Characterization of a 793 kilobase segment of the rat genome in blood pressure regulation

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Characterization of a 793 Kilobase Segment of the Rat Genome in Blood Pressure Regulation

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DEDICATION

I dedicate this to the two people who mean the most to me:

my mom, Sadhana K. Dhindaw, and Aditi Nadkarni.
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“Keep away from people who try to belittle your ambitions. Small people always do that, but the really great make you feel that you too, can become great.” - Mark Twain
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INTRODUCTION

The primary focus of this work is to identify genetic elements that control blood pressure (BP). Genetic elements can consist of but are not limited to protein and non-protein coding genes, noncoding regulatory elements as well as single nucleotide polymorphisms (SNPs). The study of these elements can lead to novel drug therapies for the treatment of chronic high BP (also known as essential hypertension (EH)) in humans. The human genetic background is largely heterogeneous making it difficult to determine which genes are controlling BP. To minimize genetic heterogeneity, this research utilizes two commonly used inbred rat strains that have contrasting BP phenotypes: the hypertensive Dahl salt-sensitive rat (S) and the normotensive Lewis (LEW) rat. Being inbred strains, theoretically the genetic makeup of every animal from one strain is >99% identical. Therefore, inherent genomic differences between the two strains are largely responsible for observed differences in BP.

The specific genomic region of interest for this research is on Rattus norvegicus chromosome 1 (RNO1) which is homologous to mouse chromosome 13 and human chromosome 5. Genetic element/s within an inferred 2.73 Megabase (Mb) region on RNO1 have been previously implicated in BP regulation using multiple S.LEW congenic strains (Joe, Garrett et al. 2003). S.LEW refers to the incorporation of a segment of the LEW rat genome (normotensive donor strain) into the S rat (hypertensive recipient strain) genetic background. By measuring the BP of each S.LEW congenic strain, the importance of the genomic segment of interest containing LEW alleles, in controlling BP can be determined. The locus of interest containing the genetic element/s controlling the quantitative trait of BP is termed: the BP quantitative trait locus (QTL). These two
congenic strains S.LEW (D1MCO4X1X3BX1) and S.LEW (D1MCO4X1X3BX2) used in this research contain a portion (793 Kilobases (Kb)) of the previously inferred 2.73 Mb BP QTL on RNO1. Evaluation of BP indicated that the presence of LEW alleles within this 793 Kb segment in the two congenic strains lowers BP significantly compared to the hypertensive S rat.

*The fundamental hypothesis of this work is that genetic variation between the S and LEW rat strains within this 793 Kb region on RNO1 is responsible for the strain differences in hypertension susceptibility.*

The research presented here specifically addresses (1) the localization of the RNO1 BP QTL to 793 Kb (2) the identification of nucleotide variations in gene coding regions between S and LEW that are likely to contribute to the observed BP differences (3) the generation of congenic substrains through breeding to capture the minimum contiguous genomic segment containing normotensive LEW alleles within the 793 Kb region and (4) the identification of any non-coding regulatory sequences present within the region using *in silico* analysis.

The 793 Kb interval contains two predicted genes: *Adamts 16 (A Disintegrin and Metalloprotease with Thrombospondin 1 Like Repeats 16)* and LOC306665 (similar to KIAA0947 protein) (Figure 4). Moreover, *Adamts16* was the only gene in which nucleotide variations were detected that resulted in amino acid changes between S and LEW forms of the protein (Figure 6). As exciting as this finding is, it does not rule out the possibility of other single nucleotide polymorphisms (SNPs) within this gene or other SNPs within noncoding segments of the 793 Kb segment contributing the observed strain differences in BP.
The results obtained from this research will serve as the foundation for future work aimed at (a) defining novel genetic factors that can be tested as contributors to the etiology of EH in humans and (b) investigating the molecular mechanisms by which nucleotide differences in the newly defined 793 Kb BP QTL region can cause hypertension.
LITERATURE REVIEW

Overview

A few areas of consideration for understanding the genetic basis of EH include (1) studying the known physiological mechanisms, (2) identifying novel genetic factors or genes contributing to the observed increase in BP and (3) application of information obtained from animal models to humans. Comparative study of the known physiological mechanisms of BP regulation between normal and hypertensive individuals or animal models, can provide insight into the pathophysiology of the organs or systems involved in BP regulation. However, a large body of evidence from genetic studies in humans and animal models indicate that genetics plays an important role in the etiology of hypertension (Soubrier and Bonnardeaux 1994; Ogihara, Katsuya et al. 2000; Yagil and Yagil 2001; Romano-Spica, Mettimano et al. 2003; Han, Hu et al. 2006). Therefore the understanding of the genetic basis of EH, by simply studying the physiology or pathophysiology of BP regulation and hypertension, is difficult to obtain. Based on the classification of hypertension in humans, extremely rare forms, resulting from single gene mutations, only account for 5-10% of the observed cases (Karet and Lifton 1997; Luft 2003). Essential hypertension, being genetically more complex, engages multiple genes and causative factors making clinical management more challenging (Filigheddu, Troffa et al. 2006; Puddu, Cravero et al. 2006). Thus, genetic studies aimed at identifying the unknown genetic factors contributing to EH are insightful.

Previously, it has been demonstrated that genetic components of distinct chromosomal regions are associated with the presence or absence of hypertension in both animal models and human association studies (Dominiczak, Negrin et al. 2000; Lee,
Padmanabhan et al. 2000; Rapp 2000; Lifton 2004; Lerman, Chade et al. 2005; Cowley 2006). Of these, a region on RNO1 and its homologous regions on human chromosome 5 (HSA5) have been implicated in BP control (Rice, Rankinen et al. 2000; Joe, Garrett et al. 2003). Additionally, there have been homologous regions of blood pressure regulation identified in mouse and humans (Wright, O'Connor et al. 1999; Rice, Rankinen et al. 2000). The region investigated on RNO1 for this research has been localized, using congenic rats, to a relatively small 793 Kb interval containing two predicted genes: Adams 16 (A Disintegrin and Metalloprotease with Thrombospondin 1 Like Repeats 16) and LOC306665 (similar to KIAA0947 protein). The following sections provide the background and pertinent framework for the research conducted.

**Blood Pressure**

The force exerted by circulating blood against blood vessel walls is defined as BP. Blood pressure is measured in units of millimeters of mercury (mmHg) as a systolic pressure (pressure when the heart contracts) and a diastolic pressure (pressure when the heart relaxes). In humans, normal BP is between 120-130 mmHg systolic and 80-90 mmHg diastolic (www.americanheart.org). Categorized as a quantitative trait, BP can be easily measured and exhibits a normal distribution within a population. The body’s ability to maintain normal BP is vital for sustaining life.

The onset of hypertension is characterized by a chronic increase in BP. A sustained increase in BP, with no definitive cause, is diagnosed as EH. Normal consequences of EH are blood vessel changes followed by renal and systemic vasoconstriction (Vicaut 2003). If left untreated, EH can lead to severe complications
such as organ damage (kidney, brain and heart), heart attack, stroke, atherosclerosis and eye abnormalities (Pickering 1997).

There are many intrinsic (e.g. genetic) as well as extrinsic factors that can cause fluctuations in BP. As a result, it becomes important to understand how BP is regulated. By doing so, it may be possible to prevent or reduce some of the complications associated with EH. Moreover understanding how BP is regulated can provide insight into how novel genetic elements may contribute to the already established mechanisms of BP control.

**Blood Pressure Regulation**

Sensitivity to environmental influences such as stress, psychosocial stimuli, diet, and lifestyle can result in BP changes. Activity can cause a rise in BP as a normal response due to physical exertion and stress. Hence, there are a number of endogenous physiological mechanisms to counteract fluctuations in BP. These mechanisms involve many organs and organ systems. A widely accepted rationale however, is that the primary control of BP lies in the kidney’s ability to maintain salt and water balance (Guyton and Hall 2006). Disruption of these mechanisms due to prolonged exposure to damaging environmental stimuli or genetic susceptibility can lead to EH. If EH is left untreated it can cause end stage renal disease and more serious complications.

Three well characterized mechanisms of BP regulation are: 1) Baroreceptor Reflex, 2) renin-angiotensin system (RAS) and 3) Aldosterone release (Guyton and Hall 2006). The Baroreceptor reflex is activated when short-term variations in BP occur. These are usually due to stress, dietary intake and environmental stimuli. Baroreceptors
are ‘pressure sensing receptors’ present in various organs and blood vessels that detect changes in blood flow and pressure. They compensate for BP changes by altering the force and speed of the heart’s contractions (cardiac output) as well as the body’s total peripheral resistance (i.e. the resistance to blood flow by blood vessels throughout the body). Lasting for only a few seconds to hours, these fluctuations are quickly normalized by the body without pharmacological intervention.

If individual genetic makeup facilitates the onset of EH, the major mechanisms that regulate BP act in concert to try and establish BP homeostasis. The renin-angiotensin system is a hormone system that is activated when a drop in BP occurs. The net effect of the RAS system is to cause an increase in BP by the production of Angiotensin II (AngII). Ang II can act as a constrictor of blood vessels leading to an increase in BP. By exerting its effects on the adrenal cortex, AngII can also cause the release of aldosterone. Aldosterone stimulates the kidneys to retain sodium and water thereby increasing BP. The release of Aldosterone is linked to RAS but is also independently regulated by serum potassium levels and atrial stretch receptors. By causing fluid retention in the kidney, aldosterone indirectly increases BP (Guyton and Hall 2006). Essential Hypertension that is due to defects in RAS or aldosterone release requires pharmacological intervention in order to maintain normal BP. The RAS system is targeted by ACE inhibitors and Ang II receptor antagonists while release of aldosterone is directly targeted by aldosterone antagonists (Turner and Boerwinkle 2003).

