.edu/cgr/dm_microarray.htm . A significant number of

the differentially expressed genes in DM-affected dogs were found to be critical for a functional immune response. A group of these genes has been listed in Table 2.

Table 2. A subset of genes important to immune function or involved in dermatologic conditions found to be up- or down-regulated in dogs with dermatomyositis

Symbol	Gene name	Gene function	Fold change	Group*
ADIPOQ	Adiponectin	Anti-inflammation	26.3 ↓	2
CCL24	Chemokine ligand 24	Inflammatory response	7.4 ↓	2
HLA-DMA	MHC, class II, DM alpha	Immune response	2.0 ↑	2
LCP2	Lymphocyte cytosolic protein 2	T-cell receptor signalling pathway	2.4 ↑	2
C1QG	Complement component 1 Q gamma	Complement activation	3.2 ↑	1
C2	Complement component 2	Complement activation	3.4 ↑	2
C1S	Complement component 1S	Complement activation	3.7 ↑	2
CCL3	Chemokine ligand 3	Inflammatory response	3.8 ↑	2
C1QG	Complement component 1 Q gamma	Complement activation	3.9 ↑	2
C1QA	Complement component 1 Q alpha	Complement activation	4.0 ↑	2
CTSS	Cathepsin S	Immune response	4.3 ↑	2
TCIRG1	T-cell, Immune regulator 1	Cellular defence response	4.4 ↑	2
IL1F8	Interleukin 1 family, member 8	Immune response	4.4 ↑	2
CD48	CD48 antigen	Defence response	4.5 ↑	2

Table 2 continued

OAS1	2',5'-oligoadenylate synthetase 1	Susceptibility to viral infection	4.8 ↑	1
IFI44	Interferon-induced protein 44	Response to virus	5.6 ↑	2
IFI16	Interferon, gamma inducible protein 16	Response to virus	5.7 ↑	2
CD16	Fc fragment of IgG	Immune response	5.7 ↑	2
CCL23	Chemokine ligand 23	Inflammatory response	7.8 ↑	2
OASL	2'-5'-oligoadenylate synthetase-like	Immune response	9.3 ↑	1
CD2	CD2 antigen	Natural killer cell activation	9.8 ↑	2
GPR65	G protein-coupled receptor 65	Immune response	11.0 ↑	2
C5R1	Complement component 5, receptor 1	Cellular defence response	11.3 ↑	2
BF	B-factor properdin	Alternative complement pathway	11.3 ↑	2
CD64	Fc fragment of IgG	Immune response	15.6 ↑	2
KRT2A	Keratin 2	Defects cause *Ichthyosis bullosa* of Siemens	20.1 ↑	2
IGLV1-51	Immunoglobulin lamba variable 1–51	Immune response	22.3 ↑	2
LY9	Lymphocyte antigen 9	Humoral defence mechanism	29.5 ↑	2
*Group 1: lesional skin from four dogs with dermatomyositis were compared to nonlesional skin from the same four dogs; Group 2: lesional skin from four dermatomyositis dogs were compared to nonlesional skin from the same dogs and normal skin from four control dogs.				

Q-RT-PCR

To verify data from probing of the microarray, three genes from which transcripts were shown to be differentially expressed on the array were selected for Q-

RT-PCR. Two genes, *ADIPOQ* and *CCL24*, were down-regulated and the third, *KRT2A,* was up-regulated. Expression patterns generated by the microarray were validated by the Q-RT-PCR experiments (Figures 8 and 9). There was no differential pattern of expression of *GAPDH* between samples in the affected and normal sample groups.

