DIFFERENTIAL ACTIVITIES OF MULTIPLE Sry PROTEINS ENCODED ON THE RAT Y CHROMOSOME

A dissertation submitted to Kent State University in cooperation with The University of Akron and North Eastern Ohio Universities College of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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December, 2008
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I would like to begin by thanking and recognizing the many individuals who have provided both the opportunity and support essential for completion of my doctoral degree. The research and experiments presented in this dissertation represent not only the work conducted by myself, but also an incalculable amount of support regularly provided by those who surrounded me, specifically my mentor Dr. Milsted and co-advisors Dr. Turner, Dr. Ely and Dr. Londraville. Direction and encouragement provided from these individuals, especially Dr. Milsted, fostered an environment that paved a road to my academic self-discovery. Completion of this work would have surly terminated with few successes without their collective advisement. Additionally, I would like to recognize my friends and colleagues, specifically, Dr. Jonathan Toot, Jeff Dunmire, Dr. Cathy Jenkins, Gail Dunphy, and Shannon Boehme. With Cathy, Jeff and Jonathan I spent hours in discussion that resulted in an experimental synergy which I believe enhanced all of our understanding and enthusiasm for our respective projects. Further, without the ever-present, day-to-day help and support provided by Gail and Shannon I think everyone in the lab would likely be less productive and mentally stable.

In addition to the individuals above I must recognize the most important factor that contributed to the success and timely completion of this work, my wife Eileen and children Cora and Connor. Without their support, understanding and continuous encouragement I may not have completed this work. Thank you everyone and I will most certainly see you in the future.
CHAPTER I

INTRODUCTION

Background and Purpose

*Sry* is the Y chromosome linked locus identified as the testis determination factor (Koopman *et al.* 1990). The gene encodes a transcription factor with a 79-80 amino acid high mobility group (HMG) box DNA binding domain that is highly conserved across mammalian species and essential for initiation of male gonadal development during early embryogenesis. Since the discovery of Sry nearly two decades ago, very little has been learned about exact mechanisms by which this protein functions as the “master switch” necessary for male organogenesis in many mammalian species (Koopman *et al.* 1991, Goodfellow & Lovell-Badge 1993). This is due in part to the fact that within the mammalian genome, *Sry* is one of few functional loci on the Y chromosome in a region that is not involved in genetic recombination, thus *Sry* is predisposed to rapidly acquire mutations (Graves 2002). Between species, regions outside the conserved HMG domain are quite variable, which has confounded research focused on identifying consistent or set regulatory and interactive mechanisms essential for function (Harley & Goodfellow 1994, Harley *et al.* 2003a). Indeed, while the identification of upstream regulators, downstream targets and potentially other cellular activities has been minimal, advancements have been achieved in understanding many biochemical and protein structural features required for function.
While most research has focused on delineating the molecular genetic functions of Sry during a brief window of expression at the genital ridge at approximately 11.5 days post coitum in mouse (~16 days in human), an increasing number of reports suggest that Sry may have additional functions beyond testis determination. These alternative roles, which include male-specific differentiation of the brain (Dewing et al. 2006), direct regulation of androgen receptor activity (Yuan et al. 2001) and male pattern baldness (Chen et al. 2007, Neves et al. 2007), all occur in postnatal and adult tissues, implying that Sry also contributes to the development and maintenance of male-specific physiology and secondary characteristics.

In accordance with these studies, is the link between Sry and hypertension in the Spontaneously Hypertensive rat/University of Akron colony (SHR/Akr) described by our group (Ely et al. 1993, Ely et al. 2007). While this research has contributed much to the understanding of blood pressure differences between the male and female phenotype, it also affords us the opportunity to examine closely the unique biochemical features of Sry that contribute to function. The most significant advantage we have is the fact that the rat Y chromosome encodes multiple functional Sry loci, while most species, such as human and mouse (which are the two species primarily used in Sry research) possess one locus. Recently Turner et al. (Turner et al. 2007) described the presence of six full length Sry loci (Sry1, Sry2, Sry3, Sry3B, Sry3B1 and Sry3C) on the SHR/Akr Y chromosome and their differential tissue-specific expression. From this work, we have learned that all identified Sry loci are expressed in vivo and that Sry2 transcripts consistently represent the majority of total transcripts in all tissues. However, the relative abundance of
transcripts generated from the remaining loci, Sry1 and the Sry3s, differs among tissues, and these shifts in transcript abundance may indicate that the proteins generated from these loci are involved in tissue specific functions.

In addition to these expression studies, we have shown that functional differences among the rat Sry proteins exist from electroporation studies where Sry1 and Sry2 expression constructs, encoding only the protein coding region, were electroporated into normotensive WKY rats (Ely et al. 2007). In these experiments Sry1 but not Sry2 increased blood pressure and tyrosine hydroxylase activity. Significantly, these results demonstrate that in vivo Sry1 and Sry2 proteins have different activities.

The research presented in this dissertation analyses the slight amino acid (aa) differences among the rat Sry proteins to determine if these changes confer altered activity at the level of the protein. The overall hypothesis to explain these functional differences is that Sry protein effects are controlled by a combination of differential levels of transcript expression and sequence variation among the proteins. In this study, I tested the hypothesis that the multiple Sry proteins encoded on the rat Y chromosome differentially activate target genes and localize differently in cells, and that these conditions are a reflection of their slightly different amino acid sequences. Additionally, I examine potential sites within the Sry protein sequence that may act as locations for posttranslational modifications. Two covalent modifications are examined. The first is phosphorylation, which in human SRY is shown to modulate SRY – DNA interactions. The second is the potential interaction of Sry with members of the small ubiquitin-like modifier (SUMO) protein family.
This introduction will present a compendium of the current research and understanding of Sry structure and function.

**Specific Aims**

**Specific Aim I:** Determine the effects that making changes to an N-terminal nuclear localization signal (nNLS) and polyglutamine (Q-rich) activation domain, have on nuclear localization.

**Hypothesis:** the hypothesis to be tested is that Sry proteins containing the weakly basic amino acid (aa) histidine (H) in the nNLS will exhibit reduced nuclear import compared to proteins with the highly basic aa arginine (R) at the same position and that the Q-rich activation domain will have little effect on nuclear localization.

**Specific Aim II:** Determine the effects of amino acid differences between Sry1/3 and Sry 2 at position 21 (arginine, R vs. histidine, H), amino acids within the nNLS, on regulating activity of a synthetic Sry-inducible luciferase reporter (pGL3/AR600) construct.

**Hypothesis:** Since the nNLS is part of the HMG box DNA binding domain, aa changes could alter protein function, and because at this position an R is conserved in most species, the presence of H will alter local protein architecture and reduce protein activity.
**Specific Aim III:** Determine the effects of modifying/deleting the Q-rich potential activation domain in Sry1, 2, 3 on activating the pGL3/AR600 luciferase reporter.

**Hypothesis:** The hypothesis to be tested is that due to a 13 aa truncation in the Q-rich motif, Sry2 proteins will exhibit reduced activity compared to Sry1 and 3, which have a longer 25 aa Q-rich region, and inclusion of this truncation or removal of this motif in Sry1 or 3 will reduce activity of these proteins.

**Specific Aim IV:** Determine if Sry proteins are posttranslationally modified by small ubiquitin like modifiers (SUMO)1, 2 and 3, and characterize Sry phosphorylation state using anti-phosphoserine and anti-phosphothreonine antibodies.

**Hypothesis:** All rat Sry proteins have potential consensus SUMO and kinase modification sites, which allow posttranslational modification of Sry proteins via sumoylation and phosphorylation.

Determining if native and mutated Sry proteins can translocate to the nucleus and transactivate a reporter construct will enhance our understanding of the biochemical and intracellular activities that may contribute to the functional variability observed *in vivo* between Sry proteins. The fact that the rat Y chromosome contains multiple functional Sry loci that exhibit differential spatial -temporal and expression level profiles affords the opportunity to examine what slight differences in aa composition among proteins confer to function. In this dissertation, I present a series of experiments that show the rat Y chromosome and the multiple Sry proteins it encodes are an invaluable tool for the characterization and discovery of Sry functions in species with one locus. In these
studies, I demonstrate that the cellular activities and localization patterns among Sry1, 2, and 3 proteins are different and suggest what these variations could indicate about the biochemical relevance of each protein in vivo.

The Y Chromosome

The determination of male phenotype is dictated by the presence of a Y chromosome and often the expression of loci such as Sry in eutherian mammals (Koopman et al. 1990, Koopman et al. 1991, Skaletsky et al. 2003). The Y chromosome is divided into two regions, the pseudoautosomal region (PAR) and non-recombining, male-specific region of the Y (MSY). This division is unique because all regions but the PAR, located at the Y chromosome telomeric regions, are not involved in crossover with the X chromosome during meiosis (Graves 1998, Charchar et al. 2003). Thus, loci within the MSY are not maintained by traditional recombination events.

Until sequencing of the human Y chromosome was completed, the evolution of the Y chromosome was believed to occur solely through the addition and attrition of X-linked and autosomal loci, resulting in maintenance of only a few functional loci linked to gender-specific developmental pathways. However, this is not the case. The history and maintenance of the loci on Y chromosome is far more complex than initially hypothesized (Graves & Erickson 1995). Research by Skaletsky et al. (Skaletsky et al. 2003) found that the MSY is divided into three sequence classes, the X-transposed, X-degenerate and ampliconic regions, which are characterized on the basis of organization and evolutionary history. Of these three classes, the most recent addition to the Y
chromosome’s MSY is thought to be the X-transposed region. On the human Y chromosome this region contains only two genes, TGIF2LY (TGF β-induced transcription factor 2- like Y) and PCDH11Y (Protocadherin 11 Y), which were transposed from the X, and are surrounded by many repeat elements.

The two largest sub-regions of the MSY are the X-degenerate and the ampliconic regions, which are thought to have evolved together early in Y chromosome history. Together these regions contain the most loci and fewest number of interspersed repeat elements. The ampliconic region accounts for approximately 25% of MSY sequence and is structurally organized into eight palindromes. Structurally the palindromes contain two arms that extend from a centrally located spacer sequence. Opposite arms contain near identical gene copies which interact with one another and recombine, thus maintaining the integrity of genes within this region intrachromosomally. Genes located within the ampliconic palindromes are expressed predominantly in the testis, and are believed to play an important role in spermatogenesis.

The final MSY region is the X-degenerate. This portion of the MSY contains functional and nonfunctional pseudogenes with high homology to X-linked loci from which they originated. Unlike the ampliconic division, this region is not organized as palindromes, thus loci are prone to mutation and degradation. Interestingly, the Sry locus, which is hypothesized to be a degenerate Y-linked homologue of the X-linked SOX3 (Sry box containing protein 3) locus, is found in this region (Graves 1998).
The Sry Protein

*Sry* (Sex-determining region of the Y chromosome), is an intronless gene that encodes a protein with a highly conserved 79-80 aa High Mobility Group (HMG) box DNA binding domain that shares homology with members of the HMG-1/HMG-2 protein families (Werner *et al.* 1995b). However, unlike members of these two families, *in vitro* Sry interacts with DNA in a sequence specific manner with a high binding affinity ($K_d \sim 10^{-9} – 10^{-10}$ nM) (Harley *et al.* 1992). Because Sry-DNA interactions occur at the minor groove rather than the major groove, it is likely that this interaction would not interfere sterically with protein-DNA interactions of other nearby DNA binding proteins. This supports the idea that Sry interacts with DNA as a complex, with a partner or with other cofactors *in vivo*. Identification of a preferred high affinity response element targeted by Sry *in vitro* (ACAATAG) (Harley *et al.* 1994), has not aided in the continued hunt for downstream targets, since this consensus is extremely common in promoter regions throughout the mammalian genome. Sry binding as a complex to response elements containing this consensus, or some variant, and perhaps a yet to be identified adjacent response element, or elements, dependent on the resulting architecture of different complexes or binding partners, may be one reason no definitive direct DNA target for Sry has been recognized or reported *in vivo*. However, evidence suggests the Sry protein likely interacts with and regulates expression of the developmental genes: SOX 1, 3, 9; DAX 1; and Mullerian Inhibiting Substance (MIS) (Goodfellow & Lovell-Badge 1993, Graves 1998).
Sry is founder of the HMG box containing (SOX) protein family, which is estimated to contain at least 20 members (Wilson & Dearden 2008). All SOX proteins contain an HMG domain that shares great sequence identity to that of Sry and many of these proteins can interact at Sry response elements with comparable binding affinities (Harley 2002). During testicular development Sry is not the only HMG box protein expressed and necessary at the genital ridge during the time of male organogenesis. Many SOX proteins, such as SOX 9 and 3 are also present (Clarkson & Harley 2002, Lovell-Badge et al. 2002). The formation of a complex, or synergistic DNA binding with a partner protein, could increase Sry binding specificity in a sea of very similar SOX proteins.

Structurally the HMG box is composed of three α helixes that form a tertiary organization that resembles a twisted or curved triangle (Werner et al. 1995a, Werner et al. 1995b). The resulting tertiary structure occurs due to the N-terminal portion of helix 1 contacting the C-terminal region of helix 3. This association occurs at what would be the apex of the triangle. Therefore, helix 1 and 3 form the body of the triangle, while most of helix 2 forms what would be the base (Figure. 1.1). Thus, the protein regions flanking the HMG domain are likely in close proximity; however currently there are only crystal structures of the HMG box. In this conformation, the Sry HMG domain facilitates DNA binding and bending. After interacting with a target DNA consensus, Sry induces a characteristic species-specific 65-80° bend (Werner et al. 1996), thus classifying Sry as an architectural transcription factor. DNA distortion is thought to occur when the base of the triangle (helix 2) interacts with the minor groove. This minor groove interaction
stabilizes Sry and allows the sides and apex of the triangle (helix 1 and 3) to act as a cantilever that compresses the DNA, inducing a bend. Alteration of DNA structure is hypothesized to enhance interactions with additional DNA binding proteins at the bend site and/or to promote interactions between distant factors positions on opposite sides of the Sry mediated kink, which could alter transcription of target genes (Pontiggia et al. 1994, Li et al. 2006, Phillips et al. 2006).

DNA binding and bending carried out by Sry is by no means the end of the HMG box story. Because Sry is a transcription factor, nuclear localization is essential. This process is facilitated by two basic aa regions (composed of primarily R and K residues) that flank the HMG box and function independently as nuclear localization sequences, the N-terminal NLS (nNLS) and C-terminal NLS (cNLS) (Sudbeck & Scherer 1997). Based on aa organization in protein primary structure, the nNLS exhibits bipartite or split organization and the cNLS is classified as a monopartite or basic cluster NLS (Boulikas 1993). Bipartite indicates the nNLS is composed of two separated aa modules that come together forming a complete NLS upon generation of protein secondary structure, while monopartite indicates the NLS is simple a stretch of basic residues. Research indicates that both NLSs are necessary for testis development to occur (Sudbeck & Scherer 1997). The current hypothesis is that while one NLS can facilitate some level of nuclear import, both are required to achieve a threshold Sry concentration within a narrow timeframe when Sry in need to initiate the male developmental cascade. Interestingly, acetylation of a K residue near the cNLS is implicated in the modulation of Sry nuclear translocation
Figure 1.1. The Sry HMG box DNA binding domain is composed of three α helixes that fold into a tertiary structure that resembles a curved or bent triangle (Werner et al. 1995b). Two nuclear localization signals are situated at the N and C-terminal extremities of the HMG box (green regions). Two aa’s C-terminal to the cNLS is an acetylation site that enhances nuclear import (Thevenet et al. 2004). In rat and mouse, Sry contains a C-terminal Q-rich activation motif not present in human.
(Thevenet et al. 2004). In this study, deacetylation of Sry resulted in perinuclear cytoplasmic Sry localization and upon acetylation Sry proteins exhibited complete nuclear accumulation. While both NLSs are needed for testicular development, the current pathways thought to mediate Sry nuclear translocation via the nNLS or cNLS are believed to be different.

The “classic” model to explain translocation of nuclear targeted proteins through the nuclear pore complex (NPC) depends on the maintenance of a steep RanGTP concentration gradient across the nuclear envelope (Gorlich & Mattaj 1996). The activities of the nuclear protein RanGEF (guanine exchange factor) and the cytoplasmic protein RanGAP (GTPase activating protein) maintain this gradient. In this model nuclear transport of NLS containing proteins is facilitated through interaction with the nuclear import receptors importin α and β (Imp α / β). In the cytoplasm, Imp α interacts directly with the NLS containing cargo protein, while Imp β binds Imp α and directs interactions with regions on the NPC, facilitating nuclear import (Boulikas 1996, Hodel et al. 2001). Once in the nucleus, RanGEF facilitates the release of cargo proteins from the importins by generating RanGTP, which binds to Imp β disrupting the complex and releasing the cargo. The RanGTP bound Imp β is then cycled back to the cytoplasm, directed by RanGTP moving down its concentration gradient (Gorlich et al. 1996, Gorlich et al. 2003). In the cytoplasm, RanGAP hydrolyzes the GTP bound by RanGTP to GDP. This induces a conformational change in the Ran protein stimulating the release of bound Imp β to the cytoplasm where it can interact with another Imp α bound cargo protein.
The cNLS of Sry is organized as a cluster of up to five basic amino acids and is structurally similar to the NLSs first identified in SV40 large T antigen (Kalderon et al. 1984). While the aa consensus of the Sry cNLS is similar to the NLS in SV40 large T antigen, nuclear import has been shown to follow a mechanism facilitated through direct interaction with Imp β (Forwood et al. 2001, Forwood et al. 2007). This direct interaction of Sry with Imp β is important because recent reports suggest elevated Imp β concentrations are linked to increased transport efficiency and import rate of directly targeted cargo proteins (Yang & Musser 2006). Therefore, Sry import speed could be modulated by Imp β concentration.

The bipartite nNLS of Sry has been shown to not interact with importin α or β in vitro (Harley 2002, Sim et al. 2005). Alternatively, the current model suggests the nNLS is part of an extended aa consensus that includes regions of the first α helix that interact with calmodulin in an ATP and Ca\textsuperscript{2+} dependent manner, facilitating nuclear import using a Ran-GTP independent pathway (Harley et al. 1996, Sweitzer & Hanover 1996, Sim et al. 2005). While this proposed method of nuclear import is not well understood, directional transport from the cytoplasmic compartment to the nucleus is thought to be driven by DNA concentration and binding (Hanover et al. 2007). Studies of NPC permeability support many aspects that would be required for this alternative import pathway to work. Briefly, recent reports suggest NPC architecture and thus permeability is directly linked to the concentration of cisternal Ca\textsuperscript{2+} within the nuclear envelope and surrounding NPC microenvironment. Significantly, the release of Ca\textsuperscript{2+} sequestered in the nuclear envelope is shown to be mediated through activation of nuclear inositol
triphosphate (IP$_3$) receptors (Moore-Nichols et al. 2002). Therefore, nuclear localization directed by interactions with Ca$^{2+}$ activated calmodulin, could possibly link Sry nuclear translocation to many signal transduction pathways.

While the concept of signal mediated regulation of Sry nuclear import is quite attractive, many questions pertaining to this unusual calmodulin regulated pathway remain unanswered, and multiple conditions exist that oppose this type of mechanism. (1) Analysis of a mutation in human Sry, occurring in the spacer region between the two basic modules of the nNLS (Figure 1.2 and 1.3), has been shown to reduce nuclear import, but not Sry DNA binding or bending. This condition of reduced nuclear accumulation resulted in abnormal testis development in the affected patient. However, the Sry-calmodulin interaction was determined to be normal (Harley et al. 2003b, Li et al. 2006). This suggests that calmodulin directed nuclear import may contribute less to Sry translocation in vivo than predicted by studies completed in vitro. (2) Considering the architecture of the HMG box, both NLSs are positioned in close proximity of each other, at the apex of the triangle. If nuclear import is facilitated through two separate, potentially competing mechanisms (Imp β and calmodulin), and both NLSs are required for most efficient nuclear translocation, then this raises the question of which mechanism is most responsible for nuclear import? It is predicted that calmodulin binding encompasses all of the Sry nNLS and at least half of α helix 1. Since in the tertiary structure of Sry, aa’s near the nNLS and cNLS interact, calmodulin could/would effectively hinder importin β binding at the cNLS. (3) Additionally, many of the aa’s predicted to be part of the calmodulin binding site in human Sry are not present in rodent
due to the lack of an N-terminal HMG box flanking region (Figure 1.2). Therefore, while a calmodulin mediated nuclear localization mechanism is promising, many questions still need to be answered and much research still needs to be conducted.

Mutation analysis of human SRY has highlighted the importance of aa conservation within the HMG box, as aa substitutions within this region are linked to XY gonadal dysgenesis often associated with reduced DNA binding and/or bending (Li et al. 2001, Mitchell & Harley 2002). While the HMG domain facilitates DNA binding, DNA bending and directs cytoplasmic Sry to the nucleus, this domain is also implicated in modulating the duration of the Sry-DNA complex (Ukiyama et al. 2001, Phillips et al. 2006). This function is conducted by a region of basic aa at the C-terminus of the HMG box, which includes the cNLS and acts as a kinetic clamp. Mutations to this region, resulting in human sex reversal where DNA binding and bending were not affected lead to the discovery of this important region. Since the Sry HMG box appears to encapsulate all functions necessary for Sry activity, many consider the HMG domain the only functionally relevant portion in this protein. This is due primarily to the fact that most sex-reversed phenotypes linked to Sry dysfunction are associated with mutations in the HMG box. Additionally, among species the regions flanking the this domain show little aa conservation. Therefore, most biochemical research conducted to identify Sry function has been with a truncated form of Sry representing only the HMG domain. Current research and the studies presented in this dissertation suggest that regions beyond the highly conserved HMG domain are just as critical to Sry function. Here I present results that support the idea of functionally relevant regions outside this domain. While
aa sequence is often not conserved in these flanking regions, cryptic regions of functional conservation are becoming evident.

Based on HMG box position, Sry proteins can be divided into two groups. Sry proteins with the HMG box located centrally or N-terminally (Fig. 1.2). In rodent species, the HMG box is located at the N-terminal extremity with only 2-4 aa flanking the HMG box. In all non-rodent species, the HMG box is located at the center of the protein. In these species the HMG box is flanked by 50-65 aa’s N-terminally and about 60 -65 aa’s C-terminally. In rodent Sry, there is no N-terminal flanking region. The significance of this region has not been determined, however S residue 33 in the human flanking region has been demonstrated to act as a PKA phosphorlyation site, which, when phosphorylated increases DNA binding affinity (Desclozeaux et al. 1998). This is the only phosphorylation site positively identified in human Sry. In most rodent species, the C-terminal region is divided into two sections, a polyglutamine (Q-rich) activation domain and a hinge region that links the HMG box to the Q-rich region. The C-terminal flanking region in non-rodent Sry only includes what is considered the hinge region in rodent Sry, as no Q-rich motif is conserved. This raises the question about the functional importance of the rodent polyglutamine region.

