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Embryonic stem cells for development of a Williams-Beuren syndrome mouse model

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**EMBRYONIC STEM CELLS FOR DEVELOPMENT OF A
WILLIAMS-BEUREN SYNDROME MOUSE MODEL**

A Thesis

Presented to

**The Faculty of the Department of Biology
San Jose State University**

In Partial Fulfillment

of the Requirements for the Degree

Master of Science

by

Katherine Gayle Carlsmith

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ABSTRACT

EMBRYONIC STEM CELLS FOR DEVELOPMENT OF A WILLIAMS-BEUREN SYNDROME MOUSE MODEL

by Katherine Gayle Carlsmith

This thesis describes the development of mouse embryonic stem (ES) cell lines to be used to develop a mouse model of Williams-Beuren syndrome (WBS), a microdeletion disorder in which individuals have a unique cognitive profile. Studying WBS may elucidate certain aspects of human neurocognitive development. DNA constructs and the Cre/*loxP* strategy of chromosome rearrangement, developed in Alan Bradley's laboratory, were used to generate these ES cell lines using standard molecular biology and tissue culture techniques. One ES cell line was developed that is targeted with one *loxP* site on either side of the proximal half of the mouse genome homologous to that area of the Williams-Beuren syndrome deletion region, and two ES cell lines were developed that are heterozygously deleted for the sequence between the *loxP* sites. These ES cell lines will be useful in generating a compound heterozygote mouse model of the complete WBS deletion region for further study.

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INTRODUCTION

One of the major challenges to be addressed by biomedical research in the next century is the determination of the cellular, molecular, and genetic mechanisms that govern cognition and behavior in humans. Among the most tractable techniques available to study this problem is the analysis of genetic anomalies that affect cognition and/or behavior.

Microdeletion syndromes provide models conducive to studying and defining specific relationships between genes and normal human development. The restricted number of genes altered by microdeletions and the specificity of their particular associated phenotypes make microdeletion syndromes good candidate models for studying gene-function relationships in cognitive development. Microdeletions almost always result in haploinsufficiency for a limited number of genes that are dosage-sensitive or in complete loss of function for genes that are imprinted (for instance Prader-Willi [MIM#176270] and Angelman [MIM#105830] syndromes). The most common microdeletion syndromes provide researchers with enough cases to be able to study the various phenotypes associated with the deletion while controlling for background genetic variation in the population.

Williams-Beuren syndrome (WBS [MIM194050]) is a human microdeletion syndrome that provides a unique opportunity to identify the roles of a defined set of genes in the formation of distinct cognitive and behavioral characters. WBS patients tend to display a genetic deletion that encompasses approximately 1.5

Mb and contains a number of genes and transcripts, most of which are now characterized and understood (Peoples et al. 2000; DeSilva et al. 2002; Merla et al. 2002; Tassabehji 2003). As of 2003, 24 genes had been assigned to the WBS critical region (WBSCR) with the functions of some experimentally defined, some inferred by motifs (Appendix 1).

Individuals affected with WBS have distinctive facial features, vascular stenoses, and cognitive deficits with a nonuniform profile. The below-average IQ scores common to WBS individuals are not the result of global retardation. Rather, these individuals exhibit specific dissociations in higher cognitive functions marked by severe deficits in visuospatial construction, while their performance in face processing is adequate and their language abilities are relatively normal (Bayes et al. 2003). WBS individuals share very similar neurological symptoms and behavioral profiles, and finding the genes associated with this phenotype might reveal molecular mechanisms underlying basic neuronal functioning. The fact that the cognitive profile in WBS individuals is uneven and their genetic defect consists of hemizyosity for a discrete number of genes makes this syndrome a good model for understanding the molecular mechanisms controlling the development and/or physiology of the brain areas responsible for these cognitive functions. Ultimately, the goal is to identify a single gene responsible for a specific defect, and then to understand the biochemical processes controlled by the under-expressed gene that governs cognition.