**Current Antihypertensive Drug Therapies**

The elucidation of established BP control mechanisms has led to the development of a variety of treatment drugs for those with EH. There are at least eight classes of
antihypertensive drugs that are available however; each person responds differently to each drug and maybe more or less susceptible to side effects (Guyton and Hall 2006). Depending on the health state of the patient (e.g. any coexisting conditions), a combination drug therapy may be prescribed that is best tolerated by the individual. Some of the more common classes of antihypertensive drugs and their effects are given below (MacMahon and Neal 2000; Neal, MacMahon et al. 2000; Brown 2001; Chobanian, Bakris et al. 2003; Nickey, Lenfant et al. 2003):

- **Diuretics-** These work by causing the kidneys to increase excretion of salt and water. This results in the bodily reduction of fluid volume and widening of blood vessels to lower BP. Commonly prescribed diuretics include thiazides, (e.g., chlorthalidone, hydrochlorothiazide, and indapamide). Thiazides can produce an overall potassium deficiency due to an increase in its excretion. As a result, a potassium supplement may be given or the following potassium-sparing diuretics are given in combination with the thiazide diuretic: amiloride, spironolactone, or triamterene.

- **Angiotensin Converting Enzyme (ACE) Inhibitors-** By blocking the production of the AngII hormone, ACE inhibitors cause vasodilation leading to lower BP and improved cardiac output. Available ACE inhibitors include benazepril, captopril, enalapril, fosinopril, lisinopril, moexipril, perindopril, quinapril, ramipril, and trandolapril.

- **Angiotensin II Receptor Blockers (ARBs)-** Unlike ACE inhibitors, ARBs act by blocking the effects of AngII on blood vessels and cells of heart. The reduced vasoconstriction results in an overall reduction in BP. Commonly prescribed ARBs include, candesartan, irbesartan, losartan, telmisartan, and valsartan.
• Calcium Channel Blockers- Muscle cells require calcium for contraction to occur. Therefore, by blocking the flow of calcium across cell membranes, muscle cells relax, blood vessels dilate and the force and rate of heart contractions are reduced leading to a lowering of BP. Calcium channel blockers include drugs known as dihydropyridines (amlodipine, felodipine, isradipine, nicardipine, nifedipine, and nisoldipine) and the nondihydropyridines diltiazem and verapamil.

• Beta Blockers- During times of stress, the sympathetic nervous system stimulates involuntary responses and causes an increase in heart rate and BP. Beta blockers inhibit the release of endogenous catecholamines (epinephrine (adrenaline) and norepinephrine (noradrenaline) in particular) and subsequent binding to adrenergic receptors which mediate the "fight or flight" stress response. There is also a lowering of BP in part by decreasing the rate and force at which the heart pumps blood into the circulation. The available beta blockers include acebutolol, atenolol, betaxolol, bisoprolol, carteolol, metoprolol, nadolol, penbutolol, pindolol, propranolol, and timolol.

• Vasodilators- These act by reducing smooth muscle tone in blood vessels. By doing so, vessels relax and widen to lower BP. There are two main drugs in this class: hydralazine and minoxidil. Due to their side effects they are administered by combined therapy with a beta blocker.

• Centrally Acting Drugs- Of all the classes, these are the most infrequently prescribed due to their side effects. They exert their effects on the brain and reduce sympathetic activity to help lower BP. The drugs are clonidine, guanabenz, guanfacine, and methyldopa.
Currently, there is no consensus on which class of antihypertensive drugs is most effective (Materson and Reda 1994; Brown 2001; 2002). When determining which antihypertensive drug should be prescribed variables such as an individual’s general health, sex, age, race; the severity of the hypertension and any additional, underlying (coexistent) conditions are taken into consideration (MacMahon and Neal 2000; Neal, MacMahon et al. 2000; Brown 2001; Chobanian, Bakris et al. 2003; Nickey, Lenfant et al. 2003). Differences in heredity and genetic makeup of each individual add to the complexity of developing novel drugs for the treatment of EH.

Despite the existence of a vast physiological control system for BP, the pathogenesis of EH remains elusive. This is partly due to the fact that it is difficult to identify which physiological mechanism has been altered in each individual. There is also the possibility that there exist other mechanisms of BP regulation that have not yet been discovered. These pathways may involve the participation of novel genes that have no obvious link to BP or EH. This supports a rationale for the existence of a genetic cause for EH being the basis for the associated physiological impairments.

**Rats as Genetic Models to Study Blood Pressure Regulation**

The physiology and disease progression of hypertension in the rat closely resembles that in humans. The use of inbred and congenic rat strains for this research has yielded intriguing insight into how individual genetic background plays a major role in the susceptibility and resistance to complex quantitative traits such as EH. The small 793 Kb genomic segment containing LEW alleles on RNO1 lowers BP on a hypertensive S rat genetic background. Consequently, genetic variation due to DNA sequence on
homologous regions in humans, particularly chromosome 5, may be vital to certain populations being more prone to developing EH and its complications.

**Genetics and Environmental Factors Affecting BP**

Individual genetic differences are largely responsible for the BP variation seen in human populations (Crews and Williams 1999; Snieder, Harshfield et al. 2003). There have been 26 candidate genes that have been studied and can be broadly categorized as being involved in the known mechanisms of BP control (Cowley 2006). However, results from these studies have mainly been associative with no mechanistic or causative links to EH. Studies reveal that interactions between environmental stressors and genetic factors can influence the onset and progression of EH (Mtabaji, Moriguchi et al. 1992; Gottdiener, Reda et al. 1994; Barlassina, Lanzani et al. 2002; Corizzato, Sega et al. 2005; Imumorin, Dong et al. 2005; Saavedra 2007). Salt sensitivity, for example, in conjunction with individual genetic backgrounds also aggravates EH progression (Dahl 1963; Campese and Karubian 1991; Van Vliet, Chafe et al. 2006).

Approximately 50 million Americans meet the definition of hypertension (Nadim, Dua et al. 2004). Many patients are on a combination drug therapy, but only a small number have been successful at maintaining a normal BP (Turner and Boerwinkle 2003). Determining the genetic components of hypertension is essential in order to create specific antihypertensive therapies suitable for individuals with varying genetic backgrounds. The current drug treatments target the known and well-established mechanisms of BP control without taking into consideration individual genetic heterogeneity. Moreover, genetic research using inbred animal models and selective
breeding strategies could bring to the forefront novel molecular targets and pathways in BP control.

**Using the Rat to Study Hypertension**

An outbred population of Sprague Dawley rats was selectively bred, on a high-salt (NaCl) diet, for sensitivity (S rats) and resistance (R rats) to hypertension by L.K Dahl (Dahl, Heine et al. 1963). Subsequently, inbred S and R rat strains were developed from Dahl's selectively bred lines by inbreeding for 20 generations (Rapp and Dene 1985). This ensured that the genetic makeup of each rat from a particular strain was nearly identical to the next. Theoretically the S rat genome contains a larger proportion of hypertension inducing alleles compared to the R rat genome. The S rats develop hypertension over time however, an increased salt intake initiates the onset and progression of hypertension at an earlier age (Dahl 1963; Dahl, Heine et al. 1963; Rapp and Dene 1985).

In addition to S and R, several inbred rat strains, including S and LEW have divergent BP states and are used as models for identifying BP regulatory genes. The LEW strain was derived from the outbred Wistar stock, in the 1950s, by Dr. Margaret Lewis (Lewis et al Wistar Institute). Derived from a different outbred population compared to R, the LEW strain is similar in being resistant to hypertension but has a different genetic constitution. Although it was not originally selectively bred for BP like the R rat, its normotensive phenotype makes it useful for comparative genetic studies with the S rat. Each inbred rat strains may have a different set of BP regulatory genes and
possibly alternative control mechanisms. These animal models serve to facilitate the characterization of the precise genetic loci controlling BP.

The genomic region(s) that are regulating a quantitative trait (e.g. BP) are termed quantitative trait loci (QTL). The phrase QTL is generally used to describe 1) a broad chromosomal region containing one or more genetic elements (genes, transcription factors, noncoding DNA, etc) controlling the trait of interest or 2) the specific genetic element (Joe B and Garrett MR. Substitution mapping: using congenic strains to detect genes controlling blood pressure. In: Cardiovascular Genomics: Gene Mining for Pharmacogenomics and Gene Therapy; Rapp 2000). Phenotypically contrasting strains, such as the hypertensive S and normotensive LEW, are used to identify genetic markers on various chromosomes that are linked to BP. QTL analysis “maps” the genetic control of a trait to different parts of a genome. Some of the initial investigations to determine linkage of genomic regions to BP were performed in the late1980’s using inbred rat strains (Hilbert, Lindpaintner et al. 1991; Jacob, Lindpaintner et al. 1991).

The genetic background of the specific hypertension and normotensive strains being studied is an important factor in determining which genomic regions are linked to BP. For example, BP QTLs on RNO1 have been associated with inbred S and LEW, Milan hypertensive and Milan normotensive strains, but not between Dahl S and Dahl R (Joe B and Garrett MR. Substitution mapping: using congenic strains to detect genes controlling blood pressure. In: Cardiovascular Genomics: Gene Mining for Pharmacogenomics and Gene Therapy; Cowley 2006). This variation mimics the diverse genetic backgrounds and distribution of EH in humans. Certain human populations
exhibit higher susceptibility to developing EH (Freeman, Freeman et al. 1983; Cooper, Rotimi et al. 1999; Grundy 2002; Scherrer, Turini et al. 2006). This could be attributed to a various set of factors such as geographic location, dietary habits or individual genetic backgrounds.

**Construction of the Initial RNO1 Congenic Strain**

The functional importance of genes and genomic regions identified by QTL analysis studies can be tested using congenic strains. The concept of developing such strains was created by Nobel prize winner G. Snell (Snell 1964). The construction of congenic strains to validate the existence of a QTL is also called substitution mapping (Joe and Garrett, 2004). The substitution of a chromosomal segment of interest from one donor strain to a recipient (background) strain can be achieved using congenic strains.

Congenic strain production involves the use of two contrasting inbred rat strains to construct an animal whose genetic background is expected to entirely resemble one of the strains (e.g. S) except for a particular region of interest introduced from the other strain (e.g. LEW). In the S.LEW strains, the S rat is background/recipient and the LEW rat is the donor strain.