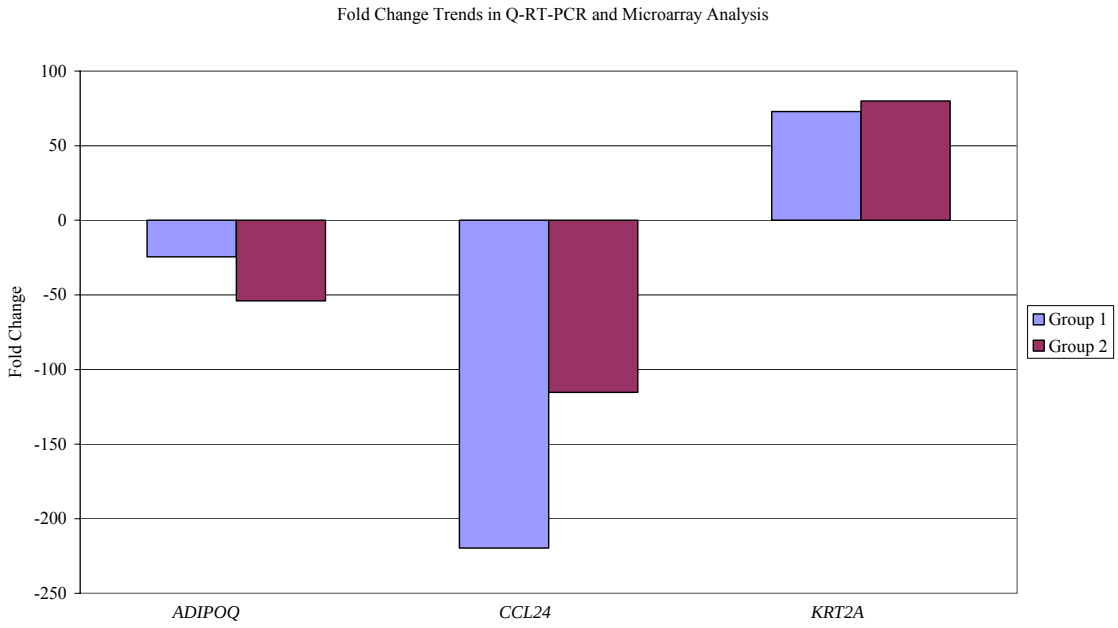

Figure 8. Significant fold change (greater than twofold and $P < 0.05$) for *ADIPOQ*, *CCL24*, and *KRT2A* for Group 2 (lesional skin from four dogs with dermatomyositis were compared to nonlesional skin from the same four dermatomyositis dogs and four normal dogs) based on microarray analysis and quantitative RT-PCR.