Correlating the microdeletion with the WBS phenotype has been difficult. The extent of the deletion on chromosome 7q11.23 varies only slightly among individuals with WBS (Bayes et al. 2003), making genotype-phenotype comparisons between individuals with different sized deletions almost impossible due to the infrequency with which such abnormal deletions occur (Osborne 1999). Careful clinical analysis of the WBS phenotype correlated with a molecular dissection of the genes affected by the deletion probably will not be able to identify specific genes controlling specific aspects of the cognitive manifestations of the syndrome. Cognition is an especially challenging phenotypic area to genetically dissect, with polygenic inheritance playing a major role in the development of mental faculties, and the inherent difficulty in recognizing the complex interactions among genes responsible for the failure of normal cognitive development. Furthermore, while some of the characteristic anomalies associated with WBS may not be manifested in all individuals, all syndromes have a variable expression in the individual making a correlation between penetrance and genotype a poor source of insight into the genetic cause of specific phenotypic characters (Morris and Mervis 2000). To date the only gene within the WBS common deletion region to have been unambiguously linked to the WBS phenotype is elastin (*ELN*). Hemizygosity for *ELN* leads to a reduced amount of elastin protein that affects arterial compliance, manifesting in WBS individuals as the various vascular problems that are present in 75% of patients (Perez Jurado 2003).

A small number of patients with smaller, atypical deletions and either a full or partial phenotype have been reported (Korenberg et al. 2000, Perez Jurado 2003). *LIMK1* hemizyosity has been implicated in visuospatial phenotypic characters in WBS. Two families with submicroscopic deletions at 7q11.23 (the smallest being 83.6 kilobases (kb) and encompassing only *ELN* and *LIMK1*) have been described in which affected members have the specific WBS cognitive profile and vascular disease, but lack other WBS features. Since *ELN* mutations are known to cause vascular disease and are not associated with cognitive abnormalities, these cases implicate *LIMK1* hemizyosity in impaired visuospatial constructive cognition (Frangiskakis et al. 1996). However, Tassabehji et al. (2003) claim that *LIMK1* haploinsufficiency alone is unlikely to cause the cognitive deficits specific to the WBS profile, though mouse studies (Meng et al. 2002) suggest that it could contribute to the phenotype. The *TFII-I* protein family members clustering at the telomeric end of the WBS common deletion region, including *GTF2I*, *GTF2IRD1* and *GTF2IRD2*, have been implicated in the WBS neurodevelopmental phenotype by rare patients with atypical deletions. This family of proteins appears to be a complex and multifunctional group of transcriptional regulators with both repressive and activation properties depending on the cellular context (Hinsley et al. 2004). Continued identification and characterization of partial deletion patients will help to focus in on the gene or genes responsible for the cognitive profile of WBS individuals. Studying these individuals' genetic mutations will help to more precisely define the critical region

of the deletion necessary for the cognitive phenotype, allow for the identification of dosage-sensitive genes and aid in the functional dissection of the WBS deletion region.

The Francke laboratory (Stanford University, Department of Genetics), in which this thesis work was completed, has undertaken an alternative approach to studying the effects of the WBS microdeletion in mammals by developing a unique mouse model of WBS in which the phenotypes are correlated with gene anomalies that are determined by the researcher. An animal model of WBS that reproduces both the genotype and phenotype of WBS will provide the foundation for a range of research opportunities, including the functional dissection of the deletion region and identification of genes outside the deletion region that are up- or down-regulated and whose changed expression may underlie the development of the phenotypes. This thesis describes the production of three transgenic mouse embryonic stem cell lines to be used in the development of a mouse model of Williams-Beuren syndrome.

BACKGROUND

Williams-Beuren Syndrome (WBS) was first reported by Williams et al. in 1961, and soon after by Beuren et al. in 1962. The reports described children with unusual facial features, growth retardation, supravulvar stenosis (SVAS), mild mental retardation, dental anomalies and friendly personalities - common features that are present in virtually all individuals with WBS (Osborne 1999). WBS is a rare, multisystem disorder occurring in 1/20,000 – 1/50,000 live births, almost always due to a *de novo* deletion of approximately 1.5 Mb at chromosome band 7q11.23 (Tassabehji 2003). Familial cases of autosomal dominant transmission have been reported, but with no proven parent of origin effect since maternal and paternal origins of deletion occur with equal frequency (Morris and Mervis 2000).