In mouse it has been demonstrated that the Q-rich motif is required for testis development and species specific protein function and is believed to facilitate protein-protein interactions with transcription modulators (Tucker & Lundrigan 1993, Miller et al. 1995, Bowles et al. 1999). In human Sry there is no Q-rich domain, however, recent
General Comparison of Structural and Functional Regions in Human, Rat and Mouse Sry Proteins

Figure 1.2. Among mammalian species, Sry exists in two general forms, those with the conserved HMG box (shaded orange) located in the center of the protein, as in human (A), and those with this region positioned at the N-terminus such as rat (B) and mouse (B). The HMG box is composed of three $\alpha$ helixes (gray blocks), two nuclear localization signals, the nNLS (red bars) and the cNLS (blue bar), and a C-terminal kinetic clamp (pink bar). Additionally, rodent Sry contains a C-terminal Q-rich activation motif (shaded yellow) that exhibits species-specific length. Both human and rodent Sry interact with SIP-1 (green bar) in the hinge region (orange bracket), which in human may facilitate roles conducted by the Q-rich region of rodent Sry. Further, Sry may act as a gene silencer through interactions with KRAB-O (purple bar) and it has been demonstrated that human Sry is phosphorylated (aqua bar) in the N-terminal HMG box flanking region not present in rodent species.
reports suggest an interaction with a PDZ domain protein termed, SRY interacting protein-1 (SIP-1) (Poulat et al. 1997). This protein-protein interaction is mediated through a small aa consensus at the C-terminal extremity of the human hinge region and is thought to facilitate interactions with transcription regulators in much the same way as the integrated activation region in mouse. While the presence of a Q-rich region and interactions with SIP-1 suggest Sry functions as an activator of transcription, early research using an Sry regulated reporter assay indicated that Sry can also function as a repressor (McElreavey et al. 1993). Recently, support for Sry transcriptional repression come from research that describes another protein-protein interaction via the hinge region with the protein KRAB-O (Oh & Lau 2005, Oh et al. 2006). This protein contains a Kruppel-associated box domain and unlike SIP-I, which associates with human Sry at the C-terminal extremity of the hinge region, interacts with an aa motif near the HMG box. Interestingly this same region interacts with SIP-I in mice and most likely other rodent species (Thevenet et al. 2005). KRAB-O is implicated with interactions to complexes containing KAP-I, which are linked to a gene silencing. The importance of this interaction is highlight by the fact that mutations in Sry that prevent interaction with KRAB-O result in XY sex reversal (Mitchell & Harley 2002).

Additionally, Sry is shown to directly bind to proteins such as Wilm’s tumor gene 1 (WT-1) (Matsuzawa-Watanabe et al. 2003) and androgen receptor (AR) (Yuan et al. 2001) through interactions mediated by the HMG box. Interestingly, the Sry-WT-1 complex is thought to enhance Sry activity by using the activation domain encoded by WT-1, which supports the concept of Sry binding DNA with a partner or as a complex,
while Sry-AR complex formation prevent AR interactions with target promoters. The direct repression of AR by Sry is implicated in reducing prostate cancer, as initiation of prostate cancer often shows reduced expression of Sry and other Y linked loci (Perinchery et al. 2000, Yuan et al. 2001). This direct interaction with AR, supports a role of Sry in maintaining adult male physiology.

While Sry is responsible for facilitating the switch from a bipotential gonad into testis (Koopman et al. 1991) it is becoming apparent that this is not the only function of Sry. Recently, Sry transcripts were identified in multiple tissues (heart, brain, kidney and adrenal gland) of adult male rats (Milsted et al. 2003). Additionally, research measuring Sry mRNA indicate unique expression profiles within testis, adrenal and kidney (Turner et al. 2007). Further, Mayer et al. (Mayer et al. 1998, Mayer et al. 2000) identified Sry transcripts in brain tissues of humans and mice. The adult expression of Sry in various tissues provides strong support for the possibility that Sry serves a role in the adult animal. Therefore, besides being involved with the development of gonads, Sry could be activating transcription of genes in other pathways. For example, Sry can increase tyrosine hydroxylase activity in vitro in PC12 cells (Milsted et al. 2004).

**Multiple Sry loci**

The Y chromosome of most eutherian mammalian species contain one Sry locus, however, in many rodent species the presence of more than one copy is not unusual. Using Southern Blot analysis the Y chromosome of certain members of the family Microtidae (vole) was determined to encode multiple Sry loci after hybridization with a
mouse HMG box probe (Bullejos et al. 1999). Interestingly, this lab also found that some species, such as Microtus cabrerae, have Sry loci in both males and females (Bullejos et al. 1997, Fernandez et al. 2002). However, due to point mutations that introduce early stop codons many of these loci were found to be nonfunctional. Using similar techniques, Nagamine (Nagamine 1994), identified multiple Sry copies in many Asian mouse and murid species, including Rattus norvegicus (the laboratory rat). Further, amplification of Sry from six African murid species, identified 2-4 Sry loci on the Y chromosome of each species examined (Lundrigan & Tucker 1997). Sequence analysis identified a conserved HMG-box, open reading frame, and evolving C-terminal region, which was thought to indicated that each copy could be functional. These results suggested that the duplication and maintenance of more than one functional copy could present some advantage over possession of a single copy, or that the additional copies could be involved in other functions.

Recently our group has described the presence of at least six Sry loci on the Y chromosome of a single laboratory rat (Rattus norvegicus) (Sry1, Sry2, Sry3, Sry3B, Sry3B1 and Sry3C), which is consistent with reports of copy number estimates in rat (Nagamine 1994, Martin 2002, Turner et al. 2007). Remarkably, recent unpublished results from our lab show the presence of a seventh locus (Sry3A) on the Y chromosome of SHR/Akr, but not WKY. Further, our lab has demonstrated that all loci are expressed in vivo and the coding regions from each locus can generate a full length potentially functional protein (Underwood 2003).
Current studies of gene duplication, suggest that duplicate genes may acquire new function more often than predicted (Wagner 1998). Research by Lynch and Force (Force et al. 1999, Lynch & Force 2000) hypothesize that if a gene with more than one function, in spatial and temporal terms, is duplicated, the resulting copies, through the accumulation of mutations to regulatory promoter elements and the coding region, may over time lose some of the functional characteristics exhibited by the parent gene. Thus only by maintaining the duplicated genes can all cellular functions be fulfilled. Therefore the multiple functions of the parental gene are divided between two or more duplicates, that eventually became specialized for that one parental function. Thus, due to subfunctionalization, duplicated genes are preserved. This suggests that the multiple, functional Sry loci of rat may be functional specialists in terms of expression and activity. Therefore the multiple Sry loci of rat may be an effective tool in the identification of Sry function during testicular development and in adult tissues.

Interestingly, the SHR/Akr Sry loci exhibit sequence differences comparable to those observed between Sry loci of different mouse genomes, all of which have a single Sry locus (Turner et al. 2007). Studies involving the use of transgenic mice have demonstrated that allelic variations between species contribute to expression level differences. These differences are reported to prevent or reduce the ability of Sry from one mouse species to induce testicular development in another species (Bowles et al. 1999, Nagamine et al. 1999). Interestingly, our lab has demonstrated, through fragment analysis, that the multiple loci of rat exhibit varying expression profiles in a single tissue
and that these profiles differ between adult tissues (Turner et al. 2007), which is consistent with expression studies in mice (Nagamine et al. 1999, Albrecht et al. 2003).

From consomic and transgenic studies in mice, it has been found that two independent factors contribute to a sex reversed phenotype; expression and protein variability. Recently an association between expression level and protein structure was shown (Albrecht et al. 2003). It has been demonstrated that mice with a truncated Q-rich domain exhibit elevated expression levels / transcript abundance compared to mice encoding Sry proteins with longer or full-length polyglutamine regions. One hypothesis to explain this phenomena is that Sry proteins with longer Q-rich domains are more efficient than shorter alleles, therefore lower titers of long Q-rich proteins are sufficient for effective initiation of testis (Lee & Taketo 2001). Interestingly, expression studies conducted in our lab with the multiple Sry loci of rat, have demonstrated that Sry2 is expressed in all tissues at higher relative transcript levels than all other loci. The Sry2 protein isoform also happens to be the shortest rat of the rat proteins and the decreased size comes from a 13 aa deletion in the rat Q-rich region.

The Rat Sry Proteins

The Sry proteins encoded on the rat Y chromosome all have the potential to express a functional protein (Underwood 2003). All are very similar. However, aa differences at regions of great functional significance, such as the nNLS, C-terminal kinetic clamp and Q-rich region, may indicate that in vivo each has a specialized
functional role. Based on primary aa sequence I have categorized these protein isoforms as Sry1, Sry2, and Sry3-like proteins.

The organization and aa sequences of rat and mouse Sry are quite similar up to the Q-rich region, which in rat is 12-26 aa in length compared to the up to 250 aa in some mice (Bowles et al. 1999). Excluding the Q-rich region, rat Sry2 shares greatest sequence identity with mouse proteins, with the most notable similarity being in an H residue at position 21 in the nNLS, while the remaining rat proteins encode a conserved R residue at this position (Figure 1.3). Additionally, rat Sry3-like proteins, which are more similar to each other than to Sry1 or 2, encode a T residue rather than a P at position 76, near the cNLS in the region thought to act as a kinetic clamp. The aa substitution is not conserved. All other mammalian species encode a P, thus this aa difference has the potential to alter the architecture of the C-terminal region of the HMG domain and perhaps Sry-DNA interaction. Among Sry3-like isoforms the remaining differences are located in the Q-rich region, however, there are no aa changes between Sry3 and Sry3C. The difference that defines Sry3 and Sry3C is located in the 5’ UTR. Sry1 shares features of both Sry2 and Sry3-like proteins. Like Sry3, Sry1 encodes an R at position 21, but similar to Sry2 contains a P at aa 76. Unique to Sry1 is an H vs. Q residue at position 38. Since this residue lies in the center of a helix 2, Sry1 proteins may interact with DNA differently.
Amino Acid Differences Among Rat, Mouse and Human Sry at the nNLS, cNLS, C-terminal Kinetic Clamp and Polyglutamine Motif

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Figure 1.3. The bipartite nNLS is composed of aa’s 6-8 and 20-22. At residue 21, rat Sry2 and mouse Sry contain an H (red) vs. a conserved R. At the cNLS (aa’s 73-79), rodent Sry encodes an H vs. R at residue 77 (red), all non-rodent sequences contain an R. Rat Sry3-like proteins encode a unique T vs. P at position 76, which may alter the architecture of the kinetic clamp (green region). The C-terminal Q-rich region (italics) of Sry2 contain a 13 aa deletion (--) and Sry3 differences (red). Gray shaded areas represent α helix 1, 2 and 3 in the underscored HMG box. Amino acids that interact with DNA are indicated with “+”. The acetylation site is highlighted yellow.
CHAPTER II

Differential Nuclear Localization and Characterization of Multiple Proteins Encoded on a Single Y Chromosome of *Rattus norvegicus*

Introduction

In most mammals, early expression of the Y-linked transcription factor *Sry* (sex-determining region Y chromosome) at the genital ridge is an essential signal for the onset of male gonadal genesis (Gubbay *et al.* 1990, Koopman *et al.* 1990). *Sry* is the founder of the SOX (Sry related box) protein family, all of which encode a conserved HMG box DNA binding domain. Based on the location of the HMG box, *Sry* proteins can be divided into two groups. In rodent species, the HMG box is located at the n-terminal extremity with only two amino acids (aa), often methionine and glutamic acid, flanking the first aa of this domain. Further, the C-terminal extremity in some rodent species, such as *Mus musculus* and *Rattus norvegicus*, consists of a polyglutamine (Q-rich) activation domain. In *Mus* the region is required for testis development and species-specific protein function (Tucker & Lundrigan 1993, Miller *et al.* 1995, Bowles *et al.* 1999). In non-rodent species, the HMG box is flanked by an N-terminal region, thus in these species this domain is found near the center of the protein. Additionally, non-rodent SRY proteins lack a C-terminal Q-rich activation domain. However, reports suggest that protein interaction with the PDZ domain protein, termed SRY interacting protein-1 (SIP-1), at
the same C-terminal region may compensate for the lack of an integrated Q-rich activation region (Poulat et al. 1997). Despite recent biochemical advances, the precise mechanisms, upstream regulators and downstream targets of Sry remain for the most part unidentified, which is consistent with Sry activities being mediated through a complex of interacting proteins rather than independently.

The HMG box facilitates two functions central to Sry activity, the first being sequence specific binding of linear DNA (Pontiggia et al. 1994) and the second being localization to the nucleus (Poulat et al. 1995, Harley et al. 1996, Sudbeck & Scherer 1997, Forwood et al. 2001, Li et al. 2001, Forwood et al. 2007). Sry - DNA interactions, unlike most DNA binding proteins, occur at the minor groove where a characteristic, species specific bend angle of about 70° is induced (Pontiggia et al. 1994, Werner et al. 1995a). Distortion of target DNA sequences is hypothesized to orient distal transcription regulatory elements that modulate activities of the preinitiation complex to facilitate mechanisms essential for normal testis development (Pontiggia et al. 1994, Phillips et al. 2006). Mutation analyses of human SRY have highlighted the importance of aa conservation within the HMG box. Substitutions in this region are linked to XY gonadal dysgenesis and are often associated with reduced DNA binding and/or bending. Despite the importance of Sry - DNA interactions, at least two mutations account for the generation of an XY female phenotype based solely on decreased Sry nuclear localization (Li et al. 2001, Plaseska-Karanfilska et al. 2007).

As with most transcription factors, Sry must efficiently crosses the nuclear pore complex (NPC) to fulfill its primary biological role as a modulator of transcription. This
process is facilitated by two nuclear localization signals (NLS) (Sudbeck & Scherer 1997). The structure of both NLSs imply that function would follow that of the classic importin α/β, Ran-dependent import pathway (Boulikas 1993, Strom & Weis 2001), however this is not the case.

The cNLS of Sry is organized as a cluster of basic amino acids similar in structure to that of the “classic” monopartite NLS first identified in SV40 large T antigen (Kalderon et al. 1984). While the consensus aa sequence of the Sry cNLS is similar to that of the NLS in SV40 large T antigen, nuclear import follows a mechanism facilitated through direct interaction with the nuclear import receptor importin β, rather than a mechanism utilizing importin α as an adapter for importin β interaction with the cargo (Forwood et al. 2001). This direct interaction with importin β could increase the rate of Sry nuclear import. Recently, elevated importin β concentration has been linked directly to increased target protein translocation to the nucleus and RanGTP cycling through the NPC (Yang & Musser 2006). Therefore, Sry nuclear accumulation could be regulated tremendously by variations in importin β concentration.

The nNLS of Sry is classified as bipartite, meaning two regions of basic amino acids separated by a nonpolar region of approximately 8-12 aa in length, come together to form a complete NLS, dependent on protein folding and secondary structure (Hodel et al. 2001). Among proteins containing NLSs this organization is not unique, and was first described in nucleoplasm (Robbins et al. 1991). However, unique to many HMG box proteins, the nNLS of Sry has been shown to be a part of an extended region including about half of the first α helix of the HMG domain that interacts with calmodulin. This
interaction is thought to facilitate an ATP and Ca\(^{2+}\) dependent mechanism of nuclear import not requiring a RanGTP gradient (Harley et al. 1996, Sim et al. 2005). While this mode of nuclear import is not well understood, directional transport from the cytoplasmic compartment to the nucleus is thought to be driven by DNA concentration and binding (Hanover et al. 2007).

Recent reports suggest NPC architecture and thus permeability is directly linked to the concentration of cisternal Ca\(^{2+}\) within the nuclear envelope and surrounding NPC microenvironment. Significantly, the release of Ca\(^{2+}\) sequestered in the nuclear envelope is believed to be mediated through activation of nuclear inositol triphosphate (IP\(_3\)) receptors (Moore-Nichols et al. 2002). Through such mechanisms, signal transduction pathways could facilitate IP\(_3\) mediated release of cisternal Ca\(^{2+}\) from the nuclear envelope, altering NPC permeability and activating calmodulin mediated nuclear translocation of Sry.

The Y chromosome of most eutherian mammalian species contain one Sry locus, however, in many rodent species the presence of more than one copy is not unusual. While the majority of research has focused on the biochemical and intracellular nature of human and mouse Sry proteins, there have no been published reports that consider these aspects in species that encode multiple Sry loci. This may be due to the fact that most species with multiple Sry copies, such as members of the family Microtidae, encode one functional copy and multiple nonfunctional pseudogenes that contain early in frame stop codons (Bullejos et al. 1997, Bullejos et al. 1999, Fernandez et al. 2002).
Recently our group has described the presence of six distinct $Sry$ loci on the $Y$ chromosome of a single laboratory rat ($Rattus norvegicus$) ($Sry1$, $Sry2$, $Sry3$, $Sry3B$, $Sry3B1$ and $Sry3C$), which is consistent with previous reports of copy number estimates in rat (Nagamine 1994, Turner et al. 2007). Interestingly, these six $Sry$ copies encoded on a single rat $Y$ chromosome show sequence differences comparable to those observed between $Sry$ loci of different mouse genomes, all of which have a single $Sry$ locus (Turner et al. 2007). Studies with transgenic mice have demonstrated that allelic variations of $Sry$ between species, which exhibit both protein and expression level differences, often prevent or reduce the capacity of Sry from one mouse species to induce testicular development in another species (Bowles et al. 1999, Nagamine et al. 1999, Albrecht et al. 2003). Interestingly, Turner et al. (Turner et al. 2007) through the use of fragment analysis, demonstrated that the multiple Sry loci of rat exhibit differential expression profiles in a single tissue and that these profiles differ between adult tissues.

At the protein level, the organization and aa sequences of rat and mouse Sry are quite similar up to the Q-rich region, which in rat is 12-26 aa in length compared to the 240 aa domain in some mice (Bowles et al. 1999). Excluding the Q-rich region, rat Sry2 shares greatest sequence identity with mouse proteins, with the most notable similarity being in the nNLS at residue 21, where both species encode an H. At this position, the remaining rat proteins encode an R, which is conserved in all mammalian species except for mouse. Additionally, rat Sry3-like proteins encode an aa difference at position 76, in the cNLS, a T rather than a P, that is not conserved in Sry proteins of any other
mammalian species and has the potential to alter the architecture of the C-terminal extremity of the HMG domain.

Traditionally Sry is viewed as having one primary function, testis determination. However, there is mounting evidence that Sry, like many SOX proteins (Weiss et al. 2003, Du et al. 2007, Neves et al. 2007), may have more than one function. Recent studies have proposed that in addition to functioning as the testis determination factor, Sry may also play a role in initiating sex-specific development, maintaining male-specific physiology and secondary characteristics throughout life (Salas-Cortes et al. 1999, Perinchery et al. 2000, Yuan et al. 2001, Teebi et al. 2004, Milsted et al. 2004, Dewing et al. 2006, Chen et al. 2007). The fact that multiple Sry loci have been selectively maintained on the rat Y chromosome, after at least two duplication events (Turner et al. 2007), suggests that all are required to generate and maintain the male phenotype. The duplication-degeneration-complementation model and subfunctionalization hypothesis proposed by Lynch and Force (Force et al. 1999, Lynch & Force 2000), predicts that duplication of developmental genes having multiple mutable functions or exhibiting unique temporal-spatial expression and activity, will often result in preservation of the duplicated genes due to fixation of degenerative mutations that eliminated one or more ancestral functions in each duplicate. Therefore, this hypothesis suggests that duplicated genes persists only because collectively all copies can facilitate the activities conducted previously by the single ancestral gene (Lynch & Force 2000, Ward & Durrett 2004). The fact that all six Sry proteins have been preserved on the rat Y chromosome, show novel aa substitution within the nNLS, cNLS and polyglutamine regions, all protein
regions of great functional significance, and exhibit unique spatial and temporal expression profiles, may indicate subfunctional specialization is at work. If this is the case, the multiple Sry proteins encoded on the Y chromosome of rat may be indispensable tools for the identification of Sry functions beyond testis determination in species with one locus. In the present study we have begun to investigate aa differences encoded in multiple Sry proteins of rat to determine if these aa substitutions contribute to altered protein function. Here we show for the first time that the open reading frames of each copy, when cloned into an eukaryotic expression construct, can generate a full length protein and that one of these aa differences leads to decreased nuclear accumulation of Sry2 proteins. Furthermore, these experiments suggest that the relative nuclear localization “strength” of the cNLS exceeds that of the nNLS.

Methods

Expression Vectors

To demonstrate that each Sry locus can generate a full length protein, the protein coding regions of Sry1 (GenBank: AY157669), Sry2 (GenBank: AY157670), Sry3 (GenBank: AY157672) Sry3B (GenBank: AY157996), Sry3B1 (GenBank: AY157997) and Sry3C (GenBank: AY157671) were subcloned cloned into the expression vector pcDNA3.1- (Invitrogen) from pCR® 4-TOPO clones encoding each (Turner et al. 2007, Underwood 2003).

To evaluate differences in Sry subcellular localization, chimeric and mutant constructs encoding single amino acid differences, deletions and directed mutations were
prepared. Two questions were addressed: (1) does a glutamine rich (Q-rich) region outside the conserved HMG Box DNA binding domain contribute to localization differences of each Sry protein and (2) does residue 21, a weakly basic H (histidine) encoded in Sry2 decrease the efficiency by which this protein localizes to the nucleus, compared to all other Sry proteins which encode an R (arginine).

Wild type rat Sry1, Sry2, and Sry3 fusion proteins containing a carboxy terminal c-Myc epitope and histidine tag were generated by amplifying the open reading frames of Sry1, Sry2, and Sry3 using primers L-BamH1/KozakSry and R-Not1-StopcodonSry (Table 2.1) from the pcDNA3.1- clones described above (Figure 2.1). Amplicons generated from these reactions began at the Sry start codon (underscored in L-BamH1/KozakSry primer), encoded a 5’ Bam H1 and 3’ Not 1 cleavage sites for cloning (bold), a Kozak translation initiation sequence (red text) and elimination of the endogenous Sry stop codon (underscored in R-Not1-StopcodonSry primer) to facilitate generation of the fusion tagged proteins (Table 2.1). These amplicons were cloned into the pEF1/Myc-His vector (Invitrogen) producing pEF1/Sry1, pEF1/Sry2, and pEF1/Sry3 expression constructs (Figure 2.1). Sry proteins generated from these constructs allowed us to determine if the naturally occurring proteins localize differently to the nucleus when transiently transfected into CHO cells. To identify the specific regions or residues that contribute to these differences, chimeric Sry proteins were generated. Using the primers set described above, the coding regions of Sry1 and Sry2 were PCR amplified and amplicons were cleaved at base 93 using the restriction endonuclease Eco RI.
Organization of native, chimeric, truncated and site-specifically mutated Sry expression constructs

Figure 2.1 Native Sry1 and 3 proteins are 169 aa in length while Sry2 is 156 aa due to an internal 13 aa deletion within the Q-rich region. All proteins contain two NLSs, shown above in Sry1/3; the bipartite nNLS (green regions) monopartite (black cross hashed). Chimeras, Sry1/3(del) and Sry2(+QR), were generated by cleaving Sry1/3 and Sry2 amplicons with Cvi QI, followed by ligations of 5’ fragments of Sry1/3 with 3’ fragments from Sry2 or vise versa. The same procedure was conducted to produce Sry1(H21) and Sry2(R21) chimeras using Eco RI to generate the 5’ and 3’ fragments. Truncations to Sry1, 2 and 3(-QR) and Sry1(HMGbox) were produced by PCR as were site-specific mutations to generate Sry1(20A22), Sry1(78A79) and Sry1(NoNLS).
This cleavage produced a 93 bp 5’ restriction fragment encoding the H21/R21 difference and a 417 bp 3’ restriction fragment from Sry1 and a 378 bp 3’ restriction fragment from Sry2 (a smaller fragment was generated due the 39 bp deletion encoded by Sry2). These restriction fragments were separated by electrophoresis on 1.5% agarose gels and extracted using a Zymoclean™ Gel DNA Recovery kit (Zymo Research Corp.). Sry1(H21) was produced by ligating 5’ fragments of Sry2 to the 3’ fragment of Sry1, while Sry2(R21) was generated by ligating the 5’ fragments from Sry1 to the 3’ fragment of Sry2 using T4 DNA ligase (Promega). After these ligations the chimeric coding regions were cleaved again with Bam HI and Not I to facilitate ligation into pEF1/c-Myc-His vectors, also opened with Bam HI and Not I.