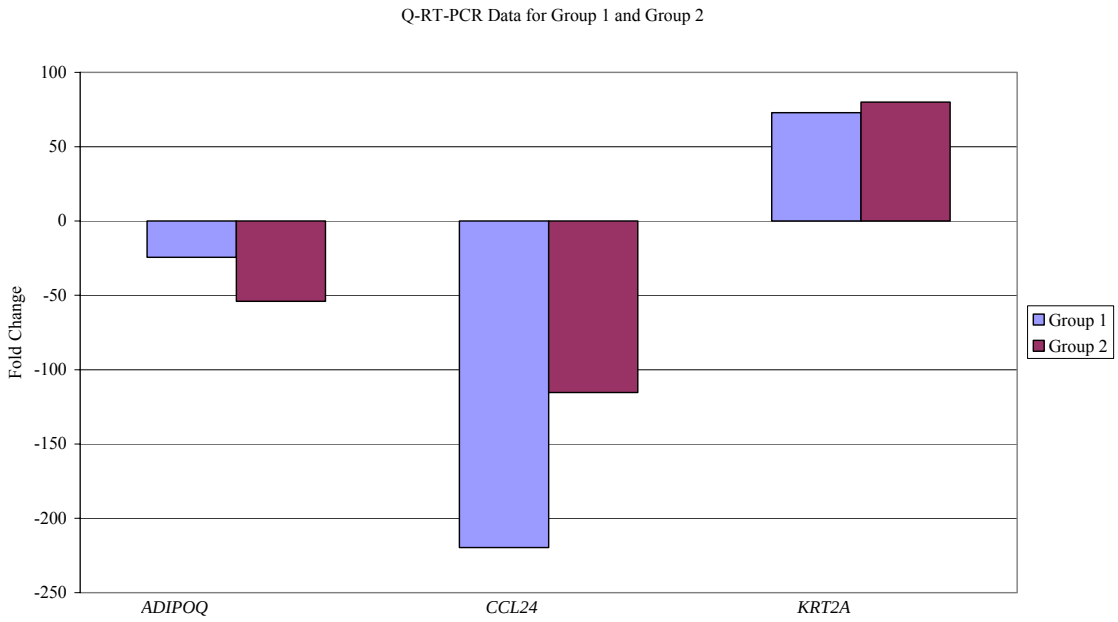

Figure 9. Significant fold change (greater than twofold and $P < 0.05$) for *ADIPOQ*, *CCL24*, and *KRT2A* for Group 1 (lesional skin from four dogs with dermatomyositis were compared to nonlesional skin from the same four dogs) and Group 2 (lesional skin from four dogs with dermatomyositis were compared to nonlesional skin from the same four dermatomyositis dogs and four normal dogs) based on quantitative RT-PCR.

Western Blot and Immunofluorescence

Western blot and immunofluorescence analyses were carried out using sera from four Shetland Sheepdogs with DM, three normal Shetland Sheepdogs, and three normal non-Shetland Sheepdogs to determine whether sera from affected dogs contain antibodies recognizing tissue proteins (i.e. normal canine skeletal muscle, normal canine skin, rat skeletal muscle, rat liver). All sera appeared to contain antibodies recognizing proteins on the blot, but there were no obvious differences in banding patterns between the sera from dogs with DM and normal dogs.

The immunofluorescence analysis showed that muscle cells stained with the sera of both control and affected animals. Small blood vessel sections were also stained on skin sections, and again there was no obvious difference in the staining patterns or intensities between the sera of control versus affected dogs.

Immunohistochemistry for CD3

Analysis of CD3 stained tissue sections revealed a mild T-cell infiltrate in DM lesions compared to normal dog skin. A mild infiltrate of lymphocytes, some staining positively with CD3 immunohistochemical stain, were present in the superficial dermis. There was no dramatic T-cell activation in the dog skin lesions, in contrary to that reported in humans (Caproni *et al.*, 2004).

Discussion

To identify candidate genes and pathways involved in DM, transcript profiles were generated for affected and normal dogs through microarray analysis. This revealed differential expression of 285 genes. A previous linkage analysis study suggested that a locus affecting the canine DM phenotype may be located near marker FH3570 on chromosome 35 (CFA 35) (Clark *et al.*, 2005). Unfortunately, no differentially expressed genes were located on CFA35.

A significant number of the differentially expressed genes in DM-affected dogs are critical for a functional immune response (Table 2), supporting the hypothesis that DM is an immune-mediated disease. Many of these genes are also implicated in human autoimmune disorders, such as lupus erythematosus, X-linked agammaglobulinemia type 1, muscular dystrophy, and complement deficiencies (i.e., C1q, C2, and C1s). The

different levels of mRNAs in our dogs, along with the association of these genes and/or the differential expression of these genes in human autoimmune diseases, lends further support that this is an autoimmune disorder in the dog despite the absence of disease specific autoantibodies. In humans, lupus erythematosus has been associated with defects in *FCGR3A* and *C1QA* (Kyogoku *et al.*, 2002; Racila *et al.*, 2003). More specifically, single nucleotide polymorphisms in *C1QA* have been associated with subacute cutaneous lupus erythematosus (Racila *et al.*, 2003). The dogs in this study exhibited an up-regulation of both *FCGR3A* and *C1QA*. An up-regulation in *BTK* was also observed in DM-affected dogs. This is intriguing because in humans, mutations in *BTK* are the cause of X-linked agammaglobulinemia type 1, a humoral immunodeficiency disease that leads to defects in the maturation of B-cells. A lack of mature B-cells may lead to recurrent bacterial infections such as dermatitis (Aghamohammadi *et al.*, 2006), which is a common occurrence in DM-affected dogs. Another gene of importance that is up-regulated in DM-affected dogs is *SGCA*. In humans, defects in *SGCA* are a cause of childhood autosomal recessive muscular dystrophy and limb-girdle muscular dystrophy type 2D (Romero *et al.*, 1994; Angelini *et al.*, 1998). An important gene, *ADIPOQ*, was down-regulated by 26.3 fold in DM-affected dogs. ADIPOQ has a similar crystal structure to the tumor necrosis factor (TNF) family, a group of proteins important in the control of inflammation, adaptive immunity, and apoptosis. ADIPOQ is also a close homolog of the complement protein C1q (Shapiro and Scherer, 1998). *C1QA* and several other complement genes were differentially regulated in affected dogs. Interestingly, humans with complement

deficiencies may develop several disorders including DM (Agnello, 1978; Ross and Densen, 1984).