The WBS Phenotype

The physical aspects of the typical WBS phenotype generally include a characteristic dysmorphic face, cardiovascular and connective tissue abnormalities, growth retardation, short adult stature and an abnormal gait, and can include hyperacusis and transient infantile hypercalcemia (Osborne 1999; Tassabehji 2003). The facial features associated with WBS have been described as “elfin” and include a stellate pattern of the iris, flat nasal bridge, short up-turned nose with anteverted nostrils, long philtrum, full lips and lower cheeks,

small or absent teeth, periorbital fullness and a small chin. Cardiovascular abnormalities occur in around 80% of individuals, presenting as generalized arteriopathy leading to vascular stenoses, most commonly supraaortic stenosis (SVAS) or peripheral pulmonary stenosis (PPS). SVAS is the greatest life-threatening element of WBS but can be corrected with surgery. Hyperacusis (sensitivity to sound) in WBS is typified by heightened emotional responses to noise and music, either attraction or aversion, especially to sounds like sirens, vacuum cleaners and thunder. Other common physical manifestations include dental anomalies (malocclusion), musculoskeletal abnormalities, impaired visual acuity and a low, hoarse voice. WBS individuals tend to have multiple developmental motor disabilities that affect balance, strength, coordination and motor planning.

WBS individuals share a range of characteristic personality traits (Bellugi et al. 1999; Mervis et al. 1999; Osborne 1999; Tassabehji 2003). They have a gregarious personality characterized by a lack of fear with strangers, over friendliness and charismatic speech rich in vocabulary. WBS individuals tend to be hyper-reactive and experience anxiety and simple phobias, and 70% suffer from attention deficit and hyperactivity disorder.

The WBS cognitive profile (WBSCP) is characterized by mild mental retardation camouflaging an uneven cognitive profile (Tassabehji 2003). In general, standard Full Scale IQ scores among WBS individuals range from 40–100, with means around 60 (Bellugi et al. 1999). The hallmark of the WBSCP

is a fractionated pattern of cognitive phenotypes characterized by profound impairment in certain cognitive areas (e.g., conceptual reasoning, problem solving, motor control, arithmetic and spatial cognition), combined with relative strengths in other complex mental abilities (e.g., language, music, face processing, and social interaction) (Tassabehji 2003).

Visuo-spatial cognition difficulties in WBS individuals seem to be especially acute at the global level of spatial organization (Tassabehji 2003). They have difficulty integrating details, which they perceive well, into a whole picture, similar to how they process faces (relying on components or features rather than the configural or holistic approach normally used to recognize faces). They are significantly more impaired than their Down syndrome (DS) age- and IQ-matched counterparts across all age ranges examined in studies that look at spatial cognition. Their abilities in facial recognition surface as a strength in their cognitive repertoire, however, with strong performance across different face-processing tasks in stark contrast to their performance in spatial tasks such as judging the orientation of lines (Bellugi et al. 1999).

Young children with WBS show significant delay in expressive and receptive language skills, but once language is acquired, this ability tends to become another relative strength in their cognitive profile (Bellugi et al. 1999; Osborne 1999). Adolescents and adults with WBS far outperform their DS counterparts in language skills at all levels when matched in age and IQ, though they are similarly delayed at younger ages.

There is mounting evidence that the superficially intact abilities of language and face processing in WBS depend on atypical cognitive and brain processes which are served by atypical neuronal processes (Tassabehji 2003). Recent studies in these areas on younger individuals with WBS appear to support the model that the human brain is not specialized early in life, and only becomes increasingly specialized and localized during development. This is in contrast to the modular theory of brain function, supporters of which have long cited the seemingly modular cognitive profile in WBS as evidence (Bellugi 2000). Instead of seeing the stronger cognitive skills in WBS as intact, it is suggested that WBS language and face processing show delay and/or deviance from normal trajectories throughout development.

The WBS phenotype also includes idiosyncratic brain morphology and processing. Neuroanatomical, structural, and functional MRI (fMRI) studies are showing that WBS individuals have abnormal neural organization, and that the origin of the mental and cognitive phenotypes of the disorder may rest in the interference of normal neurodevelopment (Tassabehji 2003). The WBS brain is smaller than normal, with an enlarged cerebellum relative to a small cerebrum, but the limbic structures of the temporal lobe appear normal (Galaburda et al. 2002). Neuronal abnormalities are associated with WBS including small neurons and increased cell-packing density in the left hemisphere of the primary visual cortex (Tassabehji 2003). These abnormalities may be related to the visuo-spatial deficits, though visuo-spatial functions mainly involve the right hemisphere

while the left hemisphere is concerned with visual object recognition. During cognitive testing WBS individuals were reported to have biochemical abnormalities in the left cerebellum, which may be related to their cognitive weaknesses.