There is a 13 amino acid (aa) deletion in Sry2 at the c-terminus of the protein; this deletion is not present in either Sry1 or Sry3-like proteins. Constructs Sry1(del), Sry3(del), Sry2(+QR), Sry1(-QR), Sry2(-QR) and Sry3(-QR) and were produced to determine if deletions in this region effect protein localization into the nucleus. In addition to these vectors an expression construct encoding only the HMG box DNA binding domain, Sry1(HMGbox), was generated to address whether the 61 aa’s found in the protein sequence that links the HMG box to the C-terminal Q-rich region of Sry contributes to nuclear localization. Sry1(-QR), Sry2(-QR) and Sry3(-QR) proteins are 142 aa in length and do not encode the Q-rich region. Constructs encoding these proteins were generated through PCR amplification of wild type pEF1/Sry1, Sry2, and Sry3 vectors using the L- SryKozakBam HI and R-SryXba-QR primer set (Table 2.1). Sry1(HMGbox) constructs, generates an 84 aa protein and was produced by amplifying
pEF1/Sry1 with primers L- SryKozakBam HI and R-SryXbaBoxOnly (Table 2.1).

Amplicons were digested with *Bam* HI and *Xba* I, gel extracted (Zymo Research Corp.) and ligated into *Bam* HI/*Xba* I opened pEF1/c-Myc-His vectors. Sry1 and Sry3 proteins containing the 13 aa deletion encoded by Sry2, Sry1(del) and Sry3(del), were produced by amplifying the coding regions of Sry1, Sry2, and Sry3 wild type constructs followed by restriction digestions with *Cvi* QI, cleaving each amplicon at base pair 294. These digests result in 294bp 5’ restriction fragments, 216bp 3’ fragments (Sry1 and Sry3) and 177bp 3’ fragments Sry2. All restriction fragments were gel extracted (Zymo Research Corp.) after agarose gel electrophoresis. Expression constructs pEF1/Sry1(del) and Sry3(del) were generated by ligating the 5’ fragments of Sry1 or Sry3 to 3’ fragments of Sry2, producing Sry1 and Sry3 like proteins containing the 13 aa truncation found in the Sry2 Q-rich region. To generate the pEF1/Sry2(+QR) construct, the 5’ restriction fragment of Sry2 was ligated to 3’ fragments of Sry1 or Sry3, which encodes a Sry2-like protein without the 13 aa deletion of native Sry2. Following ligation, the chimeric coding regions were digested with *Bam* HI/*Not* I to facilitate insertion into *Bam* HI/*Not* I digested pEF1/c-Myc-His vectors (Figure2.1).

To further investigate the contributions of both the N-terminal and C-terminal NLSs (nNLS and cNLS), native Sry1 sequences were site specifically mutated at residues resides 20, 21 and 22 (RRK to AAA) and/or residues 78 and 79 (RR to AA). These mutations replace the basic aa core of each NLS with non-polar alanine (A) residues. To
Table 2.1. Primers Used to Generate Native, Truncated, and Site-Specifically Mutated pef1/Sry Expression Constructs. The annealing location of each primer is based on the nucleotide sequence of Sry1 (Accession AY157669) and assumes the “A” of the translation start (highlighted yellow) as base number one.

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Priming site on Sry1</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-BamH1/KozakSry</td>
<td>5’-ctaggatcgaaccatggaggccatgtcaag</td>
<td>1-18</td>
</tr>
<tr>
<td>R-Not1-StopcodonSry</td>
<td>5’-ctagggcgcgtctggaactgtgtgctgct</td>
<td>491-507</td>
</tr>
<tr>
<td>R-SryXba-QR</td>
<td>5’-ctctagatgggtatccagg</td>
<td>415-429</td>
</tr>
<tr>
<td>R-SryXbaBoxOnly</td>
<td>5’-ctctagactgtggcactttaacc</td>
<td>236-252</td>
</tr>
<tr>
<td>F-nNLSsitemut</td>
<td>5’-ccgtggagaggccgagtctggtgaacag</td>
<td>48-78</td>
</tr>
<tr>
<td>R-nNLSsitemut</td>
<td>5’-ccacccataatgtcatggcctctgtgtagggtctcactt</td>
<td>10-47</td>
</tr>
<tr>
<td>F-cNLSsitemut</td>
<td>5’-tataaatatcagctgtgtggtggttzaattgaccc</td>
<td>214-247</td>
</tr>
<tr>
<td>R-cNLSsitemut</td>
<td>5’-ggttggatatt tctctctgttgtgtgcctctcagct</td>
<td>184-213</td>
</tr>
</tbody>
</table>
produce these directed changes, PCR using Phusion™ high-fidelity DNA polymerase was conducted as described by New England BioLabs®. Briefly, two 5’ phosphorylated primer sets were employed: F-nNLsSitemut, R-nNLsSitemut and L-cNLsSitemut, R-cNLsSitemut, which encode the mutation (underlined) in the forward primer (Table 2.1), and were used to amplify the entire 6.71 kb expression construct (see Appendix Figure A.1). Mutated amplicons were then re-circularized using T4 DNA ligase. This procedure was conducted three times to produce constructs encoding mutations to only the nNLs (Sry1-20A22), only the cNLs (Sry1-78A79) and to both NLs (Sry1 NoNLs). All pEF1/c-Myc-His based constructs were transformed into *E. coli* TAM-1 competent cells (Active Motif) and each construct was sequenced to confirm the presence of the expected native or modified DNA sequences on an ABI 3130xl genetic analyzer using BigDye Terminator sequencing chemistry (Applied Biosystems).

**Cell Culture, Transfection and Nuclear extraction**

Chinese Hamster Ovary (CHO) cells were cultured on 100mm plates (Nunc™) in HAM’s F12K medium (Sigma) supplemented with 10 mM HEPES and 10% fetal bovine serum (Atlanta Biologicals) in a humidified atmosphere at 37°C with 5% CO₂. CHO cells grown to approximately 1 x 10⁵ cells/cm² were transfected with plasmid DNA using ExGen500 transfection reagent (Fermentas) as described by the manufacturer. Briefly, 7.5 µg plasmid DNA was allowed to interact for 10 min with 25 µl (6 equivalents) ExGen500 and 150 mM NaCl was used to bring the total reaction volume to 1 mL. Complexes were applied to CHO in 9 mL fresh medium and centrifuged at 280 x g 5 min.
Cells were then incubated 24 hr under normal growth conditions. Following incubation, all medium was removed, plates were washed with PBS and the cells were trypsinized, pelleted (280 x g, 5 min.) and total proteins lysates were collected or cytoplasmic and nuclear proteins were separated using the reagents and protocol supplied in the ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas). Fractions generated were quantified using Thermo Biosciences Comassie Plus Bradford Assay (Thermo Fisher Scientific Inc.) and then subjected to SDS PAGE and Western blot.

CHO (1.25 x 10^4 cells/cm^2) used for immunocytochemistry were seeded onto LabTek™ 16 well glass chamber slides (Nunc™). After 24 hr cells were transiently transfected with 300 ng plasmid DNA using 1 µl (6 equivalents) ExGen500 in 20 µl total volume and incubated an additional 24 hr (see Appendix).

**Immunocytochemistry (ICC)**

CHO were fixed for 5 min in ice cold methanol. After fixation, cells were washed in sterile PBS and incubated 25 min in blocking solution (PBS containing 10% normal rabbit serum and 3% nonfat dry milk). Primary antibodies, goat anti-Myc epitope (Bethyl Laboratories, Inc.) or goat anti-mouse Sry E-19 (Santa Cruz Biotechnology, Inc.), were diluted 1:450 and 1:100 respectively in diluted blocking solution (1.5% normal rabbit serum, 0.6% nonfat milk) and incubated 1 hr at room temperature. After four washes, the secondary antibody, a rabbit anti-goat IgG-Cy3™ conjugate (Sigma-Aldrich, Inc.), was diluted 1:3500 in blocker diluted to 3% normal rabbit serum, 1.2% nonfat milk and incubated 45 min at 37°C. Following these incubations VectaSheild mounting
medium containing DAPI (Vector Laboratories) was applied and images were captured using a broad range excitation filter (530 – 550 nm) on an Olympus BX60 with DP71 digital camera and DP Controller software. Controls showing antibody specificity include CHO transfected as described above with Sry1 incubated with normal goat serum or PBS in place of a primary antibody. Each experiment was conducted three times, and in each trial all expression constructs were transfected into two wells where at least 12-25 stained cells were observed per transfection.

**SDS PAGE and Western Blotting**

Cytoplasmic (20 µg), nuclear extracts (20 µg) or total protein lysates (35 µg) were separated on 13.5% polyacrylamide gels and proteins were immobilized on PVDF membranes using semidry transfer techniques. Membranes were blocked 1hr at room temperature in PBS containing 5% nonfat dry milk and 0.1% Tween-20. Primary antibodies used to detect Sry proteins include a goat anti-mouse Sry E-19 (Santa Cruz Biotechnology, Inc.) and a goat anti-Myc epitope (Bethyl Laboratories, Inc.) Laboratories), both antibodies were diluted in blocking solution at 1:300 and 1:1000 respectively and incubated at least 1hr at room temperature. Following two washes in PBS, the secondary antibody, donkey anti-goat HRP conjugate (Bethyl Laboratories, Inc. Laboratories) was diluted 1:3000 in blocking solution and incubated 1-2hr at room temperature. Bands were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific Inc.). Each blot was exposed 5 -10 min and recorded with a Kodak 2200 Gel Logic Imaging system. In all western blots “MT” control lanes
represent CHO cell protein extracts obtained from cells transfected with an expression vector containing no insert.

Results

All six Sry loci can generate a potentially active full length protein.

CHO cells transiently transfected with pcDNA3.1- constructs encoding the protein coding regions of native Sry1, 2, 3, 3B, 3B1 and 3C were collected, and total protein was extracted and Western blotted. Figure 2.2 shows that the protein coding regions of all loci can express a potentially active full length protein.

Differential intracellular localization of Sry proteins.

To begin to understand if slight aa differences among the Sry loci in Rattus norvegicus are associated with differences in subcellular distribution, we transiently transfected native Sry1, Sry2, and Sry3 expression constructs into CHO cells. The native pEF1/Sry fusion proteins generated contain a C-terminal Myc epitope which was used to detect these proteins with an anti-Myc primary antibody (Bethyl Laboratories, Inc.) by immunocytochemistry (ICC). Figure 2.3A, shows that the cellular localization of Sry1 and Sry3 is nuclear, while Sry2 proteins show both nuclear and cytoplasmic distributions. The same Sry distributions were observed when an anti-mouse Sry primary antibody (Santa Cruz Biotechnology, Inc.) was used in place of the anti-Myc primary (data not shown). Elimination of either the anti-Sry or anti-Myc epitope primary antibody, resulted in no staining as shown in Figure 2.3A. In addition to ICC, nuclear and cytoplasmic
extractions of transiently transfected CHO followed by Western blots against the Myc epitope also reveal that Sry1 and Sry3 localize to the nucleus while native Sry2 is both nuclear and cytoplasmic (Figure 2.4; lane 3, 5 and 6). Proteins extracted from non-transfected cells show the absence of an immunoreactive band in both nuclear and cytoplasmic fractions (Figure 2.4, lane 12), as expected in the female CHO cells.

**The glutamine rich region of rat Sry does not contribute to nuclear localization.**

Following transient transfections with chimeric Sry1(del), which encodes Sry1-like proteins containing the Sry2 deletion; Sry2(+QR), which encodes Sry2-like proteins with a full length Q-rich region; and truncated Sry1(-QR) and Sry3(-QR) proteins, which have no Q-rich region, it was determined that all maintained the same subcellular distributions observed in CHO cells transiently transfected with native Sry proteins (Figure 2.5A). Western blot analysis of cytoplasmic and nuclear extracts from CHO cells transfected with Sry1(del) and Sry1(-QR) confirm the results obtained through ICC. Both of these chimeric proteins localize to the nucleus, as seen in Figure 2.4, lanes 2 and 4. Therefore, complete removal of the Q-rich region, as in all Sry(-QR) proteins, the addition of a full length Q-rich region to Sry2, as in Sry2(+QR), or the incorporation of the truncated native Sry2 Q-rich region into Sry1-like proteins (Sry1 del) has no effect on nuclear import.
Western blot of all six Sry proteins encoded on the rat Y chromosome

Figure 2.2. The Y chromosome of rat encodes at least six Sry genes, all capable of expressing a potentially active protein. CHO cells transiently transfected with constructs encoding native Sry1, 2, 3, 3B, 3B1 and 3C were collected and total protein extracted. The western blot above shows detection of all six Sry proteins. Sry1 and the Sry3-like proteins are 169-170 aa in length and ~ 20 – 21kD, while Sry2 which contains a 13 aa deletion, is 156aa in length and ~ 18 kD. The “MT” lane represents CHO cell extracts obtained from cells transfected with an empty vector. Each lane was loaded with 35 µg of CHO cell lysate and Sry was detected using enhanced chemiluminescence after incubation with a goat anti-mouse Sry E-19 (Santa Cruz Biotechnology, Inc.) primary antibody (1:300) and a donkey and goat IgG, HRP conjugate (Bethyl) (1:3000).
The presence of an histidine residue at position 21 within the N-terminal NLS of Sry2 decreases nuclear import.

While addition of a full length Q-rich region in Sry2 (+QR) resulted in the same nuclear and cytoplasmic accumulations observed with native Sry2, the replacement of H21 with an R, as in chimeric Sry2(R21) proteins, results in ICC staining exhibiting complete localization to the nucleus. Additionally, chimeric Sry1(H21) proteins that contain an H at position 21 rather than the native R, exhibit staining in both the cytoplasm and nucleus of transiently transfected CHO cells, a localization pattern typically observed in CHO cells transfected with native Sry2 (Figure 2.3B). Nuclear and cytoplasmic extracts of Sry1(H21) and Sry2(R21) subjected to western blot analysis and detected with the same anti-Myc primary antibody (Bethyl Laboratories, Inc.) used for ICC staining, show similar profiles (Figure 2.4, lanes 10 and 11). Sry1(H21) proteins are detected robustly in both nuclear and cytoplasmic fractions, as would be expected if an H residue within the nNLS leads to attenuated nuclear accumulation. Replacement of H21 with an R in Sry2, results in elevated nuclear accumulation of Sry2(R21) and decreased accumulation in the cytoplasm.

The pEF1/Sry1(HMGbox) construct encodes 84 aa’s representing the complete HMG box DNA binding domain of Sry1 and two additional aa’s that flank this domain in the native protein plus the Myc epitope and histidine tag encoded by the expression
Figure 2.3. **Frame A**, CHO cells transiently transfected with native Sry1 and Sry3 proteins localize exclusively to the nucleus while native Sry2 localizes to both the nucleus and cytoplasm, negative control: preimmune goat sera used in place of primary antibody shows no staining. **Frame B**, Sry1 proteins containing Sry deletion shown nuclear staining. Sry2(R21) encodes the R of Sry1 at residue 21 rather than H at this position while Sry1(H21) contains the H of native Sry2. Resulting staining patterns show that an R is at position 21 within the nNLS of Sry, leads to more nuclear localize, as Sry1-like proteins containing H show staining typically observed with native Sry2. All images were captured at 400 X magnification and yellow bars = about 50 µm.
Western Blot of Nuclear and Cytoplasmic Extracts of Native, Chimeric, Truncated and Mutated Sry Proteins

Figure 2.4. Western blots of nuclear and cytoplasmic extracts. Each lane was loaded with 20 µg total protein and detected using an anti-Myc antibody (see methods). Due to naturally occurring differences and introduced mutations proteins exhibit predicted changes in molecular weight. Lane 1: Sry1(HMGbox), lane 2: Sry1(del), lane 3: Sry2, lane 4: Sry(-QR), lane 5: Sry1, lane 6: Sry3, lane 7: Sry1(20A22), lane 8: Sry1(78A79), lane 9: Sry1(NoNLS), lane 10: Sry1(H21), lane 11: Sry2(R21), lane 12: negative control (MT).
vector. CHO cells transiently transfected with this construct generate ICC staining patterns similar to native Sry2, as Sry1(HMGbox) proteins are detectable in the nucleus and weakly in the cytoplasm (Figure 2.5A). This result is unusual due to the fact that this protein contains an unmodified Sry1/3-based R21 containing nNLS and complete cNLS. Western blots of nuclear/cytoplasmic extracts confirm results obtained from the immunocytochemical studies (Figure 2.4, lane 1) showing immunoreactive bands in both subcellular compartments.

**Site-directed mutations to both NLSs illustrates that the cNLS in rat Sry is most responsible for Sry localization to the nucleus**

To better understand the contributions to nuclear import of rat Sry by the nNLS and cNLC, we generated mutated Sry1 proteins site-specifically modified to replace basic, tandemly oriented R and K residues with nonpolar A (alanine) residues. In Sry1(20A22), the native nNLS containing an R at positions 20 and 21 and a K at position 22 was mutated to A residues. CHO cells transiently transfected with this construct and stained as described above, show Sry distributions equivalent to native Sry2, as most Sry is localized to the nucleus, and like Sry2 staining also occurs within the cytoplasm. Alternatively, when the cNLS was mutated by replacing R78 and 79 with A residues, Sry1(78A79), the staining pattern is reversed. Most Sry accumulated in the cytoplasm. Additionally, when these mutations were made at both sites within the same construct, as in Sry1(NoNLS), ICC staining was also primarily cytoplasmic (Figure 2.5B).
Immunocytochemical Analysis of Truncated and Mutated Sry Proteins

Figure 2.4. Truncated Sry1 with no Q-rich region, (-QR), shown nuclear staining. Chimeric Sry2 proteins with a full length Q-rich region of Sry1 or 3, (+QR), exhibit staining similar to native Sry2, indicating the Q-rich region is not involved with nuclear localization. However, staining of Sry1(HMGbox) are similar to that of native Sry2.

Mutations to the nNLS, Sry1(20A22), leads to staining similar to native Sry2. Mutating cNLS residues 78 and 79 leads to cytoplasmic staining, as do mutations to both nNLS and the cNLS. This may indicate that the cNLS, a confirmed importin β interaction site is, responsible for the majority of nuclear importation of rat Sry. All images were captured at 400 X magnification and yellow scale bars = about 50 µm.
Western blots of nuclear/cytoplasmic extracts, confirmed the results obtained through ICC (Figure 2.4, lanes 7, 8 and 9). However, even with both NLSs mutated, Sry proteins were detected in the nucleus at reduced levels.

Discussion

Here, we show that nuclear import of rat Sry2 is reduced compared to Sry1 and 3 proteins (Figure 2.3A). These localization differences seem to be directly linked to the presence or absence of an H residue at position 21 in the nNLS. Through the use of chimeric Sry1(H21) proteins we rule out the possibility that Sry2 localization patterns are a byproduct of protein regions beyond nNLS, since chimeric Sry1 proteins also accumulated in both cytoplasmic and nuclear compartments. In addition to these findings, when H21 is replaced with an R as in Sry2(R21), protein localization is primarily nuclear. Notably, Sry1(20A22) mutant proteins, in which the C-terminal portion of the bipartite nNLS (20RRK22) was mutated to A residues, intracellular distributions are very similar to Sry2 and Sry1(H21). While the existence of an H within an NLS is not unusual (Boulikas 1993), it has been reported that NLSs which harbor an H residue may be “weak” compared to NLSs encoding more basic residues, which often leads to reduced nuclear translocation (Boulikas 1996, Kaiser et al. 2004). Therefore, an NLS encoding H would be detrimental to proteins with functions that are exclusively nuclear. However, if the activities of a protein are required in both cytoplasmic and nuclear compartments, presence of H in an NLS may facilitate such spatial requirements. In many PKC isoforms, the presence of H in NLSs is hypothesized to ensure distribution
to both intracellular compartments (Boulikas 1996). Thus, in PKC, weak NLSs would be advantageous to cellular requirements where kinase function is required in both locations. Significantly, Thevenet et al. reported that in the eight-week human male embryonic gonad, some Sry is found in the cytoplasm of somatic cells, however this cytoplasmic accumulation was considered dislocation as Sry acetylation stimulated nuclear import (Thevenet et al. 2004). Further, SOX9 proteins have also been observed to localized and accumulate in the cytoplasm of germinal cells in mouse during the time of gonadal genesis (Morais da Silva et al. 1996). Perhaps this indicates the function of Sry and other SOX proteins is dependent on the ability to flood the nucleus producing high nuclear concentrations in a very short time frame. In mouse and rat, H21 and deacetylation may contribute to Sry accumulation in the cytoplasm until some threshold concentration is generated. Subsequent acetylation may acts as the switch for rapid nuclear localization allowing a required threshold level of nuclear Sry accumulate when needed. Because only rodent Sry encode H21, this may represent an ancestral mechanism. Interestingly, in mouse temporal nuclear accumulation and activity is under more stringent regulatory control than in other spices.

Histidine with a $pK_a$ of $\sim 6$ compared to R and K residues with $pK_a$ values of 12.5 and 10.5 respectively, is weakly basic ($\sim 3.8\%$ of Hs would be protonated at pH 7.4) thus the presences of H within a highly basic motif could reduce NLS interaction with corresponding acidic contact sites of nuclear transport receptors (Boulikas 1996). Therefore, aa composition based on NLS pH may be critical for Ran dependent import processes. However, the nNLS is thought to act as part of the calmodulin binding site.
While calmodulin binding sites typically contain stretches of basic aa, interactions by calmodulin are reported to be less sequence specific and is believed to rely predominantly on protein conformation (Hanover et al. 2007). Accordingly, H21 must alter the architecture of the first Sry α helix in such a way that calmodulin interaction is retarded. Similar reports supporting this idea describe missense mutations in SRY and SOX9 which lead to XY sex reversal and/or campomelic dysplasia due to reduced calmodulin mediated nuclear import (Argentaro et al. 2003, Sim et al. 2005). In accordance with the above studies, Sudbeck and Scherer (Sudbeck & Scherer 1997) found that mutating R76 with leucine in the nNLS of human SRY (which corresponds to H21 in rat), also decreases nuclear accumulation. Collectively, these results suggest that calmodulin/ Sry interactions require R at position 21 in rat and position 76 in human to effectively mediate nNLS nuclear import of Sry, as replacement of R21 with H, L, or A results in partial cytoplasmic accumulation.

If these aa differences alter the architecture of rat Sry2 enough to potentially deter calmodulin binding, do these proteins exhibit reduced DNA binding? Analysis of human mutations that result in abnormal gonadal development examined an R-P mutation at aa 76 (H21 rat) and found only a slight reduction to DNA binding affinity (Battiloro et al. 1997, Mitchell & Harley 2002, Harley et al. 2003b). Therefore, abnormal testis development was presumed to be a result of reduced nuclear import. Since an R-H difference at this position in rat Sry2 is a conserved change (both aa’s are basic), one would predict that a P might alter protein activity more than the conserved H in many rodent specie. Preliminary DNA binding studies from our lab, comparing wild type rat
Sry2 binding with wild type rat Sry1, shows that the proteins shift about the same amount of free probe, indicating no obvious reduction in DNA binding by Sry2 that encodes an H versus an R at position 21 (data not shown).