The initial RNO1 congenic strain S.LEW (D1MCO4) was constructed using a detailed breeding protocol (Saad, Garrett et al. 2001), by crossing an S rat with a LEW rat. This resulted in a heterozygous (first filial) F1 population. The F1 animals were then backcrossed to the recipient strain (S). The animals were genotyped using polymorphic microsatellite markers flanking the end of the region used for linkage analysis to select
only those animals having the correct incorporation of donor (LEW) alleles. Once selected, those animals were again backcrossed to the recipient (S) strain. This was repeated for 8-10 generations. Each backcross to the recipient S rat, while selecting for specific donor (LEW) alleles, resulted in 50% of the loci outside of the region of interest being identical to the recipient (S) strain. After 8-10 generations, the background of the developing S.LEW congenic strain nears >99% of the recipient (S) strain (Rapp 1982; Rapp 2000). Finally, a male and female rat selected by genotyping for the target donor (LEW) alleles were mated. A percentage of the offspring (~25%) from this cross were homozygous for the LEW alleles being selected for. These rats were subsequently inbred (brother-sister bred) to maintain the newly derived congenic strain (Rapp 2000; Lazar, Moreno et al. 2005). The hypothesis is that the incorporation of the LEW alleles onto an entirely hypertensive genetic background would result in a lowering of BP in the S.LEW congenic compared to the S rat. If the BP of the S.LEW strain is lower than that of the S rat, it would provide evidence for the existence of a BP lowering genetic element within the LEW region of the S.LEW congenic strain. The above described breeding scheme was originally carried out to develop the initial parental congenic strain on RNO1 (S.LEW(D1MCO4)) (Saad, Garrett et al. 2001).

S.LEW Chromosome 1 Congenic Substrains

The BP QTL region on RNO1 is one among sixteen identified in the S rat to be statistically linked to BP as demonstrated by linkage analysis of an F2 population derived from S and LEW rats (Gu, Dene et al. 1996; Garrett, Dene et al. 1998). The original RNO1 BP QTL identified covered a large chromosomal segment containing LEW alleles.
captured in the S.LEW(D1MCO4) congenic strain (Figure 1). This strain was found to have a significantly lower BP compared to the S Rat (Saad, Garrett et al. 2001). To determine exactly which LEW alleles are essential for lowering BP, congenic substrains were derived from the original S.LEW congenic strain through breeding. Natural recombinations occurred to generate animals with shorter LEW segments on the S background (Figure 1). After over ten years of research on the RNO1 congenic substrains shown in Figure 1, the results presented in this thesis narrow down the essential BP regulating alleles to a 793 Kb region. The two strains S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2), which are the focus of this thesis, represent congenic animals which have LEW alleles in a 793 Kb segment that are required for a significant BP lowering effect compared to the S rat. These strains encompass the smallest segment of LEW alleles for this RNO1 segment thus far.
Figure 1: Rat Chromosome 1 Congenic Substrains

The graph shown at the top depicts the results of a linkage analysis on a F2(S x LEW) population. The y-axis shows a logarithm of odds (lod) score, which is a statistical measure of linkage. The x-axis is aligned with both a cytogenic map and a linkage map depicting microsatellite markers. Distances between the markers are given in centiMorgans (cM). The dotted and solid lines represent suggestive and significant linkage, respectively. S.LEW congenic strains developed over the past ten years that contained chromosomal segments from the LEW genetic background are shown. Shaded rectangles in (red or gray) represent LEW alleles and the white rectangles represent areas of recombination. Red horizontal rectangles indicate that the strain had statistically significant lowering of BP compared to the S rat. The gray rectangles indicate no significance difference in BP. The vertical rectangular bar highlights the 2.73 Mb interval that was previously inferred to be the BP QTL (Joe, Garrett et al. 2003). Data from the S.LEW(D1Mco4x1x3B) substrain is unpublished previous data from the laboratory. The last two strains S.LEW (D1Mco4x1x3BX1 and D1Mco4x1x3BX2) are the focus of this thesis.
From Congenic Strains to Causative Genetic Elements

An intriguing question to consider is what are the genomic differences between the congenic substrain and the S rat? Theoretically, the only difference between a congenic strain (S.LEW) and the recipient background strain (S) is the incorporation of donor (LEW) alleles at the region of interest. Hence, identifying the genetic differences between S and LEW alleles in that region would assist in understanding the genetic basis for strain variation in BP.

Conducting DNA sequence analysis aids in the identification of candidate nucleotide variants between S and LEW in the region of interest. QTL intervals may contain several genes and numerous single nucleotide polymorphisms (SNPs). These SNPs may reside in coding regions or flanking genomic DNA. The functional consequences of variants in coding regions, can be more readily assessed than those in noncoding regions (Cowley 2006; Drake, Bird et al. 2006), provided the function of the gene is known. The elusive relationship between promoter, intergenic sequence variations, gene expression levels, protein expression levels, protein function and the trait/phenotype under investigation makes noncoding DNA sequence variation difficult to study (Glazier, Nadeau et al. 2002). Coding DNA sequence, on the other hand, can be correlated to influence protein structure and function. As a result, sequence variations in coding regions are usually investigated first (Glazier, Nadeau et al. 2002).

There is some speculation however, in the field, that quantitative traits are a result of noncoding sequence variation (Mackay 2001; Korstanje and Paigen 2002). The importance of noncoding sequence variations should not be ruled out, particularly if there are no coding sequence variations or if the coding sequence variations are not causal to
functional abnormalities accounting for phenotypic variation. The generation and
classification of the congenic substrains (Figures 11 & 12) derived from
S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2) is expected to result in
substrains that contain only non-coding DNA, which will allow for testing the biological
significance of noncoding LEW alleles within the 793 Kb BP QTL.

Rat DNA sequence within candidate BP QTLs can be obtained from online
genome databases to study which genes or noncoding DNA sequences have been
The rat genomic sequence currently available is from the Brown Norway strain (Gibbs,
Weinstock et al. 2004). This strain shares sequence similarity with other rat strains and is
widely used as a reference until sequence for other rat strains become available. If a QTL
contains a gene that has a known function linked to the phenotype (disease or trait) of
interest, identifying sequence variations or gene expression differences between the two
contrasting strains is beneficial to pursue. Candidate gene approaches utilize knowledge
of genes linked to the phenotype being investigated. The advantage of using congenic
strains is that novel genes can be identified which have never before been functionally
linked to the phenotype of interest.

For the purpose of this research, congenic substrains were phenotyped for BP and
those with significantly lower BP compared to the S rat were used for further genetic
analysis. Congenic strains are unique genetic models that allow for the strain specific
study of select chromosomal segments in isolation on a homogenous genetic background
without any artificial disruption of genes. They provide a means by which to isolate and
study the genetic elements on each chromosome that are responsible for the variations in
disease susceptibility. For this work, they have been beneficial in identifying a small genomic region on RNO1 controlling BP. It is hypothesized that variations in this region between S and LEW rat strains are responsible for their differences in BP. The data presented here is encouraging to further investigate homologous regions on human chromosome 5 for drug therapy targets for EH.

As detailed in the results section of this thesis, the two S.LEW congenic strains being studied have two genes within the QTL. The presence of coding sequence variations in *Adams16* between S and LEW, make it a stronger candidate in regulating BP compared to LOC306665. ADAMTS (A Disintegrin-like and Metalloprotease with Thrombospondin motifs) proteins are a 19 member gene family and were first discovered in 1997 (Kuno, Iizasa et al. 1997; Kuno, Kanada et al. 1997). These genes have been shown to be involved in a variety of diseases such as cancer, connective tissue disorders, arthritis, and angiogenesis (Tang 2001; Apte 2004; Jones and Riley 2005; Porter, Clark et al. 2005; Wight 2005; Jones 2006).

Much about the workings and molecular functions of ADAMTSs is unknown and currently under investigation. A recent study indicates that ADAMTS16 has metalloproteinase activity (Gao et al 2007). Research over the past few years has uncovered a proprotein convertase enzyme to be at the forefront of ADAMTS processing. Furin is a ubiquitously expressed enzyme that cleaves proteins from an inactive to an active state (Somerville, Jungers et al. 2004; Somerville, Longpre et al. 2004; Wang, Tortorella et al. 2004; Koo, Longpre et al. 2006). Furin cleaves after the recognition sequence RX1X2R, where X can be any amino acid. Most human ADAMTS proteins contain clear conserved furin cleavage sites. The sites are less conserved in ADAMTS10.
and ADAMTS12 but prodomain removal has been shown to be via a furin mediated process (Cal, Arguelles et al. 2001; Somerville, Jungers et al. 2004; Porter, Clark et al. 2005). The conserved furin cleavage sites for human ADAMTS homologues are also present in the rat orthologues. On the basis of this combined evidence, it is commonly believed that furin cleavage of the pro-domain might occur for all ADAMTS proteins across species. Cleavage of ADAMTS16 specifically by furin has not been shown, however preliminary results presented in this thesis suggests that furin cleavage of Adamts16 may occur in the rat (Figure 10).

**Noncoding Conserved Regulatory Genomic Sequences**

It is important to consider the role of noncoding DNA sequence variants within the 793 Kb region. For example, variations in regulatory elements that are present within noncoding regions could affect the functionality of genes within the QTL region as well as outside. Single nucleotide polymorphisms that are present in noncoding regions that are conserved between rat, mouse and humans may also contribute to BP regulation.

The long-term availability of information on coding DNA sequence has led to the identification and prediction of gene function. However considering that approximately 90% of the mammalian genome is thought to be noncoding DNA sequence (Pennacchio and Rubin 2001), the significance of these regions is also important to investigate. Nucleotide polymorphisms in noncoding sequence have been associated with and in some cases found to play a major role in traits that have a significant genetic component. Examples include diabetes, hypertension, thrombophilia, and atherosclerosis (Ye, Eriksson et al. 1996; Nguyen 2000; Ono, Mannami et al. 2002; Love-Gregory, Wasson et al. 2004; Garrett, Meng et al. 2005; Konishi, Izawa et al. 2006). Furthermore, highly
conserved noncoding sequences are shown to play a role in mammalian development (Woolfe, Goodson et al. 2005). Hence determining conserved segments within the noncoding region of the 793 Kb QTL, provides the means to identify common signatures that could have functional consequences.