Functional MRI studies have shown that the WBS brain tends to use atypical auditory processing (Tassabehji 2003). Significantly unusual neuronal processing of music and noise has been observed in WBS individuals, with reduced activation in the temporal lobes which support music and noise processing, coupled with increased activation in the cerebellum and amygdala, which is in accordance with the observed heightened emotional response to certain sounds (Blood et al. 1999). Auditory processing is suggested to be carried out by different neural systems in WBS compared with normal brains because the network of activation is more diffuse throughout the WBS brain during music processing, which supports the idea that their atypical reactions to sound are based in abnormal neural functioning (Tassabehji 2003).

Molecular Mechanisms of the WBS Deletion

WBS results from a microdeletion at chromosome band 7q11.23, which is thought to be caused by unequal crossover during meiosis between misaligned low-copy repeat sequences (LCRs) that flank the WBS deletion (Figure 1) (Baumer et al. 1998; Valero et al. 2000). These LCRs span approximately 400 kb, exhibit >95% sequence identity, and occur in the same and opposite

orientations (Osborne et al. 2001). They are composed of smaller duplicons containing unique sequence elements from the internal border regions of the deletion, as well as transcribed genes, conserved pseudogenes with nearly identical genomic structure, pseudogenes corresponding to their ancestral progenitors found at other sites on chromosome 7, and putative telomere-associated repeats (Osborne et al 2001; Tassabehji 2003). The majority of deletions span a genomic region of approximately 1.5 Mb, with the common breakpoints clustering within the LCR regions, resulting in remarkably homogeneous deletions (Valero et al. 2000); 98% of WBS individuals studied have the same breakpoints (Morris and Mervis 2000). The fact that there are two telomeric LCRs on human chromosome 7 (Figure 5) makes it possible for two common deletions to occur, though the deletion between the two LCRs closest to each other comprise the majority of cases and deletions between the outermost LCRs include only one additional gene, *STAG3L2*. Misalignment and unequal meiotic recombination of DNA sequences positioned in direct orientation with each other can occur interchromosomally, between two misaligned chromosomes and result in a deletion, as seen in WBS, and a duplication, which has not yet been reported (Figure 2A). They can also occur intrachromosomally, within one chromosome and result in a deletion event (Figure 2B). Misalignment and unequal cross-over between DNA sequences in inverted orientation relative to each other can result in an inversion of the intervening region (Osborne et al. 2001). The inversion has been reported as having an incidence of 5% in the

normal population (Hobart et al. 2004), and has been suggested to contribute to the mechanism underlying WBS, resulting in a predisposition not only to WBS-causing microdeletions, but also to translocations (Osborne et al. 2001).

Current Clinical and Animal Model WBS Research

WBS research is using clinical, psychological, and molecular analysis of patients with both typical and atypical WBS, alongside mouse models, to address the question of which genes in the critical region are dosage sensitive and contribute to the specific features of human cognition and behavior (Tassabehji 2003). Patients with typical WBS have provided information about the range and penetrance of the spectrum of phenotypes, and insight into the molecular mechanisms that underlie WBS. Patients with atypical WBS and smaller deletions have helped to narrow the critical region and have allowed researchers to begin to functionally dissect the deletion region. Deletions at 7q11.23 that extend past the critical region have also been identified in WBS individuals who exhibit more severe phenotypes; however no credible cases of WBS have been reported in which there is no deletion at 7q11.23 (Tassabehji 2003). WBS is a rare disorder, and finding atypical individuals is difficult because of their extreme rarity and because clinicians are unfamiliar with the syndrome's physical and behavioral manifestations and so can easily misdiagnose individuals who have mild or partial phenotypes. Furthermore, despite the commonly defined

phenotypic features, there is a wide range of clinical variability between patients (Valero et al. 2000).