For normal testis formation both the nNLS and cNLS are required for efficient localization into the nucleus. Unlike the nNLS, the cNLS is classified as being monopartite and is composed of a short stretch of up to five basic amino acids and has been shown to mediate nuclear import via interaction with the import receptor, importin β (Forwood et al. 2001, Harley et al. 2003b). In rat (and mouse) the cNLS, like the nNLS contains an H residue, which based on results from the nNLS of Sry2, was thought to indicate that this NLS is “weak” (Figure 1.3). To determine the overall contribution to nuclear localization conferred by the cNLS, we generated the site-directed Sry1(78A79) mutant that replaced R78 and R79 with the aliphatic aa A. Transiently transfected CHO cells expressing this construct, showed that the majority of Sry is cytoplasmic (Figure 2.5B). Additionally, mutating both NLS motifs in Sry1(NoNLS) resulted in the same staining patterns obtained when only the cNLS was mutate; most staining occurred in the cytoplasm. Surprisingly some staining remained in the nucleus. However this is not unusual, many reports describe consistent weak nuclear accumulation of Sry proteins with mutated NLSs, this is thought to be the result of weak elements within the HMG domain and flanking regions contributing to nuclear import (Harley et al. 1996, Sudbeck & Scherer 1997, Harley et al. 2003b). Because the cNLS mutation resulted in very high levels of cytoplasmic accumulation, and mutation to nNLS resulted in Sry2-like localization patterns, we took this to mean that the cNLS is most responsible for rat Sry
nuclear localization, which is in contrast to previous reports suggesting the nNLS constitutes the “stronger” of the two NLSs (Poulat et al. 1995).

Consistent with results that separately analyzed human missense mutations in the cNLS, which mutate R133 to W or G; mutating the corresponding residue in rat (R78) to A lead to greatly reduced nuclear accumulation (Li et al. 2001, Harley et al. 2003b, Plaseska-Karanfilska et al. 2007). It is apparent that position 78 in rat and the corresponding position 133 in human, requires the presence of an R to facilitate cNLS function. Similar to R21/R76 mutation at the nNLS, mutations at R78/R133 of cNLS are reported to not alter DNA binding, but reduce Sry nuclear import (Li et al. 2001, Harley et al. 2003b). Further, it has been proposed that in addition to acting as an NLS, the aa surrounding the cNLS also act as a kinetic clamp which prolongs Sry / DNA interactions and in turn the active lifespan of a resulting sex-specific pre-initiation complex (Phillips et al. 2006). Sequence analysis of aa comprising the clamp region show remarkable conservation, however, in all rat Sry3-like proteins there is an aa substitution at residue 76, which replaces a conserved P residue with T (Figure 1.3). While this aa change did not interfere with nuclear localization in this study, it could alter Sry3 protein function by altering DNA interactions. In human mutations at this location, replacement of P with R, results in abnormal testicular development (Phillips et al. 2006). So why has the rat Y chromosome maintained multiple Sry3-like loci? Recent studies from our lab have shown that all members of the Sry3-like group are expressed. Further, certain members of this group exhibit variable expression patterns between tissues. This indicates that Sry3-like proteins may be involved in tissue-specific functions that Sry1 and Sry2 are not.
Perhaps this aa difference enhances HMG box mediated protein-protein interactions such as those describe between with androgen receptor and Wilms’ tumor 1 (Yuan et al. 2001, Matsuzawa-Watanabe et al. 2003).

In addition to analyzing the activity of both NLSs encoded in all rat Sry proteins, we also examined the possibility that regions outside the HMG box contribute to nuclear localization. Using chimeric and truncated Sry proteins (Sry1(del), Sry2(+QR), Sry1(-QR) and Sry1HMGbox) we show that the 25 aa Q-rich region of rat Sry and differences within this region do not contribute to, or alter nuclear localization (Figures 2.3B, 2.4, and 2.5A), as inferred by studies of the large polyglutamine regions of huntingtin and atrophin-1 (Ross et al. 1999). The only protein in this group to exhibit reduced nuclear accumulation was Sry1(HMGbox). This result was unexpected and is unusual due to the fact that this protein contains unmodified Sry1/3-like NLSs. One explanation for this result could be that importin β requires more than two aa down stream, outside the HMG box to properly stabilize the interaction. Being that the cNLC of rodent Sry contains only two tandem R residues and one H, while in all non-rodent Sry proteins there exists at least 5 strongly basic aa (R and K) within or near the site of importin β interaction.

Perhaps the combination of a potentially weak cNLS in rat due to a low number of basic residues and local truncation near this site destabilized importin β binding and reduced nuclear import. Another explanation could be that this truncation interferes with calmodulin interaction sites near the cNLS that have been proposed to contribute to a binding synergy between calmodulin and importin β (Harley et al. 2003b).
In summary, these studies demonstrate that the coding regions of all rat Sry genes can express a potentially functional protein. Additionally, we found that due to slight aa differences among these proteins, nuclear localization is altered, specifically in Sry2 proteins due the presence of an H at position 21 in the nNLS. Further, these results indicate that a full length Sry protein is required for studies of cellular localization, as truncated Sry HMG box proteins with both NLSs intact exhibited reduced nuclear accumulation. Finally, these studies demonstrate that the cNLS is primarily responsible for rat Sry nuclear translocation.
CHAPTER III

Functional Aspects of Multiple Sry Loci

Introduction

Sry has long been established as the Y-linked testis determination factor in eutherian mammalian species (Gubbay et al. 1990, Koopman et al. 1990). While early expression at the primordial genital ridge is essential for male testicular development, many reports indicate that Sry may be involved in functions beyond that of testis determination (Perinchery et al. 2000, Teebi et al. 2004, Chen et al. 2007). Supporting these alternative roles are studies that describe expression of Sry in adult male testis and brain (Mayer et al. 1998, Mayer et al. 2000). Additionally, Sry has been implicated in the regulation of tyrosine hydroxylase in the adrenal medulla and brain, and androgen receptor in the prostate gland (Yuan et al. 2001, Milsted et al. 2004, Dewing et al. 2006). Collectively these studies indicate that expression of Sry after initiation of testis differentiation may be necessary for the development and maintenance of normal male physiology and perhaps behavior.

To date, all biochemical and functional studies of Sry have been conducted with species whose Y chromosome encodes a single Sry locus, predominantly human and mouse. While these studies have established a fundamental understanding of Sry structure and function relationships, the fact that these species possess only one locus
may inadvertently hinder the identification of alternative Sry activities. The Y chromosomes of most mammalian species contains one Sry locus, however the Y chromosome of some rodent species have been identified to contain multiple Sry loci (Nagamine 1994, Bullejos et al. 1997, Bullejos et al. 1999, Fernandez et al. 2002). In many of these species, the presence of early in frame termination codons suggests that most copies are non-functional. This is not the case in the laboratory rat (Rattus norvegicus), a species which maintains multiple Sry loci on a single Y chromosome. Early research by Nagamine using Southern blot hybridization, predicted that the rat Y chromosome may encode 4-5 Sry copies (Nagamine 1994). In agreement with Nagamine’s findings we have recently described the presence of at least six distinct full length Sry loci on the rat Y chromosome, designated Sry1, 2, 3, 3B, 3B1 and 3C based on sequence differences. Furthermore, we have shown that all identified copies are expressed at different levels in more than one adult tissue (Turner et al. 2007).

Rat Sry proteins are very similar to mouse, as the high mobility group (HMG) box DNA binding domain is positioned at the N-terminus in both species. At the C-terminal end, rat Sry contains a comparatively short, \( \leq 26 \) amino acid (aa) vs. \( \leq 240 \) aa in Mus musculus (GenBank: AAC53444), polyglutamine region that is linked to the C-terminus extremity of the HMG box by a 61 aa hinge region. While the general organization of the six rat Sry proteins is quite similar to mouse, the primary structure of these proteins are unique. Excluding the polyglutamine (Q-rich) region, rat Sry2 shares the greatest aa sequence identity with mouse, while the remaining five proteins exhibit varying degrees of sequence divergence. Based on these aa differences, Sry3-like proteins are most
different from mouse while Sry1 bridges the gap between Sry2 and Sry3-like proteins. In fact, the Sry proteins of rat exhibit as much sequence divergence among isoforms, as individual Sry proteins do between mouse species (Turner et al. 2007).

Of greater importance than simply showing aa variation between rat and mouse Sry is the fact that many of these differences are localized in regions that have been shown to directly modulate protein function. Recently, we have shown that a single aa difference at position 21 (H vs. R), in the HMG box region that functions as an N-terminal nuclear localization signal through an interaction with calmodulin (Harley et al. 1996, Sim et al. 2005, Forwood et al. 2007), reduces nuclear import of Sry2 in transiently transfected CHO cells (Chapter II). Interestingly, at this position, mouse also encodes an H, while in human Sry and all other mammalian species there is conservation of an R residue, as in rat Sry1 and 3-like proteins. Additionally, in the C-terminal extremity of the HMG box, Sry3-like proteins contain a T rather than a P at position 76, a substitution that is not found in Sry of any other mammalian species examined. The C-terminal portion of the HMG box is involved in three functions necessary for efficient Sry activity; (1) nuclear localization through interaction with importin β (Sudbeck & Scherer 1997, Li et al. 2001, Mitchell & Harley 2002, Harley et al. 2003b), (2) DNA binding / bending (Werner et al. 1995a, Werner et al. 1995b) and (3) acting as a kinetic clamp which is thought to enhance the duration Sry remains bound to target DNA sequences (Ukiyama et al. 2001, Li et al. 2006, Phillips et al. 2006). Therefore, this substitution may alter Sry3-like protein architecture, and thus activity, in this region. However, we have demonstrated that this difference has no effect on nuclear localization, as
immunocytochemical analysis of Sry3 proteins consistently shows complete nuclear accumulation.

Of the aa differences found among rat Sry proteins, the most pronounced is located in the C-terminal Q-rich region. Within this region, Sry2 proteins contain half the number of Q residues compared to Sry1 and 3-like proteins due to a 13 aa deletion. In *Mus musculus* maintenance of a full length Q-rich domain is required for testicular development, and introduced deletions within this domain have been shown to inhibit testicular development in XX transgenic mice (Bowles *et al.* 1999). Because the C-terminal portion of non-rodent Sry proteins does not contain a Q-rich activation motif, the relevance of this region has been questioned. However, a conserved aa consensus at the C-terminus of human Sry that interacts with a PDZ domain factor, SIP-1 (Sry interacting protein 1), may facilitate the biochemical activities achieved by the Q-rich region in rodents via interaction with an adapter protein (Poulat *et al.* 1997).

Considering that the rat Y chromosome has selectively maintained at least six Sry loci that encode proteins with unique aa substitutions at regions of great functional significance, implies that together all copies are required to facilitate the functions usually fulfilled by one Sry locus. In previous reports we have illustrated that all copies are expressed *in vivo* at different levels in more than one adult tissue, that the coding region of each locus can generate a full length protein and that these proteins localize into the nucleus differently (Turner *et al.* 2007, Underwood *et al.* 2008). In the current study we demonstrate that differences in native Sry1, 2 and 3-like proteins lead to differential activation of a luciferase reporter containing an Sry inducible synthetic promoter.
Further, through the use of chimeric, truncated and site-specifically mutated Sry proteins, we examine contributions conferred by different protein regions and amino acid substitutions among rat Sry proteins. Moreover, we show that the Q-rich region in rat Sry is essential for activity, since truncated proteins with no polyglutamine region, or proteins consisting of only the HMG domain, exhibited near baseline luciferase activity. Thus, Sry function is dependent on more than just a conserved HMGbox DNA binding domain (Sanchez-Moreno et al. 2008).

Methods

Expression Constructs and Reporters.

The firefly luciferase reporter construct, pGL3/AR600, encoding sequence spanning from the translation start site to 590 bases up-stream in the rat androgen receptor 5' UTR, and was generated through amplification of genomic DNA isolated from a single ♀ SHR/y rat with primers R-ARNco (5' - gtaccatggtttagcttgtctagcttccacc) and L-ARSma600 (5' - cacccgggttaactccctttggctga). Amplicons were cleaved using the restriction endonucleases Nco I and Sma I, followed by electrophoresis on 1% agarose gels. The resulting restriction fragments were then extracted using a Zymoclean™ Gel DNA Recovery kit (Zymo Research Corp.). Using T4 DNA ligase (Promega) the restriction fragments were inserted into pGL3 vectors (Promega) also opened with Nco I and Sma I. Reports demonstrating direct Sry inhibition of AR proteins and the presence of tandem punitive Sry response elements lead to the selection of the sequences used to construct this reporter.
Native rat Sry1, Sry2, and Sry3 fusion proteins containing a carboxy terminal c-Myc epitope and histidine tag and all chimeric, truncated and mutated expression constructs (Figure 3.1) derived from these native vectors were generated as described in CHAPTER II. In addition to the fusion proteins described previously, pEF1/Sry1, 2, and 3 control constructs were generated that maintain the endogenous Sry stop codon using the following primer set: BamH1/KozakSry and R-Not1+StopcodonSry (5'-ctagcggccgc tctagttgaaactgtgc). These expression constructs encode Sry proteins with no c-Myc epitope or His tag and were used to determine if the addition of the fusion tag interfered with assay results. All pEF1/c-Myc-His based effector constructs and the pGL3/AR600 reporter were transformed into E. coli TAM-1 competent cells (Active Motif) and each construct was sequenced to confirm the presence of the expected native or modified DNA sequences on an ABI 3130xl genetic analyzer using BigDye Terminator sequencing chemistry (Applied Biosystems).

**Growth and Cotransfection of CHO Cells.**

Chinese Hamster Ovary (CHO) cells were cultured on 100mm plates (Nunc™) to approximately 50% confluence in HAM’s F12K medium (Sigma) supplemented with 10mM HEPES and 10% fetal bovine serum (Atlanta Biologicals) in a humidified atmosphere at 37°C and 5% CO2. Prior to transfection, ~ 25,000 cells (6.6 x 10³ cells/cm²) were seeded to 24 well cassettes (COSTAR) and incubated overnight. Each well was transiently cotransfected with 50 ng effector plasmid, 500 ng firefly luciferase reporter (pGL3/AR600), and 500 pg of control construct, phRL-null Renilla, (Promega)
Figure 3.1. Native Sry1 and 3 proteins are 169 aa in length while Sry2 is 156 aa due to an internal 13 aa deletion within the Q-rich region. All proteins contain two NLSs, shown above in Sry1/3; the bipartite nNLS (green regions) monopartite (black cross hashed). Chimeras, Sry1/3(del) and Sry2(+QR), were generated by cleaving Sry1/3 and Sry2 amplicons with Cvi QI, followed by ligations of 5’ fragments of Sry1/3 with 3’ fragments from Sry2 or vise versa. The same procedure was conducted to produce Sry1(H21) and Sry2(R21) chimeras using Eco RI to generate the 5’ and 3’ fragments. Truncations to Sry1, 2 and 3(-QR) and Sry1(HMGbox) were produced by PCR as were site-specific mutations to generate Sry1(20A22), Sry1(78A79) and Sry1(NoNLS).
that served as a conditional normalizer, using Superfect transfection reagent (Qiagen) following the manufacture’s protocol. Briefly, transfection complexes were formed by combining 550ng total DNA, 1.2 µL Superfect and serum free HAM’s F12K to bring the total reaction volume to 25 µL. Following a 10 min incubation at room temperature complete medium containing 10% FBS was added to stop complex formation, bring the total volume to 300 µL, which was applied directly to a single well after removing the old medium. After a 4hr incubation, complexes were removed and fresh complete medium was applied. 24 hrs later cells were processed for luciferase activity using the reagents and protocol provided in The Dual-Luciferase® Reporter (DRL™) Assay System (Promega). Luciferase activity of both experimental and control reporters was measured on a Turner Biosystems 20/20" luminometer. Firefly:Renilla ratios obtained were used to calculate pGL3/AR600 reporter activity of each Sry effector construct relative to reporter activity obtained from CHO transfected with an empty vector, pEF1/(MT), negative control. Data reported represent means ±SEM of three trials conducted in triplicate with each Sry effector construct. Statistical analysis was performed by using: One-way ANOVA and a post-hoc Student-Newman-Kuels test and Student’s t-test where applicable. Analyses were run on SigmaStat software (Jandel Scientific, San Rafael, CA) with significance assumed at p<0.05.
Results

Sry1, 2 and 3 proteins exhibit different activities.

From these trials we demonstrate that: (1) the synthetic experimental reporter is a useful tool to measure Sry activity, (2) all native rat Sry proteins increase experimental reporter activity above that of baseline MT control groups and (3) luciferase activity of Sry effectors was elevated differentially and significantly (p<0.0001), with Sry2 proteins exhibiting the lowest level of activity (2.23 ± 0.08), Sry1 with moderate activity (3.42 ± 0.11) and Sry3 showing the most robust elevation in activity (4.31 ± 0.09) (Figure 3.2).

In addition to the experiments above, which used Sry fusion proteins containing a C-terminal c-Myc epitope and 6x His tag, trials were also performed with Sry1, 2 and 3 proteins lacking the Myc epitope and His tag to confirm that presence of the fusion region did not interfere with or enhance Sry activity. In all trials, results showed that tagged and non-tagged proteins were not significantly different (data not shown).

The internal deletion at the Q-rich region of Sry2 significantly reduces transcriptional activity.

Of rat Sry proteins, Sry2 is the most different due to an internal 13 aa deletion within the Q-rich potential activator motif. In Mus, this region is 10-20 times longer than rat and is required for testis determination (Bowles et al. 1999). To determine if this region is of equal importance in rat and if length variation alters Sry activity, we generated chimeric proteins in which the truncated Q-rich region of Sry2 replaced those in Sry1 and 3 (pEF1/Sry1(del) or Sry3(del)) and Sry2 proteins which contain the full
length 25aa Q-rich region of Sry1 and 3 (pEF1/Sry2(+QR)). These effectors, cotransfected as described above, show that the 13 aa deletion in Sry2 reduces protein activity, as Sry1(del) and Sry3(del) show significantly reduced luciferase activities compared to native Sry1 and 3 proteins (Sry1s, 2.16 ± 0.07 versus 3.42 ± 0.11, p< 0.001, Sry3s, 2.55 ± 0.04 versus 4.31 ± 0.09, p<0.001)(Figure 3). Interestingly, the activity of Sry1(del) was reduced to levels no different than native Sry2, while Sry3(del) exhibited significantly more luciferase activity than Sry1(del) and native Sry2 at these reduced levels (Sry3(del) vs. Sry1(del); 2.55 ± 0.04 vs. 2.16 ± 0.07, p<0.01; Sry3(del) vs. Sry2; 2.55 ± 0.04 vs. 2.23 ± 0.08, p<0.05). Furthermore, providing Sry2 with a full length 25 aa Q-rich region, as in Sry2(+QR) elevates Sry2 activity to levels not significantly different than native Sry1 proteins, and significantly higher than Sry3(del) (p<0.01). Together, these results indicate that the deletion in Sry2 reduces protein activity, and that unlike the additive activities of native Sry1 and Sry2, Sry3-like proteins are more active even with a truncated Q-rich region. This may be a reflection of a single aa difference at position 76 ,T vs. P, in the HMG box C-terminal basic tail that is thought to act as a kinetic clamp(Li et al. 2006, Phillips et al. 2006, Ukiyama et al. 2001).

Removal of the Q-rich region reduces Sry activity to near basal levels

To facilitate these experiments we generated pEF1/Sry1, 2 and 3(-QR) constructs, which encode Sry1, 2 and 3-like proteins devoid of a polyglutamine region. Additionally, reports indicate full length Sry is required for DNA binding (Sanchez-Moreno et al. 2008) . Since most Sry research has been conducted with only the HMG
box, we wanted to assay its activity. Figure 3.4 shows that removal of the Q-rich region reduces Sry activity to or near basal levels and that all truncated constructs have activities significantly lower than native Sry2 (p<0.001). The HMG box shows no significant difference in luciferase activity compared to all Sry(-QR) chimeric proteins. Of these truncated proteins, only Sry3(-QR) had activities not significantly above threshold level.

Mutations to amino acids 20-21 in the nNLS abolish Sry activity.

Since the Sry NLSs are part of the HMG domain, we wanted to determine if aa differences at these locations alter protein activity. Sry2 exhibits the lowest activity of any native Sry protein tested. To establish if the presence of an H in the nNLS contributes to the reduced activity of Sry2, we generated the Sry2(R21) and Sry1(H21) chimeric constructs. Replacing H21 with an R significantly elevated Sry2 fold activity (Sry2 vs. Sry2(R21); 2.23±0.08 vs. 2.83±0.02, p<0.01). However, exchanging the R-H in Sry1 showed no reduction in Sry1 activity (Sry1 vs. Sry1(H21); 3.42±0.134 vs. 3.30±0.195, p=0.47). Site-directed mutations that replaced R20, H21 and K22 at the nNLS with A residues [Sry1(20A22)], reduced Sry1 activity to baseline levels. While mutations at the cNLS in, Sry1(78A79), reduced activity of Sry1 to levels below Sry2, but significantly greater than baseline (MT vs Sry1(78A79), 1 vs. 1.57±0.64, p<0.001). Mutations at both NLSs resulted in baseline activity (Figure 3.5).
Luciferase Activities Generated By Native Sry1, 2 and 3 Proteins

Figure 3.2. Elevation of pGL/AR600 reporter activity after induction by native Sry1, 2 and 3 proteins (mean ± SEM). MT control established basal luciferase activities (dotted line). All Sry proteins have significantly greater activities compared to the MT control (*p<0.001). Further, Sry1, 2, and 3 show activities significantly different from each other (in all comparisons p<0.001). Significance was determined using a One-way ANOVA and post-hoc Student-Newman-Kuels test.
Luciferase Activities Generated By Sry1(del), 3(del) and Sry2(+QR) Chimeric Proteins

Figure 3.3. Adding the Sry2 truncated Q-rich region to Sry1 and Sry3 proteins significantly reduced active of Sry1(del) and Sry3(del) compared to native Sry1(*p<0.001) and Sry3(**p<0.001) respectively. Sry1(del) activity is no different than Sry2, while Sry3(del) has activities significantly higher than both Sry1(del) and Sry2 (♦p<0.001). Providing a full length Q-rich region to Sry2, as in Sry2(+QR), elevates activity to levels no different than Sry1, and significantly higher than Sry2(♦p<0.001).