Regions of DNA that can regulate the expression of genes located in neighboring areas on the same strand are termed Cis regulatory elements. They can also be binding sites for transcription factors. A recent study found that genomic sequence comparisons of evolutionary close species (primates) with humans resulted in the identification of a significant number of cis-regulatory elements while comparisons of more distant ones failed (Pennacchio and Rubin 2001). By using a computational prediction program based on an available database of previously identified cis-elements, conserved regions were identified between rodents and humans. Moreover, these regions were then tested for transcriptional activity in vivo. Noncoding regions that are conserved among species may contain regulatory sequences (e.g. silencers, enhancers, transcription factor binding sites and microRNAs) that influence gene expression. These regulatory sequences could determine the levels, tissue specificity and developmental stages of gene expression. If encoded within the 793 Kb QTL, the expression of genes both within and outside the BP QTL interval could be influenced differently between the S and LEW strains. Nucleotide variation between S and LEW within these segments may act independently or in haplotype blocks to control BP.
MATERIALS AND METHODS

Rat Strains

The Dahl salt-sensitive (SS/Jr) inbred rats were from the colony maintained at UT-HSC. The LEW/NcrI BR (LEW rat) strain was originally obtained from the Charles River Laboratories (Wilmington, MA) and maintained at UT-HSC, Toledo. Congenic substrains S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2) were previously derived from the progenitor S.LEW(D1MCO4X1X3B) (unpublished data). Congenic substrains S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2) were derived from the progenitor S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2X1) was derived from the progenitor S.LEW(D1MCO4X1X3BX2).

General Strategy for Congenic Substrain Construction

To generate congenic substrains, S.LEW D1MCO4X1X3BX1 and S.LEW D1MCO4X1X3BX2 strains were backcrossed to the S rats to obtain a heterozygous F1 population (Figure 2 and 11). The F1 animals were intercrossed to generate an F2 population of 2,652 animals that were screened for recombinants. The recombinant animals were selected by genotyping for smaller fragments of the original 793 Kb QTL on the S background.

DNA was extracted from rat tails and used for genotyping. DNA isolation was performed using the Proteinase K Digestion Wizard SV 96 Genomic DNA kit (Promega, San Luis Obispo, CA). The animals were genotyped by PCR amplification using the primer sequences specific for microsatellite markers D1Mco83 and D1Mco84 (TableIII). This allowed for the amplification of markers that define the boundaries of the LEW
alleles in the 793 BP QTL (Figure 4 & Table III). Once the recombinant animals were identified they were backcrossed to the S rat. DNA from the F1 pups was isolated and used for PCR amplification of the end polymorphic markers. The animals found to be heterozygous at an end marker were brother sister mated to fix the strains for homozygosity (Figure 2 & 11).

**Figure 2: Generation of Congenic Substrains.**

The red regions represent LEW alleles on RNO1. White rectangles are the S background; short white ends on the congenic, recombinant and heterozygous animals are regions of recombination. Animals in the F2 population are genotyped using microsatellite markers on both ends of the chromosome to determine the incorporation of S alleles. Once identified the recombinant animals are backcrossed to the S rat. The offspring are genotyped and only the ones identical to the original recombinant are bred to make both alleles homozygous.
Microsatellite Marker Design and Testing

The genomic region of interest in the congenic substrains were selected on the published rat genomic sequence at the NCBI website (www.ncbi.nlm.nih.gov). Microsatellite repeats were identified by scanning the sequence. Primers to amplify the microsatellites were designed utilizing the Primer3 software and the Oligonucleotide Properties Calculator software available at the websites www.frodo.wi.mit.edu/ and www.basic.northwestern.edu/biotools/oligocalc.html, respectively. Primers were synthesized by Integrated DNA Technologies (www.idtdna.com). DNA was isolated from S, LEW and S.LEW strains and subsequent PCR reactions were performed using the designed primers. PCR products were labeled with $^{32}$P and subjected to electrophoresis on acrylamide gels. Initial reactions were carried out using only S and LEW DNA to determine if the repeat was polymorphic. The polymorphic repeats were then used as markers to genotype DNA from tail biopsies of the S.LEW congenic substrain.

Blood Pressure Measurements

Blood pressures (systolic pressure) were measured using the tail cuff microphonic method. Male rats (n=20) from each set of congenic substrains and control S rats were matched for age and body weight. They were raised, weaned, and housed concomitantly. Animals were weaned at ~30 days of age. At 5-6 weeks of age, rats were fed a 2% NaCl (high salt) diet or kept on a 0.4% NaCl (low salt) (Harlan Teklad diet TD 94217 and TD7034) for 24-42 days. Systolic BP was measured by tail cuff for four consecutive days on each conscious rat restrained in a warmed 28°C incubator. Four consistent readings were taken on each rat, per day by two operators who were oblivious to the strain of the
rat to eliminate bias. Initial body weights, final body weights, and heart weights were also recorded. The daily average of the four readings were used for statistical analysis using one-way analysis of variance (ANOVA) followed by post hoc multiple comparisons to determine the significance of differences between strains (SPSS, Chicago, IL).

**Predicted Coding Sequence Analysis**

Predicted gene exon sequences within the 793 Kb region were obtained from the rat genome database at the NCBI website (www.ncbi.nlm.nih.gov). Forward and reverse primers were designed to flank exons using the ExonPrimer software available at (http://ihg.gsf.de/ihg/ExonPrimer.html). The primers were subsequently synthesized by Integrated DNA technologies (IDT) and tagged with M-13 sequences. Genomic DNA from S, LEW and S.LEW rats was amplified using the primers and PCR products were sequenced by MWG Biotech Inc. The sequence data was analyzed using DNA Star (DNA Star Inc) and Sequencher (Genecodes Corp) software.

**Gene Transcript Identification**

Predicted transcript sequences were obtained from both the NCBI database and Ensembl (www.ensembl.org). Primers were designed to contain the stop and start codons of each predicted transcript using the online Primer3 or Oligonucleotide Properties Calculator software. The primer sequences used for identifying transcripts for LOC306664 and LOC306665 are give in Table I. Messenger RNA (mRNA) from S and LEW kidney, liver, heart, lung, thyroid pancreas and spleen were extracted using the TRIzol Reagent (Life Technologies). The mRNA was then reverse transcribed to cDNA of the predicted transcripts using the SuperScript III First Strain Synthesis system for RT-PCR (Invitrogen). The cDNA from all the tissues was pooled and Polymerase Chain
Reaction (PCR) using Hi Fidelity Platinum Taq DNA Polymerase (Invitrogen) and gene specific primers were used to amplify the cDNA. In cases where the predicted transcript sequence was not resulting in an amplified cDNA product, forward and reverse primer combinations from each predicted transcript sequence were tested until a product was obtained. The S and LEW cDNA products were cloned into the Topo-TA cloning system (Invitrogen) using their recommended procedure. Top10 Ecoli cells from Invitrogen were transformed with the plasmids and colonies were picked and grown in 1ml Luria Broth (LB) cultures. The QIAPrep-miniprep kit (Qiagen) was used to purify the plasmid DNA. The S and LEW full length constructs for each gene was sequenced by MWG (High Point, NC) using vector and gene specific primers. The sequence data was analyzed using DNA Star (DNA Star Inc) and Sequencher (Genecodes Corp) software.
Table I: Primer Sequences used for LOC306664 and LOC306665 Transcript Identification.

<table>
<thead>
<tr>
<th>LOC306664</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer A (5’-3’)</td>
<td>ATGGAACCCCGCGGTTGC</td>
</tr>
<tr>
<td>Primer B (5’-3’)</td>
<td>ATGGGAGCCAATGCAAATGTATAC</td>
</tr>
<tr>
<td>Primer C (3’-5’)</td>
<td>TGAAGATATTTCACATGGTCCTCC</td>
</tr>
<tr>
<td>Primer D (3’-5’)</td>
<td>CTACTTTAGAACTCGGTGGTTG</td>
</tr>
<tr>
<td>Primer E (3’-5’)</td>
<td>CTCACAGGTGGACTTGGGAAC</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>LOC306665</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer A (5’-3’)</td>
<td>CTCAAAGCTATAACCTCAG</td>
</tr>
<tr>
<td>Primer B (3’-5’)</td>
<td>CATGTGTATGAACATGCAATAA</td>
</tr>
<tr>
<td>Primer C (3’-5’)</td>
<td>TAGGACTCAAGTCACAAAGG</td>
</tr>
<tr>
<td>Primer D (3’-5’)</td>
<td>GGAGAGTCTGCTGAGCCATC</td>
</tr>
</tbody>
</table>

The primers correspond to those given in Figures 7 and for predicted genes LOC306664 and LOC306665.

**Western Blot Analysis**

Western blots were performed to test the antibody for Adamts16, which was developed by Rockland Immunochemicals, PA (www.rockland-inc.com). Polyclonal C-terminal (amino acids 1094-1105) antibody and N-terminal (amino acids 97-108) antibody were developed. Abundant gene transcript was detected in the kidney therefore kidneys from male S and LEW rats were used for analysis by western blots (Figures 9 & 10). Denaturing SDS-PAGE and electroblotting to polyvinylidene fluoride membrane
was performed followed by detection of the bound antibody using enhanced chemiluminescence (Amersham Biosciences). Anti-Rabbit secondary monoclonal antibody was used at a dilution of 1/10,000.

In silico analysis

An in silico analysis entails the use of computers to search online information databases to analyze DNA sequence. Comparative analysis of DNA sequences from closely related species was also performed to gain insight into evolutionary conserved genomic segments. To conduct the in silico analysis of the 793 Kb rat BPQTL, computer software programs that scan nucleotide sequence were used to 1) identify regulatory sequences within the BP QTL and 2) conduct an inter-species genomic sequence analysis between rat, mouse and human to identify conserved regions.