Atypical Deletions

A few rare individuals with the WBS phenotype have been identified with deletions at 7q11.23 smaller than the typical 1.5 Mb. Olson et al. (1995) described one individual with an approximately 30 kb intragenic deletion of *ELN* who has SVAS and an otherwise normal phenotype (Osborne 1999). Frangiskakis et al. (1996) described two familial cases of SVAS caused by an approximately 85 kb deletion spanning *ELN* and *LIMK1*, in which 11/13 of the individuals with deletions also showed the impaired visuospatial constructive cognition associated with WBS. These cases implicate *LIMK1* as responsible for the cognitive phenotype in these families and in WBS in general. On the other hand, Tassabehji et al. (1999) later described two individuals with similar deletions spanning approximately 90 kb and spanning *ELN* and *LIMK1*, but lacking the WBSCP. These data suggest that *LIMK1* deletion is either irrelevant to the WBSCP or, at most, that it is necessary but not sufficient. Thus there is currently ambiguity regarding the role of *LIMK1* haploinsufficiency in cognitive spatial impairment (Tassabehji 2003). In the same paper Tassabehji et al. (1999) described a third individual with an approximately 800 kb deletion with a centromeric breakpoint similar to typical WBS deletions, but a telomeric breakpoint just distal to *RFC2*. This individual's physical, cognitive, and

behavioral phenotypes were normal except for severe SVAS and peripheral pulmonary arterial stenosis. This case appears to implicate genes telomeric to *RFC2* as the determinants of the WBS cognitive phenotype. Somatic mosaicism could be the cause of the observed lack of cognitive phenotype, but it is unlikely since the deletion was detected in both lymphoblasts and fibroblasts (Karmiloff-Smith et al. 2003), and mosaicism is most likely to arise by mitotic recombination, which happens at 10^{-4} times the frequency of meiotic recombination (Tassabehji et al. 1999).

Certain WBS patients have unique mutations which seem to point to genes telomeric to *STX1A* as being responsible for their phenotypes. Botta et al. (1999) described two WBS patients with atypical centromeric breakpoints distal to *STX1A*, and typical telomeric breakpoints that interrupted the *GTF2I* gene. The patients showed the full spectrum of the WBS phenotype including typical facies, delayed growth, mildly retarded psychomotor developmental milestones, an overfriendly and hyperactive disposition, and only lacked hypercalcaemia. Osborne et al. (2001) reported an individual with some typical WBS features (WBS face, malocclusion, hyperacusis, WBS-like behavioral profile and developmental delay), who was heterozygous for the WBSCR inversion polymorphism, and hypothesized that *GTF2I* may have been involved in the rearrangement, thereby contributing to the observed phenotype. Heller et al. (2003) described a third patient with a similar centromeric breakpoint and a telomeric breakpoint just distal to *GTF2I*. This patient also showed a wide range

of typical WBS features including SVAS and pulmonary valve stenosis, developmental retardation, very friendly nature, hyperacusis and characteristic facies. These three cases together appear to rule out *STX1A* and genes centromeric to it from being responsible for the WBSCP.

A report of an individual with mild WBS and an atypical deletion has supported the theory that telomeric genes are primarily responsible for the WBSCP (Gagliardi et al. 2003). The individual had SVAS, mild physical WBS characteristics, a borderline IQ, none of the typical WBS behavioral characteristics, mild psychomotor milestone delay, and lacked specific spatial and constructive impairment. The deletion had a typical centromeric breakpoint but an atypical centromeric breakpoint within intron 1 of *CYLN2*, resulting in a deletion that encompasses the centromeric portion of the WBSCR including at least the 5' portion of *CYLN2* and excluding *GTF2I*. The authors concluded that hemizyosity for *LIMK1* and *CYLN2*, while not sufficient to generate the WBSCP, may cause alterations in the cognitive profile resulting in the milder phenotype with some cognitive impairment.

While the WBS phenotype is the result of haploinsufficiency for a number of genes, the deletion of the *GTF2IRD1* and/or *GTF2I* genes located on the telomeric side of the critical region appears necessary for the unique WBS cognitive profile (Tassabehji 2003). Haploinsufficiency for *RFC2* and the genes proximal to it does not appear responsible for any of the main physical, mental and cognitive aspects of WBS, but these genes should not be ruled out from

playing a role in the variably penetrant components of the WBS phenotype. Data from the individuals with partial deletions at 7q11.23, combined with isolated cases of SVAS, have contributed to the narrowing of the critical region containing the main genetic determinants of WBS to the telomeric end of the deletion region.