All gold bars represent native Sry transactivation differences and significance was determined using a One-way ANOVA and post-hoc Student-Newman-Kuels test.
Stimulation of Transcription by Truncated Sry Proteins

Figure 3.4. Removal of the Q-rich region as in Sry1(-QR), 2(-QR), Sry3(-Q) and Sry1(HMGbox) reduces Sry activity to levels significantly below wild type Sry2 proteins (*p<0.0001). No significant difference in activity is observed between these truncated proteins. However, HMGbox, Sry1(-QR) and Sry2(-QR), have activities significantly greater than MT (***p<0.001) while the activity of Sry3(-QR) is no different than baseline. All gold bars represent native Sry transactivation differences and significance was determined using a One-way ANOVA and post-hoc Student-Newman-Kuels test.
Stimulation of Transcription by NLS Mutated Sry Proteins

Figure 3.5. Adding the H from the Sry2 nNLS to Sry1(H21) had no effect on Sry1 activities. However, providing Sry2 with an R at this position, significantly increased Sry2(R21) activity compared to native Sry2 (♦ p<0.001). However, Sry2(R21) activities are significantly less than native Sry1 and Sry1(H21) (**) p<0.001). Sry with mutations to the nNLS as in Sry1(20A22) and Sry1(NoNLS) abolished Sry activity, as these proteins shown no significant difference when compared to MT. Mutation to the cNLS, as in Sry1(78A79), significantly reduced Sry1 activity compared to native Sry1 and Sry2(* p<0.001). However, unlike mutations to the nNLS, Sry1(78A79) activity is significantly greater than MT (x p<0.001). All gold bars represent native Sry transactivation differences and significance was determined using a One-way ANOVA and post-hoc Student-Newman-Kuels test.
Discussion

The purpose of this study was to elucidate transcriptional activity differences among the rat Sry proteins and identify regions or aa differences that could contribute to altered protein function. The results from this study demonstrate that Sry1, 2 and 3 proteins differentially activate a synthetic luciferase reporter construct containing sequences from the 5’ UTR of androgen receptor (AR) when cotransfected into CHO cells (Figure 3.2). Here we show that the small Q-rich activator region is essential for rat Sry regulation of transcription. This was demonstrated by a large drop in luciferase activity with the truncated Sry1, 2 and 3(-QR) proteins that do not encode a Q-rich motif (Figure 3.4). Further, because–QR proteins contain the entire hinge region, they also encode a conserved SIP-1 binding site (Figure 1.2). SIP-1 (Sry interacting protein-1) was first demonstrated to interact with human SRY and is believed to mediate activities with other transcription modulators in much the same way the Q-rich region may in rodents. At a different location in the hinge region, mouse SIP-1 is also shown to bind to Sry and perhaps regulate similar interactions (Thevenet et al. 2005). Results from this study indicate that if SIP-1 binding occurs in rat, this interaction does not contribute to Sry activity. This is supported by results obtained from Sry1 proteins encoding only the HMG box. If SIP-1 contributed to rat Sry activity, removing the hinge region should reduce activity compared to the –QR proteins, which encode a punitive SIP-1 binding motif. However, this was not observed. The HMG box proteins exhibit promoter activity not significantly different from Sry1, 2 and 3(-QR) (Figure 3.3). However, there is the possibility that SIP-1 is not expressed in CHO cells or if expressed lacks the ability
to interact with rat Sry. In these studies we did not test this possibility, however, if expressed it is unlikely binding would be perturbed, as consensus SIP-1 interaction sites are conserved across species.

Additionally, these studies demonstrate that the deletion in Sry2 decreases activity compared to Sry1 and 3 proteins. This became apparent from studies where chimeric Sry1 and Sry3 proteins encoding the Sry2 Q-rich region activated the reporter at levels comparable to wild type Sry2. Further, exchanging the truncated Sry2 activator for a full length (25 aa) Q-rich region [Sry2(+QR)] elevated Sry2 activity to a level not different from Sry1 (Figure 3.2). These results are consistent with transgenic studies that demonstrate that this region is required for testis determination in mice, since truncation of this region prevented sex reversal from occurring in XX transgenic mice expressing truncated Sry transgenes (Bowles et al. 1999). Interestingly, an association between expression level and protein structure has been suggested (Albrecht et al. 2003, Lee & Taketo 2001). Experimentation with consomic and transgenic mice has demonstrated that many mice with a truncated Q-rich domain, exhibit elevated expression levels compared to mice encoding Sry proteins with longer or full length activator regions to compensate for a less active protein. Interestingly, expression studies conducted in our lab support this hypothesis, since we have demonstrated that Sry2 transcript expression is greater than all other rat loci in all tissues examined. Furthermore, localization studies show that Sry2 exhibits reduced levels of nuclear import compared to Sry1 and 3. These results support the concept that Sry2 expression may be elevated to compensate for its reduced nuclear accumulation and intracellular activities.
Although not specifically tested for, we found that Sry3 and Sry3(del) consistently show activities significantly greater than corresponding Sry1 proteins. This suggests that regions beyond the Q-rich activator contribute to elevated Sry3 function.

The only aa difference in Sry3 not accounted for in these studies is the P to T substitution at aa 76 in the C-terminal kinetic clamp region of the HMG box (Phillips et al. 2006, Ukiyama et al. 2001). This difference is not conserved in any other mammalian Sry protein and may alter Sry-DNA and/or Sry-protein interactions that occur through the HMG domain, such as the protein-protein interaction described between Sry and androgen receptor (Yuan et al. 2001). Mutations at this site, P - R, are shown to reduce Sry mediated testicular development in humans (Phillips et al. 2006). However if the molecular mechanisms initiated by Sry3-like proteins are required for testis development in rat this may not be the case, as rat Sry3 exhibits robust activity.

Previous work with these chimeric and mutated constructs has shown that H21 in the nNLS of Sry2 reduces nuclear localization. Further, mutating aa 20-22, representing the basic aa’s RRK, to A in Sry1 similarly reduced nuclear translocation, while mutations at the cNLS resulted in a mostly cytoplasmic distribution. In the remaining studies we determined if and how these regions affect the ability of Sry to transactivate the synthetic pGL3/AR600 luciferase reporter. Using the Sry1(H21), Sry2(R21) and Sry1(del) constructs we conclude that H21 encoded in Sry2 does not contribute to Sry activity. Proteins generated from Sry1(H21) and Sry1(del) exhibit activities that can be predicted by the presence or absents of the 13 aa Q-rich deletion. Thus, Sry1(H21) containing the full length Sry1 activator, promoted luciferase activities not significantly
different than native Sry1 (Figure 3.5). Further, Sry1(del), which contains the truncated Sry2 Q-rich region and the Sry1 nNLS, has activities no different than native Sry2 (Figure 3.3). Conversely, Sry2(R21) mediated promoter induction was significantly elevated compared to native Sry2 (Figure 3.5). This result is puzzling and should be addressed by future studies focusing on Sry1 DNA interactions and the resulting binding affinity. These results are consistent with studies of sex reversing mutations in humans occurring at aa 76, the location corresponding to H21 of rat Sry2. From this research of human mutations, the primary functional defect was determined to be reduce nuclear localization, as DNA binding affinities were only slightly affected (Mitchell & Harley 2002, Harley et al. 2003b).

Human cases of sex reversal caused by mutation of R75 to and N, are associated with large reductions in DNA binding affinity. In rat, the analogous aa position is R20 in the nNLS. Consistent with results of human mutations, is the total loss of Sry1 activity by mutations to R20, 21, and K22 as in the Sry1(20A22) construct. As mentioned above this directed mutation reduced, but did eliminate nuclear localization, however, there was no statistical difference between the activities of this protein and the empty vector control (Figure 3.5). Mutations changing R78 and K79 to A in the rat cNLS reduced Sry1 activity to levels significantly lower than Sry2. Two human mutations that change the conserved R at position 78 to either a Q or a W result in poor nuclear localization and slightly reduced DNA binding affinities, which is consistent with both our localization and transcriptional activity studies.
In summary, we show that the multiple Sry proteins of rat, due to regional and single aa differences, could regulate transcription at target promoters differentially. Further, these results demonstrate that this activity is dependent on a full length protein and the presence of a C-terminal Q-rich activation motif, which if removed reduces protein activity up to 310%.

Directed mutations of regions shown in human Sry to reduce nuclear transport or DNA binding affinities were reflected in corresponding alterations in luciferase activities. Together with previous expression and intracellular localization studies, these data support the concept that the multiple Sry proteins of rat could have unique tissue-specific functions.
CHAPTER IV

Analysis of Sry Posttranslational Modifications

Introduction

The functional characteristics of transcription factors and many other proteins are often linked directly to posttranslational modifications such as ubiquitination (Makki et al. 2008), sumoylation (Pichler & Melchior 2002), phosphorylation and acetylation. Among these modifications, phosphorylation of transcription factors is quite widespread, and the addition or deletion of a phosphate group can modulate potential activity by enhancing (or reducing) interactions with coregulators and DNA sequences. In human, Sry is phosphorylated at amino acid 33 in the N-terminal HMG box flanking region (Figure 1.2), and phosphorylation is reported to enhance DNA binding (Desclozeaux et al. 1998). In rat and mouse Sry, the phosphorylation site described in human is not present because rodent Sry proteins do not include this flanking region. However, analysis of rat Sry sequences using online analysis tools, such as the GPS and NetPhos2.0 phosphorylation prediction servers (Blom et al. 1999, Xue et al. 2005), identify up to six punitive phosphorylation sites at serine and threonine residues throughout the Sry protein. Since the multiple Sry proteins of rat exhibit great aa conservation, and aa’s adjacent to all predicted phosphorylation site are the same in each protein, it is likely that each isoform will be phosphorylated in a similar manner. Nevertheless, to determine if these
proteins are phosphorylated and to begin to understand the contribution of this modification to protein-protein interactions or to altered DNA binding affinities, it is necessary to characterize each protein’s phosphorylation state.

In addition to phosphorylation, sumoylation is another important posttranslational modification that can regulate transcription factor activity (Verger et al. 2003). Targets of this modification include proteins such as p53 and RanGAP, whose cellular activities are regulated by SUMO conjugation (Joseph et al. 2002, Rodriguez et al. 1999).

Sumoylation is a dynamic modification resulting in the covalent linkage of one or more 10-15 kD SUMO (small ubiquitin-like modifier) proteins to lysine residues of a target protein through an isopeptide bond (Pichler & Melchior 2002). The aa motif targeted by modulators of SUMO is \( \Theta K \Psi E \), where \( \Theta \) is any hydrophobic aa, \( K \) is the modified lysine residue, \( \Psi \) is any aa, and \( E \) is glutamic acid; however at this last position many alternative aa’s can exist. Like protein ubiquitination, the addition of SUMO proteins is carried out in an ATP dependent three step process utilizing SUMO-specific E1 activating, E2 conjugating and E3 ligating enzymes (Melchior et al. 2003). However, many sumoylated proteins are reported to have endogenous E3 ligase activity (Desterro et al. 1999), thus for sumoylation of many proteins only the E1 and E2 enzymes are required. Unlike most ubiquitination, which acts as tag that targets a polyubiquitinated protein to proteosomal degradation, sumoylation is linked to modulating the subcellular compartmentalization and activity of modified proteins through addition and removal of SUMO proteins in a sub-compartment dependent manner (Pichler & Melchior 2002). Additionally, sumoylated proteins are often protected from ubiquitination due to
competition for the same aa consensus, therefore sumoylation also increases protein longevity (Desterro et al. 1997).

Currently four SUMO isoforms have been identified: SUMO1, SUMO2, SUMO3 and SUMO4 (Hilgarth et al. 2004). Of these proteins, SUMO2 and SUMO3 are most similar to each other with sequence identity of over 90%. SUMO2/3 are both capable of polysumoylating target proteins (Hecker et al. 2006). The aa sequence of SUMO1 is distinct and this form is only capable of monosumoylating target proteins. Additionally SUMO-1 and SUMO-2/3 exhibit unique cellular distributions and substrates. However the mechanisms allowing substrate targeting are for the most part unidentified. For example SUMO-2/3 maybe involved in protein protection while SUMO-1 is not (Hecker et al. 2006), although very similar binding motifs are targeted. Unlike SUMO1/2/3, which are found in most mammalian tissues, SUMO4 exhibits tissue specificity. However, the function of this isoform is not know, as only transcripts have been identified in the kidney (Bohren et al. 2004).

The action and cellular longevity of many transcription factors is thought to be mediated by sumoylation. Importantly, several Sry-box related (SOX) proteins, such as SOX2, SOX3, SOX9 and SOX10 are regulated by sumoylation, where the addition of SUMO-1 leads to transcriptional inactivation and/or elevated cellular concentration (Savare et al. 2005, Girard & Goossens 2006, Hattori et al. 2006, Tsuruzoe et al. 2006). The HMG box of all mammalian Sry proteins encodes two aa motifs identified as punitive sumoylation sites by the SUMOsp sumoylation prediction server (Xue et al. 2006). Interestingly, these motifs are also a part of the nNLS and cNLS (Figure 4.1).
Perhaps sumoylation also contributes to subcellular accumulation and activity of Sry in rat tissues.

The recent work by Turner et al. (Turner et al. 2007) has demonstrated that each of the rat Sry loci is expressed in vitro. One tissue shown to express Sry is the adult testis. Repeated attempts to identify Sry proteins in the testis and other tissues using Western blots probed with a goat anti-mouse Sry (E-19) primary antibody (Santa Cruz) have been unsuccessful. However, when expression vectors encoding Sry proteins with a c-Myc epitope (pEF1/Sry constructs) were introduced into adult rats, Western blots of multiple tissue extracts using an anti-c-Myc epitope primary antibody (Bethyl), were able to detect the Sry fusion proteins. Yet, Western blots conducted under the same experimental condition, using the Sry (E-19) antibody did not detect Sry. Interestingly, the epitope for Sry (E-19) includes the Sry nNLS. These preliminary studies may indicate that a modification occurs at or near the N-terminus of rat Sry that masks the epitope targeted by Sry (E-19) antibodies. Since a punitive SUMO binding motif exists in this epitope, sumoylation is a possible candidate modification. The aim of the current study is to determine if the multiple Sry proteins of rat are posttranslationally modified by sumoylation and phosphorylation. This will establish the ground work for future studies focused on possible posttranslational regulation of Sry protein function.
Methods

Production and Isolation of recombinant prokaryotic Sry proteins

The open reading frame of Sry3B1 was subcloned from Sry3B1/pCDNA3.1 constructs into the prokaryotic expression vector pIVEX2.4 (a gift from Dr. D. Stroup) with the SrySmaRT and SryNcoLT primer set (see Appendix). Constructs were then transformed into E. coli BL21 (De3) (Lucigen). Sry proteins generated from this construct encode an N-terminal 6X histidine tag used for protein purification. Growth of 20mL E. coli BL21 (DE3) cultures was monitored. When cell density was at $A_{600} = 0.8$, Sry protein synthesis was induced with addition of isopropyl-1-thio-β-D-galactopyranoside (IPTG) to a final concentration of 0.75 mM. Two hours after induction cells were collected by centrifugation at 10,000 x g for 10 min (4°C). Pellets were resuspended and lysed in 2 mL lysis buffer (500mM NaCl, 20mM NaHPO$_4$, 25mM Imidizole, 2.0 mg/ml lysozyme, 1mM PMSF, 0.5 mµM Leupeptin, 1X CelLytic B (Sigma), 10 U DNAse I, 25mM MgCl, pH 7.6) for 15 min at room temperature. Lysates were then diluted with six volumes phosphate buffered imidizole (PBI) (500mM NaCl, 25mM Imidizole, 20mM Na$_2$HPO$_4$) followed by addition of 75μl Ni Sepharose 6 Fast Flow beads(GE Healthcare). After 45-60 min incubation at 23°C on a rotator, the Ni resin was collect by centrifugation at 500 x g for 2 min and the supernatant was decanted. The Ni beads were then washed four times with 1.5 ml PBI. After removing the final wash, Sry proteins were eluted with addition of 500 μL elution buffer (PBI buffer containing 500mM imidizole). Isolation of Sry was confirmed by SDS PAGE and Western blot analysis. Proteins were aliquoted and stored at -80°C in elution buffer.
Eukaryotic Expression Construct pEF1/Sry3B1

Sry3B1 fusion proteins containing a carboxy terminal c-Myc epitope and histidine tag were generated by amplifying the open reading frames of Sry3B1 using primers L-BamH1/KozakSry (5’-ctgatcgaaccatggggccatgtaaag) and R-Not1-StopcodonSry (5’-ctagcggccgcgtggaacttgtgctgct), from the pcDNA3.1- clones described previously (Figure 2.1). Amplicons generated began at the Sry translation start codon (underscored in L-BamH1/KozakSry primer), encoded a 5’ Bam H1 and 3’ Not 1 cleavage sites for cloning (bold), a Kozak sequence for efficient translation (red text) and elimination of the endogenous Sry stop codon (underscored in R-Not1-StopcodonSry primer) to facilitate generation of the His-tagged fusion protein (Table 2.1; Figure 2.1). These amplicons were cloned into the pEF1/Myc-His vector (Invitrogen), producing the pEF1/Sry3B1 expression construct. This construct was transformed into E. coli TAM-1 competent cells (Active Motif) and the identity of each clone was confirmed by sequencing on an ABI 3130xl genetic analyzer using BigDye Terminator sequencing chemistry (Applied Biosystems).

Cell culture and transient transfections

pEF1/Sry3B1 plasmid DNA was prepared for transfection using endotoxin free Maxipreps (Qiagen). Chinese Hamster Ovary (CHO) cells were cultured on 6 well cassettes (Falcon) in HAM’s F12K medium (Sigma) supplemented with 10mM HEPES and 10% fetal bovine serum (Atlanta Biologicals) in a humidified atmosphere at 37°C and 5% CO₂. Cells were grown to approximately 50-70% (~1.5x10⁵ cells/cm²)
confluence and then transiently transfected with 3 µg plasmid DNA using ExGen500 transfection reagent (Fermentas) following the manufacturer’s suggested procedure. During all trials a single control well was transfected with the pEF1/MT negative control, that contains no insert. 24 hrs post-transfection cells were collected and centrifugation at 300 x g for 5mins at 4°C followed by three washes with 4°C PBS supplemented with 1mM PMSF, 20mM NaFl, 50µM leupeptin and 1µM pepstatin A. Pellets were stored at -80°C. Sry fusion proteins were batch purified using Nickel resin (GE Healthcare) as described above.

**Generation of anti-Sry124, an anti-rat Sry antibody**

Antibodies against rat Sry peptide were generated in rabbits by Sigma Genosys using Peptide 1 (DWARAAHQSSKNQKS) corresponding to amino acids 116-130 of Sry1. Immune rabbit sera was applied to an affinity column containing Peptide 1 covalently linked to cyanogen bromide-activated Sepharose 4B (GE Healthcare). Purified anti-Sry124 antibodies were aliquoted and stored at -20°C.

**SDS PAGE and Western blot analysis**

Proteins were quantified using a Comassie Plus or Bicinchoninic Acid protein assay following provided protocols (Thermo/Pierce). Samples were prepared for electrophoresis by denaturation for 5 mins at 95°C in reducing sample buffer (10% glycerol, 2% SDS, 50mM DTT, 62.5mM Tris-HCl, 0.0003% Bromophenol blue, pH 6.8) and 35µg of each was separated on 13.5% SDS-polyacrylamide mini gels and transferred.
to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked in PBS containing 4% (w/v) nonfat dry milk and 0.1% Tween-20, unless protein phosphorylation was to be detected. Then membranes were blocked in Zymed Blocking buffer (Zymed), as milk contains phosphorylated proteins such as caseins. Western Blot analysis was performed using anti-Sry antibodies Sry E-19 (1:300) (Santa Cruz), anti-Myc epitope (1:5000) (Invitrogen), anti-Sry124 (1:500), monoclonal anti-phosphoserine (1:400) (AbCam) and monoclonal anti-phosphothreonine (1:250) (Zymed) primary antibodies. After incubation with the secondary antibody (see APPENDIX) immunoreactive protein bands were detected and captured on a Kodak Gel Logic 2200 gel documentation system after addition of SuperSignal West Pico chemiluminescent substrate (Thermo).

**Magnetic DNA Affinity Purification of Sry Proteins**

Double stranded biotinylated oligonucleotides containing a single Sry response element were generated by annealing equal molar amounts (15µM) of the following oligonucleotides (5’ biotin-agcttcagtcctaaacaatagtagatga and the compliment 5’-tcatctactattttaggactgaagct) at 85°C for 5 mins in buffer B1 (50mM NaCl, 10mM Tris pH 8, 1mM EDTA), and cooling to room temperature (RT) over 2 hrs. The double stranded oligo nucleotides were then conjugated to Strepavidin MagneSphere® Paramagnetic Particles (SA PMP) (Promega) in a 20 min reaction at RT followed by three washes in buffer B2 (500mM NaCl, 10mM Tris pH 8, 1mM EDTA). CHO cells transfected with Sry expression constructs and *E. coli* BL21 (DE3) cells expressing Sry were separately lysed, purified and total protein content estimated as described above.
Total protein (35 µg) was then added to 500 µL buffer B3 (10mM Tris pH8, 40mM KCl, 3% glycerol, 0.05% NP 40, 1mM EDTA, 1mM PMSF, 1mM DTT, 50µM leupeptin, 1µM pepstatin, 20mM NaF), containing 100 µL SA PMP conjugated oligos and incubated overnight at 4°C on a rotator. Unbound proteins were removed from the complexes and bound Sry was collected by addition of 1X SDS sample buffer and analyzed by Western blot. Purified histidine-tagged Sry1, 2 and 3B1 proteins used in these tests were first desalted and then incubated with the SA PMP DNA conjugates in B3 buffer.

**Sumoylation**

Analysis of Sry protein sequence using the SUMOplot online sumoylation prediction server (Xue et al. 2006), identified two possible SUMO interaction sites in rat Sry proteins. Based on this prediction and current literature that demonstrated SUMO regulation and interactions with other SOX proteins, we tested to see if Sry is also a target of this posttranslational modification. Sry proteins were isolated and purified from CHO cells and *E. coli* BL21 (DE3) as described above. Potential Sry interactions with SUMO-1, SUMO-2 and SUMO-3 were tested using the protocol, reagents, enzymes and anti-SUMO-1, 2 and 3 antibodies included with a BIO MOL International Sumoylation Kit. In all reactions ~ 0.5-2 µg of Ni Sepharose 6 Fast Flow purified and desalted Sry was used. The only alteration to the procedure was use of the provided antibodies at 1:1500 vs. the recommended 1:4000 dilution. After optimization all reactions were
conducted at least two times with Sry3B1 proteins generated in CHO cells and Sry1 and 2 in proteins produced in *E. coli* BL21 (DE3) cells.

**Results**

**Sumoylation**

Analysis of all rat Sry protein sequences using the SUMOplot server (Xue *et al.* 2006), identified two potential SUMO target sites (aa 5-8 and 80-83) (Figure 4.1). Isolated Sry proteins generated in both CHO cells and *E. coli* BL21 (DE3) did not interact with any of the three SUMO isoforms. Western blots were determined valid by the presence of strong antibody interactions with unbound SUMO proteins and substrate bound forms interacting with the RanGAP1 positive control protein (Figure 4.2). This indicated that (1) all enzymes, buffers and other components of the kit performed properly during each reaction and (2) the blotting protocol was optimized. Thus, rat Sry is not a target of direct sumoylation by addition of any of these three SUMO proteins. These results were obtained from two trials using Sry3B1 generated in CHO cells and two trial with Sry1 and Sry2 isolated from *E.coli* BL21 (DE3) with each SUMO isoform.

**Detection of Immunoreactive Sry in Cultured Cells and Tissues**

Sry generated and isolated from CHO cells and *E. coli* BL21 (DE3) is readily detectable on Western blots using a goat anti-mouse Sry (E-19) antibody (Santa Cruz) (Figure 4.3). The exact epitope targeted by this antibody is proprietary information (Santa Cruz); however, it is thought to be the first 19 aa of rat and mouse Sry (Figure
4.1). Only Sry proteins generated in bacteria were detectable with the affinity purified rabbit anti-Sry124 antisera (Figure 4.3). This was taken to mean that at the epitope targeted by anti-Sry124, there may be posttranslational differences between Sry produced in a eukaryotic vs a prokaryotic system. Sequence analysis of this epitope found a probable phosphorylation site at serine 124 (Table 4.1).