To query homologous regions of the rat sequence in mouse and human, the 793 Kb BPQTL was divided into three regions (Figure 3). The QTL region was also searched for microRNAs using the miRBase available at http://microrna.sanger.ac.uk/.
Figure 3: Division of the 793 Kb QTL for *in silico* analysis

The microsatellite markers D1Mco86 and D1Mco85 denote the limits of the QTL. The gray boxes represent the regions of recombination. Markers D1Mco83 and D1Mco84 are the microsatellite markers corresponding to LEW alleles in the S.LEW congenic rat strains. Blue boxes represent the genomic sequence of the two predicted genes (LOC306664 and LOC306665) within the region.
RESULTS

Localization of a BP Regulatory Segment to 793 Kb on RNO1

The chromosome 1 area presented in this thesis was previously named BP QTL2 (Saad, Garrett et al. 2001). It was fine-mapped to a 2.73 Mb interval flanked by the microsatellite markers D1Rat211 and D1Rat12 (Joe, Garrett et al. 2003). With the development of the S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2), polymorphic microsatellite makers between D1Rat211 and D1Rat12 were used to genotype the strains. Subsequently, the BPs of the two strains were measured and shown to be significantly lower compared to the S rat (Table II). As a result, the QTL2 region has been narrowed to a 793 Kb segment (Figure 1 & 4). The microsatellite markers of this region and their physical coordinates are listed in Table III. Due to a discrepancy in the online annotation of the rat genome, the RNO1 region is also mapped to chromosome 17 (www.ncbi.nlm.nih.gov). However, genotyping data for the RNO1 congenic strains clearly shows that this fragment is incorrectly placed in the online database. The correct location is on rat chromosome 1 between the previously published markers D1Mco4 and D1Mco8 (Table III & Figure1).

The chromosomal segment in the two S.LEW congenic strains S.LEW (D1MCO4X1X3BX1 & D1MCO4X1X3BX2) contains BP lowering alleles from the normotensive LEW rat. To delineate the boundary of the LEW alleles in the S.LEW congenic strains, primers were designed to amplify microsatellite markers between D1Rat211 and D1Rat12, the previously inferred QTL (Joe, Garrett et al. 2003). The QTL had been captured partially in the progenitor D1MCO4X1X3B strain (Figures 1 & 4). The polymorphic markers between S and LEW were used to genotype the two congenic
substrains S.LEW (D1MCO4X1X3BX1 & D1MCO4X1X3BX2). The end markers delineating the LEW alleles are D1Mco83 and D1Mco84 and the closest S alleles begin at D1Mco86 and D1Mco85 (Table III & Figure 4). The 793 Kb congenic interval, which denotes the BP controlling genomic locus, resides between D1Mco86 and D1Mco85 (Figure 4). Surprisingly, a closer look at this region, revealed that there are only two complete predicted genes and the first two exons of a gene whose record has been discontinued: LOC306664 (Adamts16), LOC306665 and two exons from LOC502107 respectively (Figure 4). The S.LEW (D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2) congenic strains generated serve as models that have been naturally created through breeding to test the effect of a small genomic segment on a hypertension susceptible genetic background in lowering BP.
Figure 4: Mapping and Localization of the RNO1 BPQTL to 793 Kb.

The previously inferred RNO1 2.73 Mb BP QTL is indicated in green. This was partially captured in the S.LEW(D1MCO4X1X3B) progenitor strain from which S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2) were derived. Microsatellite markers are shown along with the predicted genes within the region (blue boxes) on the physical map. The third gene, LOC502107, in the proximity of D1Mco84 is no longer predicted to be a gene (NCBI and Ensembl). The congenic strains all have significant BP effects and are shown underneath the RNO1 axis. LEW alleles are shaded in red. The white rectangles at the ends of the congenic strains represent regions containing the recombination break points of the donor RNO1 strain.

**Phenotypic Characterization of Congenic Strains S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2)**

The first step towards characterizing these strains involved measuring their BPs compared to the S rat. Twenty male rats from each congenic strain and S were either kept on a 2% or 0.4% NaCl diet for four weeks or six weeks respectively. Blood pressures were measured along with heart weights, which often corroborates well with BP, and found to be significantly lower in both the congenic strains compared to the S rat irrespective of dietary salt content (Table II).

The BP of the congenic strains S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2) on a high salt (2% NaCl) diet were significantly lower by
24 and 18 mmHg respectively than that of the hypertensive S rat (Table II). The blood pressures of the two strains on low salt (0.3% NaCl) diet were also significantly lower by 18 ad 14 mmHg respectively. The presence of dietary salt had no adverse effects on the BP of the congenic strains, which remained significantly lower than the S rat. This data provides evidence for the observed BP effect to be elicited by the alleles present with the 793 Kb QTL.

Table II: S.LEW Congenic Strains are Resistant to Developing Hypertension.

Blood pressure and heart weights were significantly lower in the congenic strains than the S rat both on a 2% and 0.3% NaCl diet. *The single asterisk represents combined averages of two separate experiments done on different days. **Effect = Congenic value – S value. Values in [brackets] indicated standard error of the mean. Negative values indicate a decrease in the congenic BP compared to the S rat. The number of rats in each group ranged from 20 to 30. Only male rats were studied.

<table>
<thead>
<tr>
<th>Dietary Salt: 2% NaCl</th>
<th></th>
<th>Heart Weight (g)</th>
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<tbody>
<tr>
<td></td>
<td>Blood Pressure (mm Hg)</td>
<td>S</td>
</tr>
<tr>
<td>Congenic Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.LEW(D1MCO4x1x3Bx1)*</td>
<td>215 [2.96] 191 [3.62]</td>
<td>-24</td>
</tr>
<tr>
<td>S.LEW(D1MCO4x1x3Bx2)*</td>
<td>215 [2.96] 197 [2.12]</td>
<td>-18</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>(b) Dietary Salt: 0.3% NaCl</th>
<th></th>
<th>Heart Weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Blood Pressure (mm Hg)</td>
<td>S</td>
</tr>
<tr>
<td>Congenic Strain</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.LEW(D1MCO4x1x3Bx1)</td>
<td>190 [5.44] 172 [1.94]</td>
<td>-18</td>
</tr>
<tr>
<td>S.LEW(D1MCO4x1x3Bx2)</td>
<td>190 [5.44] 176 [2.17]</td>
<td>-14</td>
</tr>
</tbody>
</table>
Coding Sequence Comparison between S and LEW in the Newly Defined 793 Kb RNO1 BP QTL2

To identify the specific genetic differences that account for the S rat being susceptible to hypertension, sequence analysis of all the exons within the QTL interval was carried out. Primers were designed to amplify the predicted exons and RTPCR was performed using DNA isolated from the S and S.LEW congenic strains. Sequence analysis revealed two SNPs resulting in amino acid changes in only one gene. Seven SNPs were detected within LOC306664: Adams16, but only the ones at position 820 and 3508 resulted in nonsynonymous variations between S and LEW (Figure 6). The SNPs at positions 820 and 3508 result in predicted amino acid changes from Pro (LEW rat) to Ser (S rat) and Thr (LEW) to Ser (S rat) respectively in the predicted protein product of Adams16. Interestingly, there were no nonsynonymous nucleotide changes (in LOC306665 or the first two exons of LOC502107 (which is no longer annotated as a gene) between S and LEW (Figure 5).

The presence of only two nonsynonymous SNPs between S and LEW within coding regions of the QTL warrants further investigation. Figure 6, below, depicts where the two amino acid variations are located in the predicted domain organization of the Adams16 protein. The first variant is a serine to proline (S to LEW) change and lies in an interdomain region of the predicted polypeptide. The second amino acid variant from a serine in the S rat to a threonine in the LEW rat is within a thrombospondin domain of the predicted polypeptide (Figure 6). It is intriguing to note that the nucleotide variant at position 820 (amino acid 274) resides in a potential furin cleavage site and variant 3508
is within a thrombospondin type 1 repeat (TSR) domain (amino acid 1170). This raises the possibility that resulting structural changes affect the function and processing of the protein in the hypertensive S rat. It is a challenge to determine the effects of the amino acid variations on protein function.

**Figure 5: The RNO1 793 Kb Blood Pressure Regulatory Region.**

The RNO1 BP QTL region contains two complete predicted genes. Exons are shown as blue boxes. Intrinsic regions are indicated by the black line connecting the exons. Sequencing of coding regions revealed no coding sequence variations except in LOC306664 (Adams16). The exon numbers where the two variations were found are given in the gene (exon 5 and exon 23).
Figure 6: Coding Sequence Variation in LOC306664 (Adamts16).

LOC306664 (Adamts16) is located on RNO1 at 2560022-2689574 bp. It was the only gene in which coding sequence variations were found between the hypertensive S and normotensive LEW rat. The figure shows the gene (top), transcript (middle) and protein (bottom) organization of Adamts 16. The exon number, transcript and amino acid location are given for all seven SNPs. Pink arrows denote the location of nonsynonymous SNPs both in the transcript as well as the protein. The chromatograms of the SNPs resulting in amino acid changes are shown. At the bottom of the figure is the predicted domain organization of the protein.
Evidence for the Existence of Gene Transcripts In Vivo

The genes present within the 793 Kb BP regulatory region are novel genes that have not been implicated in BP regulation. There has been no evidence that a transcript or protein product of any of the genes within the QTL exist in vivo. For LOC306664 (Adamts16) there were different transcript predictions at the Ensembl and NCBI databases. To identify which prediction resulted in the amplification of a transcript, primers were designed to flank the start and stop codons of each predicted transcript. cDNA was pooled from six different organs and RT-PCR was performed using the primers (Figure 7). None of the primer combinations from the predicted transcripts resulted in a product. However, when the forward and reverse primers from each prediction were combined a transcript product of 3675 bp in size was identified for LOC306664. The S and LEW product were gel extracted and purified. They were subsequently cloned into TOPO-TA cloning vector and sequenced. The full-length transcript size was found to be 3666 bp. The gene transcript variants found by sequencing of the predicted gene exons at position 820 and 3508 were confirmed. A full-length transcript for LOC306665 using the available predictions could not be detected. However, a small fragment has been amplified using primers flanking predicted exon 13 to exon 20 (Figure 8).
Figure 7: Identification and Expression of an Adamts16 Transcript:

The Ensembl and NCBI databases have differing predictions for the Adamts16 transcript. Primers that were designed to test which prediction resulted in an expressed transcript are indicated by boxes. Using the primer combinations shown below and mixed cDNA from seven different rat tissues, a transcript of the expected 3675bp has been identified. However after sequencing of the cDNA the actual size was found to be 3666bp. The true Adamts16 transcript size results from a combination of the Ensembl and NCBI rat genome database annotations.