Mouse Models

Knockout mice have been made in which single genes from the WBSCR have been either partially or completely deleted. Knockout mice allow researchers to observe behavioral and cognitive phenotypes associated with reduced or eliminated gene expression. They also make it possible to compare levels of gene expression between normal and hemizygous or null mice not only for the gene knocked out, but also for any other genes that can be screened using microarray technology. This approach can illuminate disrupted downstream regulatory effects of the knocked out gene, helping to elucidate the effects of the WBS microdeletion both inside and outside the critical region. Single gene knockout mice can also be bred together to create double, triple etc. knockouts to study additive phenotypes. Current mouse models that contribute to our understanding of WBS include single gene knockouts for *Eln*, *Limk1*, *Cyln2*, *Gtf2ird1* and *Fzd9*.

Mice that lack elastin were generated by Li et al. (1998) by deleting 4.0 kb of the promoter and exon 1 of the *Eln* gene, resulting in a null allele. *Eln*^{-/-} mice

were found to die after birth due to subendothelial accumulation and reorientation of proliferating smooth muscle cells, in both systemic and pulmonary arteries, that eventually obliterated the vascular lumen. *Eln*^{+/-} mice did not have SVAS (normal gross appearance and life expectancy), despite having abnormal elastic lamellae in the aorta at birth, but they were hypertensive and had altered arterial compliance. Interestingly, hypertension is often seen in WBS, indicating that humans with WBS may use a similar mechanism to compensate for the results of elastin-deficiency (Tassabehji 2003). In this context, hypertension may be an important adaptive response to maintain cardiovascular function and the compensatory increase in the lamellar units results in an increased risk of obstructive vascular disease in humans. Li et al. (1998) concluded that elastin is a molecular determinant of late arterial morphogenesis, stabilizing arterial structure by regulating proliferation and organization of vascular smooth muscle. The *Eln* knockout mouse model uncovered a regulatory function for elastin in arterial development and confirmed that the pathophysiological mechanism for SVAS is haploinsufficiency through lesions in elastic arteries.

The *Limk1* gene was knocked out by Meng et al. (2002), resulting in mice that do not express *Limk1* messenger RNA or protein. *Limk1* encodes a serine/threonine kinase, specifically expressed in neuronal tissue, which regulates actin reorganization through phosphorylation and inactivation of ADF/cofilin; actin cytoskeletal reorganization is essential for various cell activities including the directional movement of neurons and neurite outgrowth. Therefore

actin remodeling may be essential to the normal establishment of the dendritic spines that make up the majority of synaptic connections within the hippocampus, and which are associated with the formation and maintenance of memory and learning (Tassabehji 2003). *Limk*^{-/-} mice are viable with a grossly normal nervous system, possibly as a result of *Limk2* or *Tesk* activity (both able to regulate cofilin) providing some of the missing function, though they show significantly reduced ADF/cofilin phosphorylation, and lack normal dendritic spine morphology and synaptic function including long-term hippocampal potentiation (a form of a synaptic plasticity considered critical to learning and memory) (Meng et al. 2002). *Limk1*^{-/-} mice display mildly altered fear responses and deficiencies in spatial learning in behavioral tests, but their relevance to the cognitive defects in humans with WBS is not yet clear (Tassabehji 2003).

Mice haploinsufficient for *Cyln2* were generated by Hoogenraad et al. (2000) using a gene-targeting approach; the authors showed that mice had features reminiscent of WBS that included mild growth retardation, mild brain abnormalities, hippocampal synaptic dysfunction and certain motor coordination impairments. Although the encoded CLIP-115 protein is not essential for life (*Cyln2*^{-/-} mice are viable), the growth deficiency in these mice indicates that this highly conserved protein, expressed in dendrites and cell bodies of many neurons in the brain, may be necessary for normal development. CLIP-115 is a member of a family of cytoplasmic linker proteins (CLIPs) that specifically associate with the ends of growing microtubules and is thought to play a role in

the formation and/or turnover of gap junctions (Tassabehji 2003). CLIPs are involved in regulating microtubule dynamics and establishing interactions between microtubule tips and various cellular structures, including cargoes destined for transport by dynein. Microtubule dynamics in *Cyln2^{-/-}* mice were largely unaffected apart from an increased accumulation of CLIP-170 and dynein at microtubule tips (Hoogenraad et al. 2000). This protein redistribution may be significant in disrupting dynein motor regulation and, together with the loss of CLIP-115-specific functions, could underlie the neurodevelopmental abnormalities seen in the mice. Perhaps this is also the case in WBS since there is some overlap in the neurological phenotypes.