**Sry Phosphorylation**

Results from an online phosphorylation prediction tool, NetPhos2.0 server (Blom et al. 1999), identified up to six potential phosphorylation sites, five at serine residues and two at threonine residues in the Sry3B1 protein (Figure 4.1). The only predicted phosphorylation difference among Sry proteins is in Sry3-like proteins which encode a threonine residue at position 76 rather than a proline as in Sry1 and Sry2. Western blots of Sry3B1 proteins which contain all possible phosphorylation sites, probed with anti-phosphoserine and anti-phosphothreonine antibodies With Sry generated in CHO cells, show that Sry is potentially phosphorylated at one or more serine residues; however, phosphorylation at threonine residues was not detected (Figure 4.4). Further, Western blots of Sry proteins produced in *E. coli* BL21 (DE3) cells showed no immunoreactive protein with either the anti-phosphoserine or anti-phosphothreonine antibody (Figure 4.4).
DNA Affinity Purification

To begin to understand if phosphorylation state has an effect on Sry-DNA interactions, DNA affinity purification was conducted. From these studies it was found that all Sry generated in CHO cells, and thus believed to be phosphorylated, was captured by the DNA oligo bait containing a single Sry response element. Under the same experimental conditions, approximately half as much bacterially expressed Sry was able to be captured compared to Sry generated in CHO (Figure 4.5). At first this was thought to indicate that phosphorylation of rat Sry enhances DNA binding. However, further analysis did not support this assumption and the observed reduction in the capture rate of Sry was identified to be the result of the N-terminal histidine tag encoded on the bacterially generated Sry proteins.
Prediction of Phosphorylation Sites, the SUMO Binding Site, and Epitopes of Sry(E-19) and anti-Sry124 antibodies in the Sry3B1 Protein

Figure 4.1. All rat Sry proteins contain at least 6 potential phosphorylation sites at serine and threonine residues. Sry3-like proteins have one additional phosphorylation site at residue 76, which is T. Above is a NetPhos2.0 prediction server image (Blom et al. 1999) of predicted serine and threonine phosphorylation sites in Sry3B1. I have added the aa positions. Additionally, the provided image of Sry3B1 demonstrates the relative locations of the potential phosphorylation sites, predicted sumoylation sites (green bars), the predicted epitope for the Sry(E-19) antibody (black bar) and the predicted epitope of anti-Sry124 antisera (red bar). The blue area represents the HMG box and the orange region is the Q-rich activator.
Western Blot of Sry3B1 Sumoylation Reaction with SUMO-1

Figure 4.2. This Western blot is a representative of all Sumoylation reactions with Sry3B1 expressed in CHO and Sry1 and 2 expressed in BL21(DE3) cells. Each conjugation reaction was completed twice with SUMO-1, 2, and 3 proteins. Lane 1, reaction with SUMO-1 and Sry3B1. The band at lane 1, band B is thought to be an artifact of the sumoylation reaction and not a SUMO-1 interaction with Sry as this band is consistently observed in reaction without addition of Sry. Lane 2, negative control, contains RanGAP1 target, SUMO-1 and no ATP or Mg. Lane 3, positive control, showing RanGAP1 sumoylation (lane 3, band A). In all lanes arrow C represents free unbound SUMO-1.
Analysis of Sry isolated from *E. coli* BL21 (DE3) and CHO Cells with goat anti-mouse Sry (E-19) and anti-Sry124 antibodies

Figure 4.3. Bacterially expressed Sry detected by Sry (E-19) (Santa Cruz) (lane 1), anti-Sry124 antisera (lane 2) and a negative control (lysate obtained from *E. coli* BL21 cells not transformed with Sry and probed with anti-Sry124, lane 3). Lane 4, Sry expressed in CHO cell detected with Sry (E-19). Lane 5, negative control probed with Sry (E-19) (CHO cell lysate transfected the MT control vector). Lane 6, Sry expressed in CHO cells and probed with anti-Sry124 shows no reactive band. These results indicate that in a eukaryotic system rat Sry may be posttranslationally modified at or near aa 124, within the epitope targeted by anti-Sry124.
Rat Sry is Phosphorylated at Serine But Not Threonine Residues

Figure 4.4. Sry3B1 proteins purified from transiently transfected CHO cells and Western blotted show that Sry is potentially phosphorylated at one or more serine residues and is not phosphorylated at threonine residues. Lane 1, the positive control shows Sry3B1 as detected by the anti-mouse Sry(E-19) (Santa Cruz) antibody. Lane 2, an immunoreactive protein at the same estimated molecular weight as Sry3B1 probed with an anti-phosphoserine primary antibody (AbCam). Lane 3, no detectable reaction using the anti-phosphothreonine antibody (Zymed). An additional three trials using Sry3B1 produced in *E. coli* BL21 (DE3) yielded no detectable phosphoamino acids. These results indicate that in a eukaryotic system rat Sry may be posttranslationally modified at serine residues.
Western Blot of DNA Affinity Captured Sry Proteins Expressed Bacterially in E. coli BL21 (DE3) and in Transiently Transfected CHO Cells

Figure 4.5. Lanes 1 and 3 represent protein fractions of Sry not captured (unbound) by immobilized DNA oligonucleotides encoding an Sry response element. Lanes 2 and 4 show Sry proteins that interacted with the DNA oligonucleotides and eluted. Both Sry expressed in CHO cells and bacterial cells interact with the Sry response elements. However, when equal amounts of protein are added to each reaction, all Sry expressed in CHO interacts with the DNA while interactions of Sry expressed in E. coli BL21 (DE3), containing an N-terminal histidine tag, are less robust as indicated by the presence of Sry in the unbound fraction (lane 1).
Table 4.1. Comparison of antibody reactivity to Sry3B1 proteins expressed in *E. coli* and CHO cells. The goat anti-mouse Sry (E-19) antibody interacts with Sry near the N-terminus. Western blots probed with this primary antibody detect Sry generated in both eukaryotic and prokaryotic systems. However, the anti-Sry124 antibody, which interacts with Sry at an epitope within the following sequence, DWARAHQ$\text{SSKNQKS}$, located in the hinge region between aa’s 116-130, is only able to interact with Sry generated in a prokaryotic system. This may indicate that Sry isolated from CHO cells is posttranslationally modified within this region. One predicted modification is phosphorylation at S residue 124, which is shaded red in the sequence above.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Sry3B1 extracted from <em>E.coli</em> BL21 (DE3)</th>
<th>Sry3B1 isolated from transfected CHO cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sry(E19)</td>
<td>Sry is detected</td>
<td>Sry is detected</td>
</tr>
<tr>
<td>anti-Sry124</td>
<td>Sry is detected</td>
<td>Sry is not detected</td>
</tr>
</tbody>
</table>
Discussion

While sumoylation of SOX proteins has been reported, this interaction occurs at binding motifs in regions flanking the HMG box having slightly different aa motifs. In Sry this site (xKxP) contains a proline while all SOX motifs (xKxE) contain a glutamic acid at the most C-terminal position. This difference may have contributed to results obtained in these studies, which demonstrate that rat Sry is not a substrate for SUMO modification. Sumoylation reactions with Sry3B1 proteins expressed in CHO cells and Sry1 and 2 expressed in *E. coli* BL21(DE3) were not reactive with any of the three SUMO isoforms. However, positive and negative control reactions involving RanGAP1, showed positive reactions in the presence of ATP and no SUMO conjugation in reactions not containing ATP or Mg. This indicates that the experimental procedures and reactions conducted were valid. Because the mechanisms involved with SUMO conjugation are thought to be conserved between species, it is unlikely that the human SUMO proteins used in these studies would not interact with rat Sry.

These studies show that rat Sry proteins are potentially phosphorylated at serine but not threonine residues. While the number and location of phosphorylation sites is yet to be determined, predictions from the NetPhos2.0 server (Figure 4.1) and western blots with anti-Sry124 antisera, which detect Sry proteins isolated from and BL21(DE3) E. coli cells but not CHO, indicate possible locations could be serine residues 124 and/or 130. In these studies, it was found that anti-Sry124 can interact with Sry produced in bacteria, but not in CHO cells (Table 4.1). One explanation for these results is Sry expressed in CHO may be phosphorylated within the epitope targeted by this antibody, and the
modification perturbs anti-Sry124 binding; the assumption being that a eukaryotic cell will readily phosphorylate a eukaryotic protein and a prokaryotic cell will not (Figure 4.3). These residues and over 10 aa’s flanking these positions are conserved in all rat Sry protein isoforms, therefore any functional change induced by a modification would likely affect each protein equally. Of the punitive phosphorylation sites identified, only one, T76 in all rat Sry3-like proteins, has the potential to differentially regulate Sry3 function. However, from these studies, there is no evidence that threonine residues are phosphorylated (Figure 4.4).

Because previous reports indicate that human Sry is phosphorylated only in the N-terminal HMG box flanking region, the results from this study are the first to suggest that between species phosphorylation at alternative locations may occur. Further, while Sry aa conservation between species is limited in region outside the HMG box, this result supports the concept that functionally significant cryptic motifs are possibly conserved.
CHAPTER V

CONCLUSION

Earlier work by Turner et al. (Turner et al. 2007) identified multiple Sry loci on the rat Y chromosome that exhibit expression level differences in more than one adult tissue. This work led to the question: could the differences among the Sry proteins alter function? The overall purpose of this research was to identify and characterize differences in protein structure that could potentially modify intercellular localization and activities of the multiple Sry proteins encoded on the rat Y chromosome. Collectively, the results of these studies support the overall hypothesis: the multiple Sry proteins encoded on the rat Y chromosome differentially activate target genes, and localize differently in cells and that these conditions are a reflection of their slightly different amino acid sequences. Below, an overview of the results directed by the four specific aims is summarized. Following this section, a discussion of what the current results potentially mean in conjunction with earlier expression studies from our lab about Sry activity in rats. Following the discussion will be a presentation of future directions.

Results of indirect immunoflorescent studies with wild type, chimeric and mutated Sry1, 2 and 3 proteins support the hypothesis for Aim I. These studies show that among the three Sry protein groups Sry2 exhibits reduced nuclear import and this is a
result of an H vs. R at position 21 in the nNLS. Additional tests with chimeric and Q-rich truncated Sry1/3 constructs showed that the Sry2 deletion and the entire Q-rich motif contribute little to Sry cellular distribution. Further, site directed mutations to the nNLS and cNLS demonstrate that both NLSs are required for complete nuclear localization. However in rat, mutations to the cNLS reduce nuclear import more than mutations to the nNLS. Thus, under the same experimental conditions nuclear targeting of Sry is directed mostly through the cNLS. These results support the concept of rat Sry nuclear localization by an importin β/Ran GTP dependent pathway rather than a calmodulin mediated mechanism.

The purpose of Aim II was to determine if differences in aa composition at the NLSs alter the ability of Sry to transactivate the luciferase reporter construct pGL3/AR600. Data collected from trials with Sry containing site-directed mutations to the nNLS and cNLS support the hypothesis for Aim II. Mutations to the nNLS, Sry1(20A22), abolished Sry function; luciferase activity mediated by these proteins showed no significant difference from the MT control. Likewise, mutating the cNLS, Sry1(78A79), significantly decreased Sry activity, however, these proteins were still active at a reduced level. These data are constituent with results from DNA binding studies conducted in other labs with human Sry containing sex-reversing mutations at the same regions. Significantly, mutations at R75 (R20 in rat) in the human nNLS prevented formation of an SRY-DNA complex (Harley et al. 2003b, Mitchell & Harley 2002), while mutations at R133 (R78) in the cNLS reduced the duration of Sry-DNA interactions but not DNA binding (Li et al. 2006, Li et al. 2001). These results suggest
that the amino acids in this region are required for Sry function as a transcriptional regulator. Mutation analysis confirms that aa at the cNLS contribute less to Sry function than amino acids mutated at the nNLS.

Analysis of the native Sry2 nNLS, encoding H21, showed that this difference has little to no significant effect on Sry activity. In these trials, Sry1 containing an H vs. an R at position 21 showed no reduction in activity. Therefore, these data suggest that in native Sry2, H21 reduces nuclear import although it has little effect on potential Sry2 transcriptional function. However, exchanging H21 with an R significantly elevated Sry2 activity, yet not to levels comparable to Sry1. Again, DNA binding studies of human Sry containing mutations at this position (R76) are consistent with the above studies of rat Sry activity. In the human mutation, DNA binding was not affected and sex reversal was considered to be a result of reduced Sry nuclear import (Mitchell & Harley 2002).

The studies proposed in Aim III were designed to test the activity of wild type and mutated Sry proteins encoding manipulations to the Q-rich activator and nuclear localization signals. Sry1 and Sry3 proteins with the 13 aa deletion of Sry2 support the concept that the deleted region is required for rat Sry function. Sry1(del) showed transcriptional activities no different from wild type Sry2. These results demonstrate that Sry2 activity is diminished by the deletion in the Q-rich region. Interestingly, while Sry3(del) showed marked reduction in activity compared to wild type Sry3, this protein chimera exhibited activities significantly above Sry1(del) and Sry2. This suggests that in Sry3 proteins, regions outside of the Q-rich activator must also contribute to activity. The most likely candidate aa difference in Sry3 that could alter protein function is the
presence of a T vs. a P at position 76 in the C-terminal kinetic clamp region of the HMG box. Work from other labs show that mutations to this aa reduce Sry activity (Phillips et al. 2006). However, in the current study this aa difference seems to enhance Sry3 protein function. Further, removal of the activator region, as in Sry1, 2, 3, and HMG box proteins, demonstrates the functional importance of this region. All of these truncated proteins exhibited the same near baseline reduction in activity. However, Sry3(-QR) protein activity was no different than basal levels. Nonetheless, these studies demonstrate that in rat, the most robust Sry protein activity is dependent on a full length Q-rich activator, and therefore these studies support the hypothesis for Aim III.

The purpose of Aim IV was to identify whether potential posttranslational modifications, phosphorylation and sumoylation, could regulate Sry protein interactive and localization differences. From these studies, it was determined that Sry is not a substrate for sumoylation, because no immunoreactive bands were detected on Western blots probing for Sry sumoylation. However, phosphorylation at one or more serine residues, possibly serine 124, was shown to occur. Therefore the hypothesis for Aim IV was partially supported. In addition, these studies demonstrated that Sry1, 2 and 3B1 can interact with and be isolated using DNA affinity techniques. However, Sry shows reduced DNA binding when an N-terminal His tag is present.

These results demonstrate that the multiple Sry proteins expressed in rat exhibit unique intracellular activities and localization patterns. Further, each locus is expressed differently in a single tissue. Between tissues this profile also changes (Turner et al. 2007). However, in most tissues, Sry2 is consistently expressed at the highest relative
level. This result is unusual in light of the current studies demonstrating that both nuclear accumulation and activity are reduced in Sry2 proteins compared to the other Srys. One possible explanation comes from research based in consomic and transgenic mouse lines. In these studies an observation has been described that indicated mice with \textit{Sry} loci encoding a truncated Q-rich activator show elevated expression levels compared to strains encoding a full length protein (Nagamine \textit{et al.} 1999, Lee \& Taketo 2001, Albrecht \textit{et al.} 2003). Therefore, in these mice transcriptional regulation may have evolved to provide elevated Sry concentrations to compensate for the reduced activity of a less robust protein. Likewise, in the rat, elevated Sry2 expression may also reflect this protein’s reduced activity and nuclear import. Alternatively, reduced expression of Sry1 and 3 could represent the relative transactivational strength of these proteins. Thus with respect to activity level, Sry1 and Sry3 proteins could have as much if not more effect on transcriptional events when expressed at lower levels, than Sry2 when expressed at elevated levels. Therefore, even a minor change in the expression level of Sry1 or Sry3-like proteins could have a tremendous physiological effect that would not be observed in the presence of large elevations in Sry2 expression. While it is more exciting to view Sry as an activator of transcriptional events, these activity differences could also work in the opposite direction to modulate transcription repression.

In the section above it is implied that these multiple Sry proteins are competing for the same limited number of target response elements. While no conclusive empirical data pertaining to DNA binding affinity has been generated for these proteins, from the activity studies it could be hypothesized that Sry2 and Sry1 may have near identical DNA
interactive characteristics. This is implied from analysis of results of activity of the chimeric Sry1(del) and Sry1(H21) proteins. Because removing the Q-rich region or exchanging R21 for H21 in Sry1, resulted in activities not significantly different from wild type Sry2 or Sry1 respectively, this indicates similar DNA interactions are occurring. Therefore, Sry1 and Sry2 may in fact compete for the same response element and interact with very similar affinities. However, Sry3 exhibits an activity profile that is not based entirely on the Q-rich region. The aa difference that may contribute to altered Sry3 activity (T76) is located in the HMG box region identified as a kinetic clamp. In human, this clamp region is shown to modulate the lifespan of the Sry DNA complex (Phillips et al. 2006). In rat this difference could alter Sry3 binding properties with DNA or other proteins. Thus Sry3-like proteins may be involved in initiating or modulating cellular cascades that are different from those regulated by Sry1 and Sry2.

Experimentation monitoring the in vivo activities associated with Sry3 has only just begun. However, preliminary data appears to support the concept that Sry3-like protein activity is involved with regulating cellular pathways that are different from Sry1 or Sry2.

Future directions

The studies presented here have demonstrated that among the multiple Sry proteins expressed in rat proteins Sry1, 2 and 3 have unique biochemical attributes that contribute to altered intracellular activity. A next plausible step would be to thoroughly examine the activities among Sry3-like proteins. In preliminary studies for this research project the transcriptional activities of Sry3B and 3B1 were briefly examined and both
seemed to function in a manner similar to Sry3. However, with the recent discovery of Sry3A in only the SHR genome, these studies needed to be completed. Additionally, the biochemical contributions of T76 in the kinetic clamp region of Sry3 proteins needs to be directly tested using site directed mutations. In addition, experiments focusing on Sry3 DNA binding affinity, bending and complex duration should be addressed. Experiments of this nature would contribute to an overall understanding of Sry structure and function. Further, analysis of protein-protein interactions through binding events facilitated by the HMG box need to be explored with each Sry protein. Using androgen receptor as a target protein these studies could identify differences in protein binding affinity among the rat Sry proteins and could elucidate altered Sry activates. Finally, to begin to understand how Sry regulates the activation or repression of a target promoter we must identify coregulators that assemble at the promoter with Sry. To identify these potential protein interactions, techniques such as Southwestern blotting and chromatin immunoprecipitation should be employed.
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Graves PE, Erickson RP. 1995. Sequence variation in the 5', putative promoter of Sry and its possible relevance to the C57BL/6J-YDOM sex reversal. *Biochem Biophys Res Commun* 208: 624-28


Harley VR, Clarkson MJ, Argentaro A. 2003a. The molecular action and regulation of the testis-determining factors, SRY (sex-determining region on the Y chromosome) and SOX9 [SRY-related high-mobility group (HMG) box 9]. *Endocr Rev* 24: 466-87


Lee CH, Taketo T. 2001. Low levels of Sry transcripts cannot be the sole cause of B6-Y(TIR) sex reversal. *Genesis* 30: 7-11


Werner MH, Bianchi ME, Gronenborn AM, Clore GM. 1995a. NMR spectroscopic analysis of the DNA conformation induced by the human testis determining factor SRY. *Biochemistry* 34: 11998-2004
Cloning of pEF1/c-Myc-His Expression Constructs

A. Generation of pEF1/Sry1, 2, 3, and 3B1 His-tagged Clones subcloned from Sry1, 2, 3, 3B1/pcDNA3.1- clones (Underwood, 2003).

<table>
<thead>
<tr>
<th>Primers</th>
<th>Anneals to Sry1 at bp’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-BamH1/KozakSry</td>
<td>5’-ctagatccgaaccatggaggccatgtcaag bases 1 - 18</td>
</tr>
<tr>
<td>R-Not1-StopcodonSry</td>
<td>5’-ctagcgccgccgctgtggaactggtgctgct bases 491 - 507</td>
</tr>
</tbody>
</table>

Base pair numbering assumes the “A” of the translation start as base number one.

These primes include Bam HI and Not I cleavage site for cloning (bold); Sry amplicons have no stop codon to facilitate formation of Sry proteins with a c-Myc epitope and His tag. The left primer includes a Kozak translation initiation sequence (red) for enhanced translation.

<table>
<thead>
<tr>
<th>PCR Reaction</th>
<th>Cycler Program (PEFSRY)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>200 ng</td>
</tr>
<tr>
<td>R-primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>L-primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>5x buffer</td>
<td>1x</td>
</tr>
<tr>
<td>MgCl</td>
<td>1.75 mM</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>GoTaq</td>
<td>2.5 Units</td>
</tr>
<tr>
<td>Water</td>
<td>to 50 µL</td>
</tr>
</tbody>
</table>

All PCR reactions (other than site-directed mutations) were performed with GoTaq and the supplied 5x clear buffer containing no MgCl (Promega).

After amplification, all amplicons were cleaved with Bam HI and Not I followed by insertion into pEF1 vectors opened with the same enzymes. Prior to ligation all restriction fragments were separated on a 1% agarose gel and extracted using a Zymo Gel Extraction Kit (Zymo).

*Bam* HI and *Not* I Restriction Digests

<table>
<thead>
<tr>
<th>DNA</th>
<th>0.2-1µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>*10x Buffer D</td>
<td>1x</td>
</tr>
<tr>
<td><em>Bam</em> HI</td>
<td>12 U</td>
</tr>
</tbody>
</table>
\textit{Not I} 5 U
Water to 20 µL

Incubate reaction 2 hr at 37°C followed by 20 min heat inactivation at 70°C

*1x Buffer D: 6mM Tris-HCl, 6mM MgCl, 150mM NaCl and pH 7.9

Ligation Reactions were setup in 1:3 and 1:5 vector to insert ratios

Estimates of pmole ends were calculated with the following equation based a reaction with 75 ng open vector in 1:3 reaction:

\[(\text{ng vector} \times \text{insert length kb})/(\text{vector length kb})(3/1) = \text{ng of insert to use in reaction}\]

Example: The full-length Sry1 insert about 510bp, and the pEF1 vector is about 6,200 bp therefore the following calculation would be conducted:

\[(75\text{ng} \times 0.510\text{kb})/(6.2 \text{ kb})(3/1) = 18.9 \text{ ng insert}\]

**Ligation Reaction**
- Vector DNA 75 ng
- Insert DNA 18.9 or 31.4 ng (1:3 or 1:5)
- *10x T4 Buffer 1x
- T4 ligase 3 Wiess units
- Water to 10 µL

Incubate at 15°C overnight and heat inactivate for 20 min at 70°C.

* 10X T4 Buffer: 400mM Tris, 100mM MgCl, 100mM DTT, 5mM ATP and pH 7.8

**Transformation into TAM-1 Competent \textit{E. coli} Cells (Active Motif)**
Combine 2 µL ligation reaction with 20 µL freshly thawed TAM-1 in a 15 mL polypropylene culture tube. Incubate on ice 30 min. Heat shock cells at 42°C for 45 s. and immediately place on ice for 2 min. Add 180 µL of room temperature SOC medium and incubate at 37°C shaking at 250 RPM for 1 hr.

**Inoculate LB plates containing 75 ug/mL Ampicillin (Amp)**
- Plate A 100 µL TAM-1 transformants
- Plate B 10 µL TAM-1 and 90 µL fresh SOC

Incubate plates at 37°C overnight.

SOC medium: 2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose

**PCR Screening of Colony Forming Units (CFUs)**
- R-primer 0.2 µM (R-Not1-StopcodonSry)
L-primer 0.2 µM (L-BamH1/KozakSry)
dNTP 0.2 mM
MgCl 1.75 mM
5x green buffer 1x
GoTaq 2.5 U
DNA touch pipet tip to CFU and mix into the reaction
Water to 50 µL
10 CFUs from each plate were screened.