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Figure 8: Detection of a short Gene Product for LOC306665.

The schematic depicting the predicted gene LOC306665 is shown at the top. The size of the gene inclusive of exons is predicted to be 39,497 bp. Predicted exons are represented as light green boxes. The start and stop coordinates on RNO1 for predicted gene LOC306665 is 2771760-2811256 bps (NCBI). The predicted full length transcript size 7801 bp. Based on EST sequences from the NCBI website and transcript predictions for the gene on NCBI and Ensembl, primers were designed in an attempt to identify a transcript for LOC306665. Red arrows represent potential primers that were designed and tested. Primer sequences are given in Table I. RNA from a mixed pool of rat tissues was reverse transcribed to cDNA. This cDNA served as the template for transcript identification. PCR reactions set up with three different combinations of primers resulted in the given expected product sizes of the transcript. This data provides evidence for the existence of a transcript for LOC306665 in the rat.

Tissue Specific Expression of Adamts16 in the Rat

Tissue specific expression of Adamts16 by Reverse Transcriptase PCR using RNA from 7 different organs reveals high expression in the kidney and other important BP regulating organs (i.e. kidney, heart, brain, pituitary, lung) (Figure 9). Relative to the other tissues expression appears to be more abundant in the kidney. The expression results of Adamts16 in the rat show similar expression patterns of ADAMTS16 in human tissues (Cal, Arguelles et al. 2001).
Figure 9: Tissue Expression of Adamts16.

Shown in the top panel is a representative agarose gel of tissue expression of *Adamts16* in LEW rat tissues. There is a band of the expected size for the transcript at 3666 bp. The same pattern of expression was detected in the S rat. Actin expression (bottom panel) was used as a control.

**Evidence for an Adamts16 Protein Product**

SDS PAGE and western blotting was performed to test the antibodies custom-designed to bind the predicted Adamts16 protein (Figure 10). Kidney homogenates from S and LEW rats were used as source of antigen. The expected size of the protein product is 135 kDa. As discussed in the Literature Review section, there are potential furin cleavage sites in the Adamts16 protein. However, based on the western blot data there are two bands, one corresponding to 104 kDa and the other at 30 kDa suggesting cleavage of the full length protein in both S and LEW kidneys. Both the N and C terminal antibodies
detected a band close to 104 kDa however only the N terminal antibody recognized a band close to 30 kDa. The data presented are the best blots that were obtained using the antibodies that were developed. Kidney homogenates from an S and LEW rat were loaded on the gel at 50 and 150 micrograms. Western blot conditions were optimized to give blots with the least number of nonspecific bands. The data presented in Figure 10 are representative blots from experiments performed six times. The presence of an amino acid variant between S and LEW forms of the protein suggest that differences in BP could possibly be associated with differences in furin processing of Adamts16. More experiments need to be done to test this hypothesis as well as the validity of the antibodies.

Figure 10: Results showing Adamts16 protein expression in S and LEW Kidney

A) N-Terminal Antibody

B) C-Terminal Antibody

Shown in this figure are representative western blots using the N and C terminal. Conditions were optimized to give the best results with an antibody dilution of 1:10,000 for both Adamts16 antibodies shown in panel A and B respectively, that were developed. The amount of protein loaded in each lane is given in micrograms in subscript. Marker sizes in kDa are shown on the left side of each panel. Both antibodies detect a band close to 100 kDa and only the N terminal shows at band close to 37 kDa. The data suggests furin processing of the protein, which results in bands at the expected sizes of 104 kDa and 30 kDa. More investigation will need to be carried out before any conclusions can be made.
In Silico Analysis of the 793 Kb BP QTL.

Over 98% of the 793 Kb BPQTL is within noncoding intergenic and intragenic (intronic) segments. An in silico analysis of the region was performed to identify sequence features other than coding sequence that could be regulating BP. The first region queried is 5’upstream of LOC306664 (Adams16), the second is intergenic between the two genes, Adams16 and LOC306665, and the third region is from the 3’end of LOC306665 to the end of the 793 Kb BPQTL (Figure 3). Each region was searched for conserved segments between rat and human utilizing the regulatory visual alignment tool (rVISTA)(Loots and Ovcharenko 2004). The QTL region was also searched for microRNAs using the miRBase available at http://microrna.sanger.ac.uk/.

Encoded within the 793 Kb BP QTL, there are 794 transcription factor binding sites (tfbs) that are conserved between rat and humans. In the region 5’upstream of Adams16 (Figure 3), there are only 3 conserved transcription factor binding sites: hepatocyte nuclear binding factor 4 (HNF4_Q6), Recombination Signal-Binding Protein 1 for J-Kappa (RBJK) and RBJK_Q4. In the intergenic region of the QTL (region 2), there are 197 conserved tfbs between mouse and human and the 3’ region 3, there are 594. The 793 Kb region was also searched for microRNAs, however there were no conserved microRNAs found within the region.. There are a number of SNPs that are documented in mouse and humans available on the NCBI website. In humans there are 1,172 SNPs in region 1, 506 in region 2 and 1881 in region 3. In the mouse there are 236 in region 1, none in region 2 and 20 in region 3. This information will be particularly useful when the congenic substrains which have been generated from the two 793 Kb
strains are tested for BP. Based on the BP measurements of the new congenic substrains, the essential BP regulating regions of the 793 Kb BP QTL will be determined.

**Generation of Congenic Substrains: Genetic Models for Further Analysis.**

The development of new S.LEW congenic substrains from the existing S.LEW (D1MCO4X1X3BX1) and S.LEW (D1MCO4X3BX2) strains will aid in the further localization of chromosomal elements within the 793 Kb BP QTL that are essential BP regulation. Genetically both of the above mentioned strains appear to be identical based on genotyping using microsatellite markers. However, if one were to sequence the entire 793 Kb interval between the two congenics, the ends of the QTL may be different. When deriving these two strains the location of the crossover which occurred from the progenitor S.LEW By nature, congenic substrains are unique genetic models that allow for the strain specific study of select chromosomal segments in isolation on a homogenous genetic background without any artificial disruption of genes. Generating congenic substrains relies on the natural occurrence of meiotic crossover events to fragment the 793 Kb BP QTL. Recombination events are rare in regions less than 1 Mb. As seen in the following results, out of a total of 2,652 F2 animals generated, only three recombinant animals were identified (Figure 11). These F2 animals were genotyped at the end markers of the BP QTL (D1Mco83 and D1Mco84) and subsequently bred to fix the strains for homozygosity at chromosome 1. The breeding scheme and the number of animal pairs bred at each stage are shown in Figure 11.

The new congenic substrains are S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2) and S.LEW(D1MCO4X1X3BX2X1) (Figure 12).
These animals will serve as tools for identifying the essential genetic elements within the 793 Kb region responsible for BP control. The three new congenic substrains will potentially represent animals, which could by virtue of their unique genetic make-up, have natural protection against the development of hypertension. Depending on whether they have a significant BP effect, the BP QTL may contain only noncoding sequence as in the case with S.LEW(D1MCO4X1X3BX1X2) and S.LEW(D1MCO4X1X3BX2X1) (Figure 12).
Figure 11: Schematic Representation of the Generation of Congenic Substrains S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2) and S.LEW(D1MCO4X1X3BX2X1).

Shown above is a generalized overview of the breeding scheme for developing congenic substrains from the two progenitor S.LEW (D1MCO4X1X3BX1 and D1MCO4X1X3BX2) 793 Kb congenic strains. Presented on the right side are the numbers of animals produced at each generation. On the left is an approximate time line starting from the 3 week gestation period. Three new congenic strains have been developed and fixed for homozygosity (see Figure 12)
Figure 12: Generation of Three New RNO1 Congenic Substrains

The RNO1 BP QTL region contains two complete predicted genes. Exons are shown as blue boxes and introns are indicated by the blue line connecting the exons. Microsatellite markers denoting the QTL regions in each of the congenic substrains and the original two progenitor strains are shown. The three newly developed congenic substrains are shown below the axis. The red bars indicate LEW alleles and the white rectangular areas at the end denote regions of recombination. The size of the potential BP QTL in each new congenic substrain is given next to the strains.
DISCUSSION

Maintaining normal BP levels is vital for sustaining life. An individual’s genetic make-up is estimated to contribute 27% towards their susceptibility for essential hypertension (Hajjar, Kotchen et al. 2006). Hypertensive individuals younger than 55 years of age are four times more likely to have a family history of EH (Winnicki, Bonso et al. 2006; Winnicki, Somers et al. 2006). Human mapping studies have been successful in identifying the genes responsible for rare monogenic forms of hypertension however, the causes of EH remain a mystery. Studies utilizing animal models, particularly congenic strains, that map and link chromosomal regions to hypertension have uncovered a number of genetic suspects (Cowley 2006). This study provides evidence for the role of a 793 Kb region on the rat genome in hypertension. These data provide a foundation for the investigation of the homologous region on human chromosome 5 in BP regulation.