Autoantibodies are present in the sera of some humans with DM. Specifically, autoantibody anti-Mi2 has the strongest association with human DM, finding it almost exclusively in DM patients; however, it is present in <20% of US patients. A novel autoantibody, anti-p155, was present in 29% of patients with juvenile DM and appears to be more common than other autoantibodies (Targoff, 1993). Neither autoantibody has been specifically investigated in the dog and this is something that can be investigated in future studies. Western blot analyses were carried out to identify any antibody(ies) that may be present in the sera of affected dogs. Sera from ten dogs were blotted against normal skin and skeletal muscle from dog and mouse. Western blots did not show any obvious differences in banding patterns between the sera of normal and affected dogs. We cannot exclude the possibility that some antibodies were unable to recognize protein epitopes that were denatured by SDS during the procedure. We therefore examined whether these same sera contained autoantibodies by staining cellular structures using tissue sections. The results showed that sera from both normal and affected dogs contained antibodies that produce vascular staining of similar intensities. It is not unexpected that the sera of normal dogs contain antibodies recognizing native as well as denatured proteins because the presence of such antibodies in many animals that are apparently free of autoimmune diseases is well-documented (Yildiz *et al.*, 1980; Guilbert *et al.*, 1982; Serre *et al.*, 1987). Pathogenic autoantibodies, on the other hand, are present in the sera of human patients with DM but not normal individuals. Our results suggest

that the sera of dogs affected by DM do not contain such pathogenic antibodies. Although autoantibodies have been associated with human DM (Targoff, 1993; Arnett *et al.*, 1996; Okada *et al.*, 2003), myositis-specific autoantibodies are not commonly found in juvenile DM patients (Feldman *et al.*, 1996; Rider and Miller, 1997). If a specific gene of interest was identified, future studies may find the use of purified immunoglobulin to that protein more informative than sera.

Immunohistological staining of affected and normal skin samples was used to assess the difference in immune and inflammatory complexes in lesional canine skin. Specifically, immunohistological staining focused on CD3 population of cells that infiltrate DM skin lesions. Autoreactive T-cells in autoimmune diseases, such as type I diabetes, have been shown to infiltrate and target multiple autoantigens (Han *et al.*, 2005). Comparison was made to normal skin in order to determine if there was an infiltrate of T cells in DM lesions. CD3 positive cells were found in skin lesions of DM but not in a dramatic manner (data not shown) as has been reported in human cases of DM (Caproni *et al.*, 2004).

The potential limitation to this study relates to the data obtained from the microarray analysis. Microarray analysis showed 285 differentially regulated transcripts between skin from DM affected dogs and skin from normal dogs. This represents a rather large number of genes to investigate and it is unknown if differences in transcript levels are the primary cause(s) of the disease, or are merely secondary effects (i.e., the genetic response to the disease). Therefore, selection of genes as candidates for being the cause(s) of DM is difficult based solely on microarray data. In addition, the statistical

method used to analyze our microarray data was the Benjamin and Hochberg multiple testing correction. This is not the most stringent test that could be used. The more stringent Bonferroni multiple testing correction could be used in the future to further analyze the data. We chose to use the Benjamin and Hochberg multiple testing correction in order to identify genes of biological significance to further investigate by Q-RT-PCR and immunobiological studies.