Positive colonies were used to inoculate 5 mL LB broth liquid culture containing 75µg/mL Amp. Cultures were grown overnight at 37°C with agitation at 250 RPM. From these liquid cultures 1 mL glycerol stocks were prepared. Plasmid DNA from 2 mL of each culture was isolated using a Qiagen mini prep. Isolated plasmids were then digests with Bam HI and Not I (as above) to confirm insertion. Restriction fragments were then separated on a 1% agarose gel.

Glycerol Stocks = 20% v/v sterile glycerol added to 80% v/v liquid culture (200 mL : 800 mL); vortex and store at -80°C.

B. Generation of pEF1/Sry1+, 2+, and 3+ Constructs Not Encoding a c-Myc epitope or His tag.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Anneals to Sry1 at bp’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-BamH1/KozakSry</td>
<td>bases 1 - 18</td>
</tr>
<tr>
<td>R-Not1+StopcodonSry</td>
<td>bases 494 – 510</td>
</tr>
</tbody>
</table>

Base pair numbering assumes the “A” of the translation start as base number one.

These constructs were generated using the protocols above. Because the right primer maintains the endogenous Sry stop codon, proteins generated from these constructs contain no c-Myc epitope or His tag.

C. Generation of pEF1/Sry1(del), 3(del), and Sry2(+QR) constructs.

These constructs were produced by cleaving pEF1/Sry1, 2, and 3 amplicons with the restriction endonuclease Cvi Q1. This produced two restriction fragments; a 5’ fragment and a 3’ fragment from each amplicon. These fragments were separated on a 1.5% agarose gel and extracted using a Zymo Clean Gel Extraction kit. Following extraction all fragments were quantified on a NanoDrop ND1000 spectrophotometer and ligated with T4 DNA ligase (Fermentas).
Cvi QI Restriction Digest
DNA 0.5 µg
Cvi QI 10 Units
*Buffer 3 1x
water to 10µL

Incubate this reaction at room temperature 20min, do not heat inactivate
* 1x Buffer 3: 100mM NaCl, 50mM Tris, 10mM MgCl, 1mM DTT pH 7.8

Ligation Reaction
5’ fragment 50 ng (294 bp)
3’ fragment 50 ng (177 or 216 bp)
T4 DNA ligase 3 Wiess units
*10x buffer 1x
water to 10µL

Incubate all reactions at 15°C overnight (~16 hr); heat inactivate at 70°C for 20 min
* 10x T4 Buffer: 400mM Tris, 100mM MgCl, 100mM DTT, 5mM ATP and pH 7.8

Ligation Scheme
Sry1(del) 5’ fragment Sry1 (294bp) + 3’ fragment Sry2 (177bp)
Sry3(del) 5’ fragment Sry3 (294bp) + 3’ fragment Sry2 (177bp)
Sry2(+QR) 5’ fragment Sry2 (294bp) + 3’ fragment Sry1 or Sry3 (216bp)

These chimeric Sry amplicons were then electrophoresed on 1% agarose gels and extracted as previously described.

Following the clean-up step the chimeras were digested with Bam HI and Not I, ligated into pEF1 vectors and transformed into TAM-1 competent cells as described above in Section A.

D. Generation of pEF1/Sry1, 2, 3(-QR); and Sry1(HMGbox) constructs.

<table>
<thead>
<tr>
<th>Primers:</th>
<th>Anneals to Sry1 at bp’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-BamH1/KozakSry</td>
<td>5’-cttagatccgaaccatgagggccatgcaag bases 1 - 18</td>
</tr>
<tr>
<td>R-SryXba-QR</td>
<td>5’-ctctagatggctatccagtgg bases 415 - 429</td>
</tr>
<tr>
<td>R-SryXbaBoxOnly</td>
<td>5’-ctctagactggtgacatttaacc bases 236 – 252</td>
</tr>
</tbody>
</table>

Base pair numbering assumes the “A” of the translation start as base number one.
**PCR Reaction**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
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</thead>
<tbody>
<tr>
<td>DNA</td>
<td>200 ng</td>
</tr>
<tr>
<td>R-primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>L-primer</td>
<td>0.2 µM</td>
</tr>
<tr>
<td>5x buffer</td>
<td>1x</td>
</tr>
<tr>
<td>MgCl</td>
<td>1.5 mM</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.2 mM</td>
</tr>
<tr>
<td>GoTaq</td>
<td>2.5 Units</td>
</tr>
<tr>
<td>Water</td>
<td>to 50 µL</td>
</tr>
</tbody>
</table>

**Cycler Program (-QR)**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C</td>
<td>3 min</td>
</tr>
<tr>
<td>94°C</td>
<td>30 s</td>
</tr>
<tr>
<td>50°C</td>
<td>30 s</td>
</tr>
<tr>
<td>72°C</td>
<td>45 s</td>
</tr>
<tr>
<td>Cycles 2-4</td>
<td>repeat 34 times</td>
</tr>
<tr>
<td>72°C</td>
<td>5 min</td>
</tr>
<tr>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

Amplicons were then cleaved with **Bam HI** and **Xba I** and separation on / extracted from a 1% agarose gel. Quantification, ligation, and transformation were performed as described above.

**Restriction Digests with Bam HI and Xba I**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0.2 – 1 µg</td>
</tr>
<tr>
<td>*10x Y+Tango</td>
<td>1x</td>
</tr>
<tr>
<td><strong>Bam HI</strong></td>
<td>10 U</td>
</tr>
<tr>
<td><strong>Xba I</strong></td>
<td>10 U</td>
</tr>
<tr>
<td>Water</td>
<td>to 15 µL</td>
</tr>
</tbody>
</table>

* 1x Y+Tango Buffer (Fermentas): 33mM Tris-Acetate, 66mM potassium-acetate, 10mM magnesium acetate, 1 mg/mL BSA, pH 7.9

**E. Generation of pEF1/Sry1(H21) and Sry2(R21) constructs.**

These constructs were produced by cleaving **pEF1/Sry1** and **Sry2** amplicons generated in Section A, with the restriction endonuclease **Eco RI**. This produced two restriction fragments; a 5’ fragment and a 3’ fragment from each amplicon. These fragments were separated on a 1.5% agarose gel and extracted as above followed by quantification on a NanoDrop ND1000 spectrophotometer and ligated with T4 DNA ligase (Fermentas). Following this first ligation the chimeric coding regions were cleaved again with **Bam HI** and **Not I**, and ligated and transformed as previously described.

**Restriction Digest with Eco RI**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0.2 – 1 µg</td>
</tr>
<tr>
<td>*10x Buffer O</td>
<td>1x</td>
</tr>
<tr>
<td><strong>Eco RI</strong></td>
<td>10 U</td>
</tr>
<tr>
<td>Water</td>
<td>to 15 µL</td>
</tr>
</tbody>
</table>

* 1X Buffer O (Fermentas): 50mM Tris-HCl, 10mM MgCl, 100mM NaCl, 0.1 mg/mL BSA, pH 7.5
Ligation Reaction
5’ Fragment  50 ng (93 bp)
3’ Fragment  50 ng (328 or 417 bp)
10x buffer  1x
T4 DNA ligase  3 Wiess units
Water  to 10 µL

Incubate all reactions at 15°C overnight (~16 hr); heat inactivate at 70°C for 20 min
* 10x T4 Buffer: 400mM Tris, 100mM MgCl, 100mM DTT, 5mM ATP and pH 7.8

Ligation Scheme
Sry1(H21)  5’ fragment Sry2 (93bp) + 3’ fragment Sry1 (417bp)
Sry2(R21)  5’ fragment Sry1 (93bp) + 3’ fragment Sry2 (328bp)

These chimeric Sry amplicons were then electrophoresed on 1% agarose gels and
extracted as previously described.

Following this clean-up step the chimeras were PCR amplified as in Section A; digested
with Bam HI and Nor I, ligated into pEF1 vectors and transformed into TAM-1
competent cells as described in above in Section A.

F. Cloning of Site-Specifically Mutated Constructs pEF1/Sry1(20A22), Sry1(78A79)
and Sry1(NoNLS)

Primers  Anneals Sry1 at bp’s
F-nNLSsitemut  5’-ccgtggagagacagcagctggctcaacag bases 48 - 78
R-nNLSsitemut  5’-ccacccctaatgcattcatggggcgcttgacatggc bases 10 - 47
F-cNLSsitemut  5’-atataaatagccctcatgctggaagttgcaatccttcttcttcagctct
R-cNLSsitemut  5’-gtttggatatt ctctctctcttctctcaatccttcttcttcagctct bases 184 – 213

Base pair numbering assumes the “A” of the translation start as base number one.

Mutated portion is underlined in the forward primers.

Site Directed Mutagenesis PCR Reaction  Cycler Program (HI FI)
*5x HF Buffer  1X  98°C  3 min
R-primer  0.5 µM  98°C  30 s
F-primer  0.5 µM  68/62°C  30 s (nNLS/cNLS)
Template  500 pg  72°C  4 mins
**Phusion Pol.  0.05 U/µL= (2U/ reaction) Cycles 2-4 repeat 34 times
dNTP  0.2 mM  72°C  7 mins
Water  to 50 µL  4°C  ∞
* 5x HF (Finnzymes, New England BioLabs): contains 7.5 mM MgCl₂ = 1.5mM at 1x
** Phusion™ High-Fidelity Hot Start DNA Polymerase (Finnzymes, New England BioLabs)

Separate and Gel Extract Amplicons
Following amplification, load the entire PCR reaction onto a 1% agarose gel and electrophorese. Gel extract the amplicons following the Zymo Clean gel extraction protocol. Using the Nanodrop quantify extracted band (should be ~ 0.5 – 1µg)

Ligation Reaction
DNA 50-100 ng
T4 DNA ligase 3 Wiess units
10X buffer 1X
Water to 10 µL
Incubated at 15°C for 16 hrs.
Heat inactivate reaction at 70°C for 20 min

Transformation into E. coli TAM-1Competent Cells
Combine 0.5 and 3 µL ligation reaction with 20 µL freshly thawed TAM-1 in a 15 mL polypropylene culture tube. Incubate on ice 30 min. Heat shock cells at 42°C for 45 s. and immediately place on ice for 2 min. Add 180 µL of room temperature SOC medium and incubate at 37°C shaking at 250 RPM for 1 hr.

Inoculate four LB plates containing 75 ug/mL Amp
Plate A 100 µL from tube with 0.5 µL ligation added
Plate B 100 µL from tube with 3 µL ligation added
Plate C 20 µL TAM-1 from 0.5 µL ligation tube and 80 µL fresh SOC
Plate D 20 µL TAM-1 from 3 µL ligation tube and 80 µL fresh SOC
Incubate plates at 37°C overnight.

PCR Screening of colonies
R-primer 0.2 µM (R-Not1-StopcodonSry)
L-primer 0.2 µM (L-BamH1/KozakSry)
dNTP 0.2 mM
MgCl₂ 1.5 mM
5X buffer 1x
GoTaq 2.5 U
DNA touch pipet tip to colony and mix into the reaction
Water to 50 µL
10 CFU’s from each plate where screened using the BamHI and NotI primer set.
Cycler program used was: pEF1Sry; amplicons were then electrophoresed on a 1% agarose gel to identify positive colonies.

Positive colonies were used to inoculate 5 mL LB broth liquid culture containing 75µg/mL Amp. Cultures were grown overnight at 37°C with agitation at 250 RPM. From these liquid cultures 1 mL glycerol stocks were prepared. Plasmid DNA from 2 mL of each culture was isolated using a Qiagen mini prep. Isolated plasmids were then digest with Bam HI and Not I to confirm insertion.

These steps were repeated with each primer set to generated Sry1(20A220 and (78A79). Sry1(NoNLS) was produced by amplifying Sry1(20A22) vectors with the cNLSSitemut primer set. (See Figure A.1)
Map of Cloning Site-directed Sry1 Mutant Constructs

1. PCR amplify the entire pEF/Sry1 expression construct. Only the forward primer contains mutated bases. These mutations are flanked by at least 10 bases complimentary to the target.

2. All primers have a 5’ phosphate (pink) added to facilitate ligation of mutated amplicons.

3. Ligate with T4 DNA ligase to recircularize the mutated constructs.

Figure A.1.
Cloning of pIVEX/2.4 Bacterial Expression Constructs

G. Subcloning pIVEX/Sry1, 2, and 3B1 constructs encoding an N-terminal Histidine Tag from Sry1, 2, and 3B1/pcDNA3.1-

<table>
<thead>
<tr>
<th>Primers</th>
<th>Anneals to Sry1 at bp’s</th>
</tr>
</thead>
<tbody>
<tr>
<td>SrySmaRT</td>
<td>5’- gcgcccgggctagtggaactggtgct bases 493 - 510</td>
</tr>
<tr>
<td>SryNcoLT</td>
<td>5’- gcggccccatggaggccatgcaag bases 1 - 19</td>
</tr>
</tbody>
</table>

Base pair numbering assumes the “A” of the translation start as base number one.

PCR Reaction

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0.2-1 µg</td>
<td>94°C</td>
<td>4 min</td>
</tr>
<tr>
<td>R-primer</td>
<td>0.2 µM</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>L-primer</td>
<td>0.2 µM</td>
<td>54°C</td>
<td>45 s</td>
</tr>
<tr>
<td>5X buffer</td>
<td>1X</td>
<td>72°C</td>
<td>45 s</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.2 mM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MgCl2</td>
<td>2 mM</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>GoTaq</td>
<td>2.5 U</td>
<td>4°C</td>
<td>∞</td>
</tr>
<tr>
<td>Water</td>
<td>to 50 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cycler Program

<table>
<thead>
<tr>
<th>Component</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>94°C</td>
<td>4 min</td>
</tr>
<tr>
<td>R-primer</td>
<td>94°C</td>
<td>1 min</td>
</tr>
<tr>
<td>L-primer</td>
<td>54°C</td>
<td>45 s</td>
</tr>
<tr>
<td>5X buffer</td>
<td>72°C</td>
<td>45 s</td>
</tr>
<tr>
<td>dNTP</td>
<td></td>
<td>2 min</td>
</tr>
<tr>
<td>MgCl2</td>
<td>72°C</td>
<td>7 min</td>
</tr>
<tr>
<td>GoTaq</td>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

After amplification, all amplicons were cleaved with NcoI and SmaI followed by insertion into pIVEX2.4A vectors opened with the same enzymes. Prior to ligation all restriction fragments were separated on a 1% agarose gel and extracted using a Zymo Gel Extraction Kit (Zymo).

NcoI and SmaI Restriction Digests

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Temp</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0.2-1 µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>*10X Buffer</td>
<td>1X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NcoI</td>
<td>5 U</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SmaI</td>
<td>10 U</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>10 µL</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Incubate reaction 2 hr at 37°C followed by 20 min heat inactivation at 70°C

*1X Buffer 4 (New England Bio Labs): 20mM Tris-acetate, 10mM magnesium acetate, 50mM potassium acetate, pH 7.9

Ligation Reactions were setup at 1:5 vector to insert ratios

(ng vector x insert length kb)/(vector length kb)(3/1) = ng of insert to use in reaction
**Ligation Reaction**
Vector DNA 50 ng  
Insert DNA 35 ng (1:5)  
*10X T4 Buffer 1X*  
T4 ligase 3 Wiess units  
Water to 10 µL

Incubate at 22°C for 5hrs and heat inactivate for 20 min at 70°C.  
*10X T4 Buffer: 400mM Tris, 100mM MgCl, 100mM DTT, 5mM ATP and pH 7.8*

**Transformation into Lucigen BL21 (DE3) Competent E. coli Cells**

Combine 3 µL ligation reaction with 40 µL freshly thawed E. coli BL21 (DE3) in a 15 mL polypropylene culture tube. Incubate on ice 30 min. Heat shock cells at 42°C for 45 s. and immediately place on ice for 2 min. Add 260 µL of room temperature SOC medium and incubate at 37°C shaking at 250 RPM for 1 hr.

SOC medium: 2% bactotryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose

Continue with CFU selection, plasmid screening and stock storage as described previously in Section A.

**H. Generation of the pGL3/AR600 Luciferase Reporter Construct**

Genomic DNA used as a template was obtained from a previously isolated stock (isolated by Gail Dunphy) from a single male SHR/y rat

**Primers:**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Anneals Rat AR at bp’s</th>
<th>DNA 400 ng Genomic DNA</th>
<th>Cycler Program</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-ARNco</td>
<td>5’-gtaccatggttagcttgctcttagccacc</td>
<td>bases -22 to 1</td>
<td></td>
</tr>
<tr>
<td>L-ARSma600</td>
<td>5’- cacccgggaactccctttggtga</td>
<td>bases -590 to -474</td>
<td></td>
</tr>
</tbody>
</table>

Base pair numbering assumes the “A” of the translation start as base number one. The rat androgen receptor (AR) 5’ UTR is 998 bp in length, this construct encodes 590 bases of this regions, spanning from the translation start upstream 590 bp.
After amplification, amplicons were digested with *Nco* I and *Sma* I followed by ligation and TAM-1 transformation using protocols described above in Section A. After conformation of insertion by PCR of CFUs and restriction digests plasmid DNA was MAXI prep (Qiagen).

**NcoI and Sma I Restriction Digests**

<table>
<thead>
<tr>
<th>Component</th>
<th>Requirement</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>0.2-1µg</td>
</tr>
<tr>
<td>*10X Buffer</td>
<td>1X</td>
</tr>
<tr>
<td><em>Nco</em> I</td>
<td>5 U</td>
</tr>
<tr>
<td><em>Sma</em> I</td>
<td>10 U</td>
</tr>
<tr>
<td>Water</td>
<td>10 µL</td>
</tr>
</tbody>
</table>

**Sequencing of Clones**

<table>
<thead>
<tr>
<th>Reaction Mix</th>
<th>Cycler Program (BigDye50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5X Buffer</td>
<td>1µL</td>
</tr>
<tr>
<td>96°C</td>
<td>5 min</td>
</tr>
<tr>
<td>Primer(10µM)</td>
<td>1µL (usually T7/BGHrev)</td>
</tr>
<tr>
<td>96°C</td>
<td>10 s</td>
</tr>
<tr>
<td>BigDye Term.</td>
<td>2µL</td>
</tr>
<tr>
<td>50°C</td>
<td>5 s</td>
</tr>
<tr>
<td>DNA</td>
<td>0.2µg</td>
</tr>
<tr>
<td>60°C</td>
<td>4 min</td>
</tr>
<tr>
<td>Water</td>
<td>to 10 µL</td>
</tr>
<tr>
<td>4°C</td>
<td>∞</td>
</tr>
</tbody>
</table>

After the cycling, clean up the DNA with an EtOH precipitation

1. Add 3 µL 100 mM EDTA pH 7.8 and 2 volumes 100% chilled EtOH.
2. Let this incubate at room temperature for 15 minutes.
3. Spin down at 13,000 x g for 30 minutes.
4. Remove liquid and add 30 µL 70% EtOH, let stand one min. then centrifuge 15mins as above.
5. Decant, liquids and let tube dry ~ 45 – 60 mins at 22°C.
6. Add 28 µL HI-DI Formamide.

7. Incubate in heat block 2min at 95°C, then 2min on ice.

8. Now store the tubes at -20°C wrapped in foil until they are ready to sequence.

**Tissue Culture**

1. Vials containing CHO (Chinese Hamster Ovary) cells were removed from liquid nitrogen and thawed quickly at 37°C in an H2O bath.

2. Wash vials completely with 70% Ethanol to eliminate contamination.

3. The 1mL content of each vial was transferred to 9 mL of prewarmed (37°C) tissue culture medium (HAM’s F-12K containing L-Glu (Sigma), 10mM HEPES, 10% Fetal Bovine Serum (FBS), pH ~ 7.4) and no antibiotic or antimycotic in 100mm tissue culture plates.

4. Incubate plates in a humidified chamber at 37°C and 5% CO2 for 24 hours followed by a change of medium to remove the cryopreservative dimethyl sulphoxide (DMSO).

5. Plates were grown to ~75 – 90% confluent and then subcultured (1:8)

**Passage of CHO cells:**

1. Remove old medium, and wash cells twice with 2mL of *PBS.

2. Trypsinize cells with 1mL of warmed 1X Trypsin (0.25%) –EDTA (1mM) solution (GIBCO) in a 5 minute incubation at 37°C/5% CO2.

3. After incubation tap dish lightly to dislodge cells still attached to plate.

4. For a 1:8 split add 7mL (for a final volume with trypsin of 8mL) of complete medium to trypsinized cells. Transfer 1mL of this cell culture mix to 9mL of complete medium in a new 100mm plate.

5. For a 1:3 dilution to a 6-well cassette, add 3mL (final volume with trypsin of 4mL) of medium to trypsinized cells and transfer 230µl to each well containing 3mL of medium
   a. Example: 1:3 dilution between a 100mm plate to one 35mm well on a 6-well cassette:
i. Surface area of 100mm dish = 55cm$^2$, 35mm well = 9.5cm$^2$
ii. Surface area ratio $55cm^2/9.5cm^2 = 1:5.789$
iii. 1:3 dilution, final dilution factor $(3)(5.789) = 17.367...1:17.367$
iv. Volume added to each subculture $4mL / 17.367 = 0.230mL$

*1X PBS: 137mM NaCl, 2.7mM KCl, 100mM Na$_2$HPO$_4$, 2mM KH$_2$PO$_4$, pH 7.4 (sterile filtered 0.2 µm)

**Cryopreservation of CHO cells:**
1. Wash cells twice with 2mL of 1X PBS.
2. Trypsinize cells with 1.5mL of 1X trypsin-EDTA.
3. Stop enzymatic activity of trypsin with the application 3mL of medium containing 10% FBS.
4. Transfer cells to a sterile 14mL screw cap centrifuge tube and spin for 5 minutes at 400 x g to pellet cells.
5. Aspirate off trypsin and medium and resuspend cells in 1mL in complete medium containing 10% v/v DMSO.
6. Transfer resuspended cells to a 2mL cryo-tube and freeze cells down slowly in -70°C freezers. Once frozen (24 hours) move tubes to liquid nitrogen.

**Transient Transfection with ExGen500 Transfection Reagent (Fermantas)**

Each 6-well cassette was incubated for 24 hours after being passed as described above. These procedures produce cell densities at 24 hr at the recommended 50-70% confluence (~1.5x10$^5$ cells/cm$^2$).

**ExGen500 Transfection Protocol:**
1. Dilute 3μg DNA in 200 μL of 150mM NaCl. Vortex and spin down briefly
2. Add 9.87μL of ExGen 500 and vortex solution immediately for 20 seconds, incubate for 10 minutes at room temperature.
3. While incubating prepare the 6-well cassettes for transfection
   a. Aspirate off old medium and wash cells with 4mL PBS one time.
   b. Add 1800 mL fresh medium to each well.
4. After the complexes have formed, add 200µL to each well (1/10 the total volume of medium in each well); rock plates to evenly disperse the transfection complexes.

5. Centrifuge the cassettes for 5 minutes at 280 x g.

6. After centrifugation incubated for 24 hours and collect cells for total protein or nuclear cytoplasmic extraction.

**Transient cotransfection of CHO with pEF1/Sry effector constructs; pGL3/AR600 reporter constructs; and phRL-null Renilla (Promega) control vector.**

1. From a 100 mm plate seed ~ 25,000 cells (6.6 x 10³ cells/cm²) to each well in a 24 well cassettes (COSTAR) and incubated overnight.