Substitution Mapping Using Congenic Rats

The data presented in this thesis has a history of over a decade of studies utilizing congenic rats to finely map and localize a genomic segment on RNO1 controlling BP. Comparable work implicating 11-B hydroxylase in the pathogenesis of hypertension was previously done using the same approach (Rapp and Dahl 1971; Rapp and Dahl 1972; Cicila, Rapp et al. 1993). The extrapolation of the data obtained by studies using congenic rat strains can provide the foundation for new treatment strategies for EH. The work presented here is among the first to identify a novel genetic locus potentially controlling BP based on substitution mapping using congenic rats.
Localization and Fine Mapping of BP QTL2

BP QTL2 was localized to a 2.73 Mb region that was inferred from boundaries of multiple congenic substrains. The generation and testing of S.LEW congenic substrains that had fraction of this 2.73 Mb region incorporated as a QTL provided \textit{in vivo} data for the existence of BP controlling alleles within the region. The new BP QTL2 is now localized to 793 Kb in two S.LEW congenic substrains. Although S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2) appear identical in their genetic make-up from genotyping using microsatellite makers, this may not be the case. The region of recombination could be different between the two strains. Sequencing the ends of the BP QTL interval in both strains would reveal the strain differences at the nucleotide level. This remains to be done. The new 793 Kb BP QTL contains only two candidate genes. The finding that only Adamts16 has predicted amino acid variations between the hypertensive S and normotensive LEW strains makes it a promising target for further investigation. However there is also the possibility that noncoding regions within the QTL could be responsible for the BP effect. The characterization of congenic substrains S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2) and S.LEW(D1MCO4X1X3BX2X1), which have been generated, will aid further localization of the essential BP regulatory genetic elements within this RNO1 segment. While the nucleotide variations in Adamts16 are promising, they may not be the only genetic elements contributing to the observed BP differences between S.LEW(D1MCO4X1X3BX1 and S.LEW(D1MCO4X1X3BX2) congenic strains and S. Single nucleotide polymorphisms that reside in noncoding segments of the 793 Kb QTL could function as haplotype blocks which are associated with genetic susceptibility or
resistance to hypertension in certain populations. Further investigations will need to be conducted to determine which SNPs are associated with BP.

**Linkage Analysis versus Substitution Mapping: Conflicting Data?**

Genetic linkage analysis provided statistical evidence for the implication of RNO1 in BP. However, the location of the 793 Kb BP QTL obtained from substitution mapping (i.e. congenic substrain construction) does not coincide with the suggestive linkage confidence intervals of the LOD peaks shown in Figure 1. By virtue of being an *in vivo* model, the S.LEW congenic substrains have captured the BP QTL within the confines of their genome. Substitution mapping using congenic substrains more precisely defines the limits of a QTL while linkage analysis provides a general overview of the possible location. Initially what appears to be conflicting information from linkage analysis and substitution mapping is in reality data that concomitantly provides evidence for a chromosomal region influencing the trait of interest. The location of the 793 Kb QTL does not coincide with the RNO1 LOD peak confidence intervals (Figure 1).

Previous work from this laboratory has revealed three closely linked QTL regions on RNO1 (Saad et al 2001). It is possible for there to be multiple closely linked peaks as in RNO1 which could be linked to BP however discerning the QTL simple based on linkage analysis may not aid in the identification of a true QTL *in vivo* (Rapp 2000). If congenic strains had not been constructed, the 793 Kb region may not have been recognized as containing a distinct QTL.

An advantage to substitution mapping is that novel genes that have no obvious physiological link to BP can be discovered. The data presented in this thesis is a prime example. The 793 Kb BP QTL contains two novel genes that have no known function.
Their newly found link to a BP QTL can form the basis of a thorough investigation into their structure and function. The sequencing of the rat, mouse and human genomes has greatly facilitated the discovery of novel genes and greatly helped researchers utilizing linkage analysis and congenic strains make significant progress in gene identification for quantitative traits.

**Availability of Genome Sequence Resources**

Despite the widespread availability of genomic sequence information on databases such as NCBI, RGD, Ensembl and UCSC genome browser (http://genome.ucsc.edu), the accuracy of the genome assembly as well as gene predictions is questionable and in need of modifications. There have been a number of inaccuracies that added additional hurdles to the research conducted for this thesis. The QTL location is incorrectly placed on RNO17 in the available genome assemblies. A close look at the available genotype data and the microsatellite markers tested in this work correctly place the QTL on RNO1 instead of RNO17. The coordinates of the relevant markers are given in Table III. The 793 Kb region is correctly located within the region spanned by RNO1 markers D1Rat211 and D1Rat12.

In the event that discrepancies are found across databases, it is reasonable to utilize the information available on multiple sites and combine them to obtain information about the true prediction. With respect to the discrepancies in gene predictions for *Adamts16* (Figure 7), it was not until primers were designed to accommodate predictions on both NCBI and ENSEMBL that a full-length gene transcript was identified and biological evidence was obtained for the tissue specific expression of *Adamts16* (Figure 9). Recently the most updated version of the *Adamts16* prediction at
the NCBI rat genome resources website (Build 4.1) is in accordance with the results in this thesis. Periodic checking of databases ensures that information about any changes being made in predictions and sequences can be utilized. An example of this can be seen from the genes with the 793 Kb QTL. A portion of the predicted gene LOC502107 was initially predicted on NCBI to be within the genomic region of interest however the record was discontinued at a later date. This partially explained the inability to obtain evidence for the existence of a transcript.
Table III: Microsatellite Marker Locations on RNO1

<table>
<thead>
<tr>
<th>Markers</th>
<th>Location on RNO17 (Mb)</th>
<th>Location on RNO1</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1Rat7</td>
<td>15999443-15999615</td>
<td></td>
</tr>
<tr>
<td>D1Rat153</td>
<td>21229309-21229515</td>
<td></td>
</tr>
<tr>
<td>D1Rat121</td>
<td>21288065-21288167</td>
<td></td>
</tr>
<tr>
<td>D1Got33</td>
<td>26956572-26956707</td>
<td></td>
</tr>
<tr>
<td>D1Got35</td>
<td>27326922-27327075</td>
<td></td>
</tr>
<tr>
<td>D1Mco4</td>
<td>28864486-28864664</td>
<td></td>
</tr>
<tr>
<td>D1Rat211</td>
<td>1147797-1148037</td>
<td>29841429-29841642</td>
</tr>
<tr>
<td>D1Rat239</td>
<td>1910351-1910478</td>
<td></td>
</tr>
<tr>
<td>D1Mco86/SD43</td>
<td>2385815-2386002</td>
<td></td>
</tr>
<tr>
<td>D1Mco83/SD44</td>
<td>2398101-2398245</td>
<td></td>
</tr>
<tr>
<td>D1Rat14</td>
<td>2480947-2481129</td>
<td></td>
</tr>
<tr>
<td>SD 14</td>
<td>2562567-2562722</td>
<td></td>
</tr>
<tr>
<td>SD 17</td>
<td>2590644-2590812</td>
<td></td>
</tr>
<tr>
<td>SD 71</td>
<td>3079587-3079713</td>
<td></td>
</tr>
<tr>
<td>SD 79</td>
<td>3129707-3129826</td>
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<td>SD81</td>
<td>3155177-3155327</td>
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<tr>
<td>D1Mco82</td>
<td>2381682-2382055</td>
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<tr>
<td>D1Arb5</td>
<td>3472897-3473079</td>
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<tr>
<td>D1Rat12</td>
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<tr>
<td>D1Mco8</td>
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<td>D1Arb4</td>
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<td>D1Rat11</td>
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<td>D1Got40</td>
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<td>D1Got46</td>
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</tr>
<tr>
<td>D1Rat18</td>
<td>43747375-43747532</td>
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</tr>
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</table>

The physical map locations of microsatellite markers are given in Megabases (Mb). These markers are linked to RNO1 by genetic linkage analysis for BP using an F2(S x LEW) population. Information about these markers is available at the website: (http://hsc.utoledo.edu/depts./physiology/research/rat/marker.html). The physical map locations of each marker were obtained from the rat genome sequence assembly at the Ensembl and NCBI websites (www.ensembl.org, www.ncbi.nlm.nih.org).
Genes within the 793 Kb BP QTL

A genomic interval in humans and/or rodents of 1-3 Mb in size typically has anywhere from 10-30 genes (Gibbs, Weinstock et al. 2004; Hancock 2004). Therefore, it is unusual for a region the size of 793 Kb to have only two genes. Interestingly, HSA5 which is homologous to this region on RNO1 is heavily populated with gene deserts, large DNA tracts that are devoid of protein coding genes (Ovcharenko, Loots et al. 2005). One of the predicted genes within the 793 Kb segment, LOC306665 had no coding sequence variations between S and LEW and a full-length transcript could not be obtained. The lack of coding sequence variation does not exclude a role for the gene in BP regulation. A possibility is that the gene could be differentially expressed in one or more organs involved in BP regulation. Also the expression levels and patterns of the gene in various organs could be dependent upon the phenotypic state of the animal (i.e. normotensive, pre-hypertensive, hypertensive, severely hypertensive).

The second and perhaps more interesting candidate BP gene in the 793 Kb interval belongs to a family of genes that have a wide variety of biological functions. \textit{Adamts16} has no known function however a look at its structure compared to other ADAMTS family members can provide clues into how Adamts16 could be involved in maintaining BP at a molecular level. ADAMTS proteins have the following conserved domains: signal peptide, pro-peptide, metalloprotease, disintegrin-like, spacer domains and their signature Thrombospondin type 1 Sequence Repeat (TSR) motifs. The domains present in Adamts16 are shown in Figure 6.

The structural properties of ADAMTS genes are common in extracellular matrix proteins (Apte 2004; Porter, Clark et al. 2005). Thus, there is speculation that all of the
ADAMTS16 proteins have a role in the proper structure and functioning of the extracellular matrix, a defining feature of connective tissue (Apte 2004; Jones and Riley 2005; Malemud 2006). Extracellular matrix constitution consists of structural proteins and glycoproteins, including collagens, fibronectins, and proteoglycans. Increases in the production of extracellular matrix proteins and changes in their degradation have been associated with EH (Gibbons 1995; Jacob, Badier-Commander et al. 2001; Risler, Cruzado et al. 2005). In particular, the involvement of RAS and other processes in BP regulation may play an important role in the formation of vascular sclerosis, cardiac dysfunction, and renal damage (Cowley 2006). A gradual increase in BP, results in cumulative changes to tissue structure and function, and can eventually result in end-organ damage. A prominent pathological signature of tissue injury due to increased BP is tissue fibrosis which leads to reductions in tissue compliance and function (Struthers and MacDonald 2004; Cowley 2006). Fibrosis (or sclerosis) is a product of alterations in the extracellular matrix (Struthers and MacDonald 2004; Brown 2005). Delineating the mechanisms and creating treatment interventions to prevent or reverse these defects are of great clinical significance.