Future studies should include analysis of complement in the Shetland Sheepdog. Complement deficiencies have been documented in humans with DM (Agnello, 1978; Ross and Densen, 1984). Because of the association between DM and complement deficiencies, Hargis *et al.* determined complement concentrations in seven dogs affected with DM and 22 control dogs (1988). This revealed no differences between the affected collies and normal collies. However, there was a significant difference in the concentrations of C2 between all collies and non-collie control dogs. This is intriguing because Shetland Sheepdogs and collies are the two most common breeds to present with DM. To date, complement concentrations have not been characterized in Shetland Sheepdogs with DM. Future studies aimed to characterize the complement concentrations in affected and normal Shetland Sheepdogs, as well as in various other breeds are needed. Evaluating other breeds will help determine if there is also a deficiency in the Shetland Sheepdog and collie that may predispose these breeds to immune-related disorders. Future canine DM studies could be extremely beneficial to elucidating both canine and human DM since there is a lack of understanding of the pathogenesis of this disease.

CHAPTER V

SUMMARY

Presented herein is research on two canine traits that are models for human diseases: merle coat patterning and dermatomyositis. The availability of the sequence of the canine genome and genetic tools, such as naturally-occurring microsatellite markers and detailed linkage maps, has made the search for disease genes in the domestic dog more feasible and less time-consuming. In recent years, the study of canine hereditary diseases has revealed the genetic cause of multiple human hereditary diseases. Because of the high sequence similarity between the human and the dog, identification of disease genes in one organism can facilitate studies in the other.

Waardenburg Syndrome (WS) is an autosomal dominant auditory-pigmentation disorder in humans (1 per 40,000 live births) that accounts for 2% of all cases of congenital deafness. The phenotype of WS patients is quite similar to that observed in dogs which are heterozygous or homozygous for an allele at the merle locus. The genetic bases for all cases/types of WS have not been determined. Chapter II discusses the identification of the mutation causative for the merle phenotype in the domestic dog. A short interspersed nucleotide element (SINE) insertion at the boundary of intron 10/exon 11 of *SILV* was identified and found to segregate with the merle phenotype in multiple breeds of the dog. Because of the phenotypic similarity between dogs with merle coat patterning and humans having WS, the identification of the role of *SILV* in the merle phenotype of the dog makes it an ideal candidate gene to investigate in human WS. A genetic test was created for the merle locus that can help responsible breeders of merle

dogs prevent undesirable double merle progeny by allowing them to 1) distinguish merle dogs from non-merle dogs in light-colored dogs that show little contrast between areas of dilution and full pigmentation; 2) classify harlequin Great Danes as single or double merle and 3) identify cryptic merles.

The discovery of the mutation causative for the merle phenotype in the domestic dog also served to shed light on canine SINEs. Our knowledge of SINEs has increased greatly in recent years, largely due to the assembly of whole genome sequences. Chapter III discusses the structure of canine SINEs and the unique features of the SINE that causes merle patterning, as well as the effect of the SINE on the cDNA of *SILV* . Both chapters II and III highlight the genetic cause of merle and discuss the phenotype that is commonly found in the Shetland Sheepdog, the breed in which our initial discovery of the merle locus was made. In addition, we used the availability of these dogs, in conjunction with other genetic tools, to study DM, a dermatologic condition that occurs commonly in the Shetland Sheepdog.

Chapter IV discusses the gene transcript profiling and immunobiology of dermatomyositis, a hereditary disease that affects Shetland Sheepdogs. With the use of microarray analysis, 285 gene transcripts, many of which are involved in immune function, were found to be differentially regulated between affected and unaffected Shetland Sheepdogs. Also reported are western blot, immunohistochemistry, and immunofluorescence analyses, which demonstrate the similarity between the staining patterns of sera from normal and affected dogs. Chapter IV suggests that canine DM is a disease that may be immune-mediated, although specific disease-associated

autoantibodies were not detected. Further studies on DM could help elucidate the roles of genes which are important in immune-mediated diseases in both the dog and the human.

In conclusion, this work discusses the first mutation described in *SILV* resulting in a disease phenotype and evidence to suggest a critical role for *SILV* in normal mammalian development. Through the investigation of *SILV*, more evidence has been revealed for the role of canine SINEs in pigmentation and disease. In addition, this work also highlights important gene transcript profiling and immunobiology studies on dermatomyositis in the Shetland Sheepdog that are beneficial in understanding the pathogenesis of this disease in both the dog and the human.

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