2. Dilute 50 ng effector plasmid (pEF1/Sry or MT), 500 ng pGL3/AR600 reporter and 500 pg phRL-null Renilla normalization vector in HAM’s F12K with no FBS to a total volume of 25 µL.

3. Add 1.2 µL Superfect transfection reagent (Qiagen) (1:2 DNA to reagent dilution) following the manufacture’s protocol

4. Vortex immediately and incubate 10 min at room temperature.

5. Add 275 µL complete medium containing 10% FBS.

6. Remove old medium from the well to be transfected and add all 300 µL of complexes

7. Incubate CHO cells 4hr, remove the complexes and add fresh complete medium. Do wash cells after removing complexes.

**Growth of and Induction of E. coli BL21 (DE3) Transformed with pIVEX/Sry**

1. Start LB agar plates containing 75 µg/mL Amp with E. coli BL21 (DE3) transformed with pIVEX/Sry, incubate at 37°C overnight.

2. Select CFUs and start 5 mL Terrific Broth cultures containing 75 µg/mL Amp. Incubate ~ 8-10 hrs until OD₆₀₀ is ~0.8 – 1.0.
3. Induce pIVEX/Sry expression with addition of IPTG to a final concentration of 0.75 mM.

4. Incubate an additional 2-3hrs until OD$_{600}$ = ~ 3.0-5.0.

5. Collect cells by centrifugation at 5000 x g for 10 min.

6. Remove supernatant and freeze pellets at -20°C for future protein extraction or proceed to Ni affinity isolation of Sry

**Terrific Broth (Sigma):** Yeast Extract 24g/L, K$_2$HPO$_4$ 9.4 g/L, KH$_2$PO$_4$ 2.2 g/L, Tryptone 12 g/L, glycerol 0.8% v/v. Sterilize by autoclaving.

**Ni Sepharose 6 Fast Flow (GE Healthcare) Batch Purification of His-tagged Sry proteins generated in CHO or E. coli BL21 (DE3)**

**Phosphate Buffered Imidizole (PBI):** 20 mM Na$_2$ HPO$_4$, 500 mM NaCl, 25 mM Imidizole, pH 7.4 sterile filtered (0.45 µm).

**Elution Buffer:** 20 mM Na$_2$ HPO$_4$, 500 mM NaCl, 500 mM Imidizole, pH 7.4 sterile filtered (0.45 µm).

**Bacterial Cell Lysis Buffer:** 1X CelLytic B (Sigma), 2 mg/mL lysozyme, 0.5mM leupeptin, 1mM PMSF, 5 U DNAse I, 25mM NaF, 25mM MgCl, PBI to 5mL.

**CHO Cell/ tissue Lysis Buffer:** 1X CelLytic B (Sigma), 1X protease inhibitor cocktail containing no EDTA (Sigma), 1mM PMSF, 5 U DNAse I, 25 mM NaF, 25mM MgCl, PBI to 5mL.

1. To a pellet obtained from one 5 mL bacterial culture or one transfected 6 well tissue culture plate (CHO); add 1.5 – 2 mL of the appropriate lysis buffer.

2. Disrupt pellet with a pipet tip, and draw into a 25 gauge syringe two times. Incubate 2-15min on ice. When cells are lysed supernatant is gold and has no turbid appearance.

3. While samples lyse prepare the Ni beads (1 mL of 50% bead slurry can bind ~ 20 mg His-tagged protein).
   a. Mix bead slurry and transfer 75-100 µL to a 1.5 mL tube (cut pipet tip)
   b. Wash two times with water and two time with PBI
      i. To wash, add beads, add 1mL water or PBI, invert tube ~ 5 times.
ii. Centrifuge 2 mins at 500 x g, remove supernatant and repeat. Beads are prepared for protein capture.

4. Centrifuge lysates 5min at 13,000 x g to remove large particles.

5. Transfer supernatant to 15 ml screw cap tube and dilute with 4-6 volumes of PBI.

6. Transfer the washed bead to the diluted samples.

7. Incubate beads 1 hr at room temperature on a rotator.

8. After incubation collect beads by centrifugation at 500 x g for 5 mins. Remove and save the supernatant for analysis.

9. Add 1 mL PBI to bead/protein pellet and transfer to a 1.5 mL tube.

10. Wash beads four times with 1 mL PBI. Save each wash for analysis.

11. Elute bound proteins with 0.5 – 1 mL elution buffer. Incubate beads at room temperature on rotator for 5-10 mins.

12. Pellet the beads and collect the eluted proteins. Repeat this step two more times.

13. Store proteins at -80°C or analyze directly by SDS PAGE.

14. Proteins must be desalted prior to quantification via a protein assay and other down stream applications. BioRad B6 desalting spin columns work very well.

Cytoplasmic and Nuclear Sry Extraction From Transfected CHO with the ProteoJet Extraction Kit (Fermentas)

All buffers are provided with this kit and buffer composition is proprietary.

1. Collect transfected CHO from 2-4 wells of a six well cassette or half the cells from a 100mm plate (~2-5 x 10⁶ cells) by trypsinization. Pellet as above and wash pellet two time with 1 mL PBS.

2. Add 10 volumes (compared to size of the pellet) of Cell lysis buffer (provided with the kit) supplemented with 50 mM NaF, 2mM DTT, and protease inhibitor cocktail (Sigma).

3. Vortex 10 s, incubate on ice for 10 min and vortex again.
4. Separate the nuclei by centrifugation at 500 x g for 10 minutes at 4°C.

5. Remove the supernatant containing the cytoplasmic proteins and transfer to a new tube. (Put the remaining nuclear pellet on ice.)

6. Clear cytoplasmic proteins extract by centrifugation at 16,000 x g for 25 min at 4°C
   a. Transfer the supernatant to a new tube and store at -70°C for later analysis (this is the cytoplasmic fraction)

7. Wash the nuclear pellet two times with 500 µl of Nuclei washing buffer (provided with the kit) supplemented with 50 mM NaF, 3mM DTT, and protease inhibitor cocktail (Sigma), vortex 5 s and incubate on ice for 2 min.

8. Centrifuge at 500 x g for 10 minutes at 4°C and carefully remove supernatant (save this fraction for analysis)
   a. Do not use fixed angle centrifuge, place your 1.5 mL tube in a 15 mL culture tube and use the swinging bucket centrifuge. This technique packs the nuclei at the bottom of tube making supernatant removal possible.

9. Add 150 µl of ice cold Nuclei storage buffer (provided with the kit) supplemented with 50 mM NaF, 5 mM DTT, and protease inhibitor cocktail (Sigma) to the nuclei pellet.

10. Pipette up and down 5-10 times. Add 1/10 volume of the Nuclei lysis reagent to nuclei suspension. Vortex briefly every 1-2 mins over a 15 min period. Keep nuclei on ice between mixes.

11. Centrifuge at 16,000 x g for 15 min at 4°C and transfer the supernatant (nuclear protein extract) to a new tube. Quantify protein concentration in both the cytoplasmic and nuclear extracts and store at -70°C for later use.

**DNA Affinity Purification with Promega Strep. Avidin Para Magnetic Particles (SA PMP)**

**B1: Oligo rehydration / Annealing Buffer** – (1X) = 50mM NaCl, 10mM Tris pH 8, 1mM EDTA pH 8. Sterile filter before use

**B2: SA PMP/ Biotin Avidin conjugation buffer** – (1X) = 500mM NaCl, 10mM Tris pH 8, 1mM EDTA pH 8. Sterile filter before use

**B3: Protein/ DNA Binding Buffer** - (5X stock) = 50mM Tris pH 8, 200mM KCl, 15% glycerol, 0.25% NP 40, 5mM EDTA pH 8.
Store all of the above at room temp.

(Working 1X B3) = 10mM Tris pH 8, 40mM KCL, 3% glycerol, 0.05% NP 40, 1mM EDTA, (or 20% 5X stock) (1.5mM PMSF, 1mM DTT, Protease Inhibitor Cocktail). Make fresh each day and sterile filter before use.

**Annealing oligonucleotides**

1. 100 µM stocks of all ssOligos were made in B1
   a. i.e. biotin oligo WT = 26.7nMoles + 267µl of B1 = 100µM stock

2. Anneal equal molar amounts of biotinylated sense strand and nonbiotinylated complimentary strand in B1 buffer at 80°C for 5mins, then turn off water bath and let it cool to ~ 40°C, remove rxn and allow it to cool to room temp ~ 10mins, then store the ds oligos at-20°C
   a. 1500pmole sense + 1500pmole antisense = 15µl + 15µl (oligos) + 70µl B1 buffer, total rxn volume = 100µl

**Conjugation of biotinylated oligos to SA PMP (Promega)**

1. Suspend the SA PMP by snapping the tube and/or low speed vortexing. Allow 3mins for particles of poor quality (aggregates) to settle at the bottom of the tube. Draw out 250µl of SA PMP and place in 1.5 ml tube.

2. Wash SA PMP 4x’s with 500µl of B2 buffer to remove storage buffer

3. Perform washes by removing the buffer after all PMP are pulled to one side of the tube with a magnet, then suspend the SA PMP in new buffer with no magnetic force.

4. Dilute 9µg of the ds oligos (usually about 30µl) in B2 to a total vol of 100µl
   a. 30µl oligo + 70µl B2 (make an OD$_{260}$ reading here)

5. After the final SA PMP wash, remove all buffer and add the 100µl of diluted ds oligo.

6. Incubate 20-30mins at room temp. Every 5mins use a P200 pipette with the tip clipped, to suspend the oligos and SA PMP, mixing this way keeps you from losing some on the side of the tube. (After 30mins make an OD reading)
   a. From prior trials it has been determined that after about 15mins 60-70% of the oligo is bound to the SA PMP. After 30mins the maximum amount of oligo has been bound, which usually equals ~ 70-78% of the applied oligos, longer incubations (1hr-5hrs) do not increase oligo conjugation.
7. After conjugating SA PMP/ds oligo, wash the complex 3x’s with 300µl B2
   a. Collect 30ul of the final wash and confirm that there is no free oligo in the
      supernatant
   b. Store the complex at 4°C for up to 3 days, however, it is better to use the
      complex immediately.

Capture Sry Proteins with SA PMP complexes Cell Extracts/ Tissue

1. Prepare extracts from a cell pellet containing ~ 2.5x 10⁵ cells, or 30 - 100 mg
   tissue. Alternatively to show Sry binding use Ni purified proteins
2. Add 1000µl of fresh 1X B3 buffer to the cells/ tissue
   a. Homogenize, sonicate and / or draw cells through a 27.5 gauge needle
      multiple times on ice
   b. Clear the homogenate by centrifugation at 16,000 x g 5mins at 4°C.
   c. Or dilute 35 µg Ni purified proteins in 1mL B3 (these proteins can be
c   desalted prior to dilution or not)
3. Remove the B2 buffer from the SA PMP complex and suspend in 500µl 1X B3
4. Remove B3 from SA PMP and suspend the complexes in the cleared lysate
   (save a small fraction ,~50µl, for SDS PAGE and protein assay)
5. Incubate at room temp. 30mins

Wash Captured proteins 3x’s with 500µl B3 (save each wash as an unbound fraction)
Add 75µl 4X SDS Sample buffer to SA PMP, heat at 98°C 3mins
OR Elute with 500mM NaCl

Immunocytochemistry (ICC) of Transiently Transfected CHO Cells

Buffers:
Fixer: 100% methanol chilled to -20°C

Blocking solution: 10% Normal Rabbit Serum (NRS), 3% nonfat Carnation dry milk in
sterile filtered PBS

1º antibody incubation buffer: Blocking solution diluted so that NRS is 1.5% v/v in
sterile filtered PBS, plus the primary antibody [1:100 goat anti-mouse Sry (E-19)
(Santa Cruz) or 1:400 goat anti-c-Myc epitope (Bethyl)].
2º antibody incubation buffer: Blocking solution diluted so that NRS is 3% v/v in sterile filtered PBS, plus the secondary antibody [1:3500 rabbit anti-goat IgG Cy-3 conjugate (Sigma)].

1. Seed ~ 5x 10³ cells into a 16 well chamber slide, incubate overnight at 37º C.

2. Transfect with ExGen500:
   a. 300 ng vector, 150mM NaCl to 20 µL, 1 µL ExGen500.
   b. Remove old medium add 180 µL fresh medium plus the 20 µL of transfection complexes. Incubate overnight.

3. Wash cells 3 times with 300 µL sterile PBS and fix cells 5 minutes in 300 µL of -20º C methanol.

4. Wash with three changes of sterile PBS (~2 min soak each wash). (Dump chamber contents quickly and completely, but do not allow cells to dry between steps, use a multi-channel pipette for quick addition of new reagents). Use enough reagent to cover the cells (approximately 200-300 µl per well is adequate).

5. Incubate cells with blocking solution 25 min at room temperature on shaker. Blocking serum should be derived from the same species the secondary antibody is raised. Wash with PBS.

6. Incubate with primary antibody (E-19 @ 1:100 or Bethyl anti-c-Myc epitope @ 1:400) for 60 minutes at room temperature in PBS with 1.5% normal blocking serum (diluted blocker). After 1 hr, wash with three changes of PBS for 5 minutes each.
   a. Control wells: Replace primary antibody with PBS in two wells and preimmune serum in two additional wells. Additionally, have two wells that have not been transfected and probe with the primary antibody.

7. Incubate for 45 min. at 37ºC in the dark (a box) with rabbit anti-goat IgG Cy-3 conjugated secondary antibody (1:3500) in 3% normal blocking serum (diluted blocker). Wash with 3-4 changes of PBS.

8. Remove the plastic chambers and gasket from the slide. Add VECTA-Shield mounting medium containing DAPI and put on large cover slip made for chamber slides. Seal cover slips with clear nail polish. (Keep slides out of light as much as possible).

9. Examine using a fluorescence microscope with appropriate filters. Store slides in the dark at 4º C.
Casting 13.5% Tris-Glycine Polyacrylamide Gels with 4% Tinted Stacker

4X Resolving Gel Buffer: 1.5 M Tris pH 8.8, 0.4% SDS

4X Stacking Gel Buffer: 0.5 M Tris pH 6.8, 0.4% SDS

40% J.T. Baker Acrylamide; Acrylamide : Bis acrylamide (37.5:1)

10% Ammonium Persulfate (APS): make fresh each time (0.2 g to ~2 mL water)

TEMED (N,N,N',N'-Tetramethylethylenediamine)

Glass mini gel plates and spacers.

Most importantly the ACU Cast-O-Matic 3000 gel casting system and modeling clay.

To cast three gels assemble the plates in the following order against the back of the Cast-O-Matic. 1. Tall glass plate 2. spacers standing vertically at the edges of the glass plate. 3. A white alumina plate with the notch pointing up. Repeat three times. After all plates are in, insert the precision fit plexi-glass back. Hold the back in place and seal the sides and bottom with one continuous pencil sized role of clay. Press on clay until no gaps are seen at the junction between the clay and casting unit and the clay and back plate. If done properly this will not leak when casting gels.

To cast three gels mix the following in two 50 mL screw cap tubes.

Tube I: Resolving gel. Each 1.5 mm gel needs about 10mL of this mix, so make 30ml. Add the following reagents in this order: 12 mL water, 10 mL 40% Acrylamide [13.5%], 7.5 mL 4X Resolving gel buffer [25%] , 300µL 10% APS [~1%], 30-60 µL TEMED [~0.1%]. Vortex 10 -15 s.

Using a 10 mL pipet, transfer this solution to the plates. Fill the plates to about 1 cm from the bottom of the alumina plate notch. Make sure to tilt the caster to one side (the tall side) to allow equal volumes to fill each plate. Add water to top and let sit about 30-60 mins.

Tube II: Stacking gel. Make 20 mL: 12.8 mL water, 2 mL 40% Acrylamide [4%], 5mL 4X Stacking Gel Buffer, 200 µL 10% APS [1%], 40µL TEMED, 20µL 0.01% Bromophenol Blue. Vortex to mix.

Dump water off the resolving gel and add the stacker. Insert the combs and let stand 1hr. Then store gels in plastic wrap at 4°C. Be sure to include a KimWipe saturated with water or buffer to keep the gels from drying.
Note: Always make fresh 10% APS. Always add the APS and TEMED just before use. Once these reagents are added you have about 5 – 15 mins before the mix starts to polymerize.

SDS PAGE and Western Blotting

4X SDS Sample Buffer: 35 % glycerol, 8% SDS, 0.05% Bromophenol Blue, 200 mM Tris-HCl pH 6.8, 200 mM DTT

10X Tris Glycine Buffer (TG): 250mM Tris, 1.97 M glycine, pH 8.3

1X SDS Running Buffer: Dilute TG to 1X with water, 0.1% SDS. Do not adjust pH.

Transfer Buffer: Dilute TG to 1X with water, 20% methanol, pH ~ 8.3

Blocking Buffer I: 5% nonfat dry milk in PBS, plus 0.1% Tween-20

Washing Buffer I (PBS-T): PBS with 0.1% Tween-20

Zymed Phosphoamino acid Blocker (Zymed)

BSA Blocker: PBS-T containing 1% BSA.

SDS PAGE
1. Insert gel in electrophoresis chamber and fill middle chamber with 1X running buffer. Check for leaks. Then fill outside the chamber.

2. Mix quantified protein extracts with 4X Sample buffer [25% of total volume], and heat 3-10 min at 95 – 98°C.

3. Load up to 50 µg total protein in each well and electrophorese at 150 volts about 60 – 90mins.

4. Gel is done running when the dye front leaved the gel.

5. On a 13.5% gel Sry will migrate to about the middle.

Western Blot
1. Remove gel from plates. Assemble the following on a semidry transfer unit: transfer buffer soaked blot pad, methanol soaked PVDF membrane, the gel, blot pad. Be sure all blot pads and the PVDF are cut to the same size as the gel, and that the PVDF is near the anode and the gel is near the cathode.
2. Transfer under constant current for 2 hr. The current used is equal to the area of the blotting sandwich in cm.
   a. If probing for Sry with anti-mouse Sry (E-19) (Santa Cruz), the goat anti-cMyc epitope (Bethyl), or rabbit anti-rat Sry124 antibodies, block with buffer I.
   b. If probing for SUMO proteins with the provided rabbit anti-SUMO-1, rabbit anti-SUMO-2/3 antibodies (BIO MOL) block with the BSA blocker.
   c. If probing for phosphoamino acids, using the monoclonal anti-phosphoserine (AbCam) and monoclonal anti-phosphothreonine (Zymed) antibodies, block with the Zymed blocker (you can not use milk because caseins in the milk are often phosphorylated).

3. Remove blot and block in appropriate buffer 1 hr at room temperature or overnight at 4°C on a shaker.

4. After blocking, if using the anti-mouse Sry (E-19), the goat anti-cMyc epitope, or rabbit anti-rat Sry124 antibodies do not wash. Make a bag, add 5 mL blocker and the primary antibodies at 1:300, 1:1000, and 1:250 dilutions respectively; incubate 1 hr at room temperature.

5. For SUMO blots, wash the membrane in 200 mL PBS-T two times, 5 min each. Then make a bag add 5 mL blocker and dilute the primary rabbit anti-SUMO antibodies 1:1500 and incubate 1 hr at RT.

6. For anti-phosphoserine and anti-phosphothreonine blots, wash two times with 2X PBS-T, 5 min each wash. Make a bag add 5 mL of Zymed blocker and dilute the primary antibodies at 1:400 and 1:250 respectively, incubate 1 hr at RT.

7. After incubation with the primary antibodies blots are washed 4 times with PBS-T 5-10 min each wash.

8. Add the secondary antibodies in a bag as above containing 10 mL of the respective incubation buffer or blocker. Incubate 1-2 hrs at room temperature.
   a. Secondary antibodies:
      i. Bethyl donkey anti-goat IgG, HRP conjugate at 1:3000. Use with primary antibodies Sry(E-19) and the anti-c-Myc epitope antibody.
      ii. Sigma monoclonal anti-rabbit IgG, HRP conjugate at 1:25,000. Use with anti-SUMO and anti-ratSry124 antibodies.
      iii. Affinity Bio Reagents rabbit anti-mouse IgG, HRP conjugate at 1:1200; used with both anti-phosphoamino acid primary antibodies.

9. Wash 1-4 time in appropriate buffer 2-5 mins. Wash four times, 5 min with 2X PBS-T on blots for phosphoamino acids.
10. Make a new bag and add 2 mL of each reagent (Luminal and Hydrogen peroxide) supplied with the Thermo Super Signal West Pico Chemiluminescent Substrate pack. Incubate 5 min and capture reactive bands on the Kodak Gel Logic 2200 gel documentation system.

Note all antibody incubations occurred at room temperature and were conducted on a rotator.

**Anti-Sry124 Immunoaffinity Purification Using CnBr Activated 4B Beads Coupled to Peptide 1 (GE Healthcare)**

Buffers:
- **Hydration Buffer:** 1mM HCl, pH 2.5
- **Coupling Buffer:** 100 mM Na2HCO3, 500 mM NaCl, pH 8.3
- **Elution Buffer:** 200mM glycine pH 2.8

1. Hydrate ~450 mg activated CnBr 4B beads in 10 mL Hydration Buffer

2. Wash beads immediately on a Watman #1 filter dampened with buffer and placed in a funnel sealed to a side arm Erlenmeyer flask with parafilm.
   - a. Apply vacuum, to remove hydration buffer
   - b. Apply 350 mL fresh hydration buffer to bead cake with the vacuum on
   - c. Draw buffer thought until the bead cake is not shiny, it will look like table salt
   - d. Allow to dry an additional 1 min under vacuum

3. Hydrate ~5 µmole Peptide 1 with 2.5 mL Coupling buffer in a 5 mL tube

4. Scrape cleaned beads into the 2.5 mL coupling buffer containing peptide 1

5. Incubate 2-2.5 hrs end-over-end at room temperature.

6. Wash beads with 10mL coupling buffer

7. Block reactive, unbound sites on the beads with 10mL of 100mM Tris HCl over 2hr at room temperature (1hr on rotator and 1 hr standing)

8. Degas 10 min in side are flask

9. Pour column in one continuous motion, then run four bed volumes of sterile PBS (pH 7.5) through at 75 cm/hr = ~1.25 mL/min.
10. After running 30mL (4 bed volumes) PBS through the column sample was added (0.45 µm filtered whole serum)

11. Serum was diluted with 0.5 volumes PBS (To 10mL serum, 5 mL PBS was added) and pH was adjusted to 7.5 and applied to the column. The unbound serum / flow through was then applied to the column again.

12. After applying the serum two times the column was washed with 20 bed volumes Binding buffer (PBS)

13. After washing 3mL of elution buffer was added to the column and allowed to flow through by gravity.

14. Each fraction ~7 drops or ~420 µL was collected into a 1.5mL tube containing 105 µL neutralization buffer (1M Tris pH 9.5). The end pH the IgG is stored in is about 7.5-8.

15. Fractions from step 11 and 12 were collected and proteins detected by readings at 280 nm to monitor the effectiveness of washing. Elution fractions were also collected and measured the same way to determine which fractions contain the targeted IgG (anti-Sry 124 / GN16)

16. After elution the column was washed with 20mL of binding buffer to return the matrix pH to about 7.5-8.

17. Finally 15 mL was recirculated overnight

18. Bead regeneration was then completed by washing the beads with two column volumes 100mM Tris, 500mM NaCl pH 8.5 solution followed by a two column volume wash with Sodium acetate solution pH 4.5. This was repeated three times.

19. The column was then stored in binding buffer containing 0.02% sodium azide.