The results of this study suggest a possible role for *Adamts16* in BP. The change from a serine to proline (S to LEW) at amino acid position 274 could have influences on the tertiary structure of the protein. Moreover, this variation is at a potential furin cleavage site of the protein. The proline side group could cause steric hindrance and affect the cleavage of the protein by furin in one strain compared to the other, and thereby, through yet unidentified mechanisms, result in the observed differences in BP between the two strains. Furin, being a convertase enzyme, is known to cleave proteins in
an inactive to an active state. Improper cleavage of Adamts16 could have deleterious functional affects. Adamts16 may also have metalloprotease activity, which could be affected due to the amino acid variants in S and LEW. The second change at position 1170 from serine to threonine (S to LEW) is a conservative change. Both amino acids have hydroxyl side groups so the way in which this amino acid change could influence protein function is questionable.

An upcoming challenge is identifying and characterizing a function for *Adamts16* at the molecular level in the etiology and pathogenesis of hypertension. It is interesting to observe a relatively high expression of the transcript in the kidney (Figure 9). A significant body of research suggests that the BP effect of RNO1 genetic loci is mediated through the kidney (Rettig, Folberth et al. 1990; Rettig, Folberth et al. 1990; Rettig, Folberth et al. 1990; Guyton 1991; Guyton 1991; Churchill, Churchill et al. 2001; Clemitson, Pratt et al. 2002; Lo, Liu et al. 2002). Transplantation experiments as well as linkage analysis and substitution mapping studies provide support for the involvement of genes on RNO1 in the proper functioning of the kidney. The kidney’s ability to regulate salt and water balance in the body is essential for maintaining normal BP.

**RNO1 BP QTL and Salt**

During the initial selection of S rats from the outbred Sprague-Dawley rats, the process involved a dietary salt challenge (8% NaCl) (Iwai 1987(Dahl, Heine et al. 1962; Dahl, Heine et al. 1962; Rapp 1982)). The results from this study however, depict that the 793 Kb BP QTL is not salt dependent. Interestingly the average BP readings were significantly difference between S.LEW congenic substrains and S however the average BP measurements were slightly higher when the animals were on a high salt (2% NaCl)
diet compared to low salt (Table II). This observation coincides with the known effects of salt on elevating BP. The QTL effects not being influenced dramatically by salt implies that the alleles in the region may not have segregated during the original selection process. As noted in the literature review section, there is no BP QTL on RNO1 detected in the genetic linkage analysis of S (salt-sensitive) and R (salt resistant) rats. Both of these strains were derived from the same outbred Sprague-Dawley population. The LEW rat however was derived from the outbred Wistar population (www.rgd.org).

The presence of salt in daily food intake is an important factor that can cause increases in BP in the S rat. Irrespective of the presence of dietary salt, the S rat contains a genetic constitution that makes it prone to developing hypertension. An important question to consider is whether the coding, noncoding or a combination of nucleotide variants within the 793 Kb BP QTL are likely to be responsible for the BP differences between S and LEW.

**Noncoding Segments of the 793 Kb BP Regulatory Region**

It is estimated that approximately 97% of the human genome is composed of noncoding DNA. DNA that does not encode for proteins and resides in intergenic, promoter, intronic, and 5’ and 3’ untranslated regions is defined to be noncoding DNA. A great majority (84%) of the 793Kb genomic region is also composed of noncoding DNA making it extremely difficult to ignore. By identifying the presence of noncoding elements within the BP QTL, the first step is being taken to acknowledge that they may play a role in the susceptibility or resistance to hypertension. The mechanism by which they act is yet to be discovered. Variations in these regions could affect gene splicing, transcription, translation and gene expression. The results depict that this region is rich in
potential tfbs that are conserved in both rats and humans. Additionally, a number of SNPs have been documented in both mouse and humans.

The S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2) and S.LEW(D1MCO4X1X3BX2X1) congenic substrains, which have been developed, will help provide evidence for the role of noncoding DNA sequence in regulating BP. In particular, S.LEW(D1MCO4X1X3BX1X2) and S.LEW(D1MCO4X1X3BX2X1) have potential QTL segments that contain only noncoding segments. If one of the two strains has a significant BP lowering effect compared to the S rat, it will be the first report of a congenic rat with noncoding sequence being the genetic element controlling BP.

**The Search Continues**

The identification of novel genes, which regulate BP, will help in the development of drugs, which can be used for the management of EH. The generation of congenic substrains provides a reductionist approach towards identifying novel genes. However, a great limitation in the use of congenic strains is that the region in which a recombination occurs or the frequency of recombination cannot be manipulated. Therefore, when a QTL has been narrowed to a region <1Mb it becomes difficult to generate congenic substrains. Theoretically, the recombination fraction for a 1 centiMorgan (cM) (~2000 Kb=2 Mb) region in rodents is 1% (Ritter, Gebhardt et al. 1990). In the present study, 2,652 F2 animals were screened to obtain a recombination in a 793 Kb region. Only three recombinant congenic strains were obtained giving a recombination fraction of only 0.11%. This process requires the screening of large number of animals and may not always be the most convenient approach. However, once
made, the decision to use congenic strains to identify genes is unique and allows for the identification and characterization of novel genetic elements in human BP regulation.

Congenic strains are not manipulated in anyway by molecular biology or genetic engineering and therefore make an ideal model to study how a particular gene or genomic segment affects the phenotype of interest on a fixed homogeneous genetic background. Once the limit of resolution has been obtained for a QTL, a great amount of information can be obtained regarding the genetic control of the trait of interest. In the case of the 793 Kb BP QTL, data suggests that not only are there two novel genes that could play a role in BP related physiological mechanisms but noncoding segments of the genome may indeed have a significant role in maintaining normal levels of BP as well. The results obtained from the phenotypic characterization of S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2 ) and S.LEW(D1MCO4X1X3BX2X1) will provide evidence for a possible role of noncoding DNA in BP regulation. More widespread research on the role of noncoding segments of the genome in biological processes is needed to discover information about their function.
SUMMARY

- A blood pressure regulatory region, representing <0.027% of the rat genome, has been localized to 793 Kb of the rat genome. The homologous region on human chromosome 5 has also been linked to BP.

- Congenic rat strains, S.LEW(D1MCO4X1X3BX1) and S.LEW(D1MCO4X1X3BX2) have normotensive LEW alleles in a 793 Kb region, which confers a lowering of BP effect on the genetic background of the hypertensive S rat.

- There are two predicted genes within the region: LOC306665 and Adamts16. A full-length transcript has been identified for Adamts16. Gene expression has been detected in various organs important in BP regulation.

- Two nucleotide variations between S and LEW in coding regions of the QTL have been identified that result in amino acid changes. Both of these reside in the Adamts16 protein. However, coding sequence accounts for <2% of the region.

- Analysis of noncoding segments has identified 794 conserved transcription factor-binding sites between rats and humans. A number of SNPs are cataloged in homologous regions of mouse and human genomes, however SNP information is limited in the rat.

- A total of three new congenic substrains S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2) and S.LEW(D1MCO4X1X3BX2X1) have been developed and will be tested for BP compared to the S rat.
CONCLUSIONS

1) In the current model using S.LEW congenic strains for RNO1 BP QTL2, genetic differences within a 793 Kb region are responsible for the susceptibility of the S rat and resistance of the LEW rat to hypertension. The role of the homologous region on human chromosome 5 in BP regulation should be further investigated.

2) The study of the structure and function of ADAMTS16 should be investigated in relation to BP regulation.

3) The possibility of noncoding regulatory sequences in regulating BP should not be overlooked. The effects of noncoding sequences are not readily apparent and require thorough analysis and investigation.
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ABSTRACT

The etiology of essential hypertension (EH) is greatly impacted by individual genetic constitution. This provides the rationale to identify genes that have a role in BP regulation. Human studies are confounded by genetic heterogeneity making it difficult to conclusively identify the underlying genetic causes of EH. Alternatively, by using homozygous inbred rat strains, genetic variability and environmental influences are minimized. The Dahl salt-sensitive (S) and Lewis (LEW) inbred rat strains differ in their susceptibility to hypertension. To identify the genomic region accounting for the contrasting BP phenotype, congenic strains (S.LEW) was generated and tested for BP. S.LEW refers to the incorporation of a segment of the LEW rat genome (normotensive donor strain) into the S rat (hypertensive recipient strain) genetic background. The presence of LEW alleles lowered BP significantly in two S.LEW congenic rat strains compared to the S. These results implicate a locus on rat chromosome 1, spanning 793 kilobases (Kb), in BP regulation. This research has focused on identifying and characterizing the genetic differences between S and LEW within this region. This has been accomplished by 1) Fine resolution substitution mapping: Screening a large F2 population (n=2652) for meiotic recombination, within the 793 Kb interval, resulted in three congenic substrains: S.LEW(D1MCO4X1X3BX1X1), S.LEW(D1MCO4X1X3BX1X2 ) and S.LEW(D1MCO4X1X3BX2X1). BP measurements of these strains are anticipated to provide in vivo evidence for the further localization of crucial BP regulating segments. 2) Characterizing coding sequence variations: Coding sequence analysis of the two predicted genes within the 793 Kb region
revealed two naturally occurring amino acid changes in \textit{LOC306664 (Adamts16)} between S and LEW and 3) Utilizing \textit{in silico} analysis to detection segments within the 793 Kb region that contain regulatory elements and noncoding conserved sequences which do not encode for protein. The identification of genetic elements controlling BP can open new avenues for the treatment and management of EH.