Mice that contain a *c-myc* transgene-induced deletion of approximately 40 kb of the *Gtf2ird1* gene on both chromosome 5G homologues were reported by Durkin et al. (2001). The *Gtf2ird1^{-/-}* mice were viable and fertile with a normal lifespan, but despite the deletion, *Gtf2ird1* expression was detected, though at significantly reduced levels. The authors observed no obvious behavioral or morphological abnormalities apart from those expected due to *c-myc* over-expression, specifically increased liver cell proliferation and hepatocellular carcinomas. It remains to be seen whether any subtle neurological or behavioral abnormalities exist in these mice since these analyses were not carried out.

A *Fzd9* knock-out mouse was generated by deleting the complete open reading frame of the gene (Ranheim et al. 2005). The authors reported that *Fzd9^{-/-}* mice are viable and fertile with normal development and only a

moderately reduced lifespan, though no obvious cause was found. Both the *Fzd9*^{+/-} and *Fzd9*^{-/-} mice showed no evidence of WBS-associated developmental or morphological phenotypes; their phenotype appears to be limited to abnormalities in the development of B cell populations, though WBS individuals do not exhibit any overt immunologic or hematopoietic deficits.

Interpreting the studies of single gene knockout mouse models as a strategy for genotype-phenotype correlations in WBS faces a number of difficulties. So far, every knockout model for genes in the WBSCR except for *Eln* has failed to provide evidence for a direct gene-phenotype relationship. Furthermore, the cardiovascular aspects of WBS caused by haploinsufficiency for *ELN* constitute the only part of the WBS phenotype that has been recognized as an isolated Mendelian dominant character in families with a mutation in one of the genes in the WBSCR, which suggests that the genetic mechanisms underlying the WBS phenotype are probably more complex than a superimposed set of independent single-gene haploinsufficiency effects. Thus, while studies of single gene knockouts may provide some insight into the possible contributions each gene makes to WBS, it is not sufficient simply to knock out the deleted genes one at a time.

The behavioral phenotypes observed in mouse knockouts of single genes in the WBSCR so far are subtle, and little biochemical analysis has been performed. The overall WBS phenotype could depend in part on functional relationships between genes within the region and/or on haploinsufficiency for a

specific combination of genes in the WBSCR. The role of position effects in silencing genes flanking the deletion breakpoints and disruption of cis- or trans-regulatory effects also need to be considered (Tassabehji 2003).

Research Objectives and Experimental Approach

Currently, there is no full deletion model of WBS, nor are there deletion models of large sections of the WBSCR. The approach we have taken is to develop a unique deletion mouse model of WBS by creating two half-deletions and combining them in a compound heterozygote to model WBS (Figure 3). Using the half-deletions we will be able to assign clinical, behavioral and molecular phenotypes to sub-regions by narrowing the region responsible for the cognitive phenotype to either the centromeric or telomeric half of the deletion region, to the combined deletion of the two, or to an interaction between the deletion and the surrounding genes.

The Cre/loxP site-specific recombination system developed by Alan Bradley has made it possible to manipulate mouse embryonic stem (ES) cells to generate defined deletions, duplications and inversions extending 3 - 4 cM, and to transmit them to the mouse germ line (Figure 4) (Ramirez-Solis et al. 1995). The process begins with AB2.2 ES cells (Lexicon Genetics) derived from *Hprt*-deficient 129SvEvBrd mice and available in The Mouse Kit from Stratagene (Catalog #745002). Hypoxanthine phosphoribosyl transferase (*Hprt*) is the gene that codes for the enzyme which allows cells to use the salvage pathway for

purine synthesis when the *de novo* pathway is blocked. In the *de novo* pathway, which is normally used by cells, purine nucleotides are assembled from smaller molecules that make up their components. The salvage pathway uses *Hprt* to change related molecules into purine nucleotides by rearranging their structure. The ES cells are consecutively targeted with DNA constructs containing *loxP* sequences at the end points of a genetic interval, creating doubly-targeted cells (2lox). The targeting constructs each contain part of a *Hprt* minigene cassette, and either a puromycin or a neomycin resistance gene. The linearized targeting constructs are inserted into the ES cell genomic DNA via homologous recombination. Transient expression of *Cre* recombinase is then used to induce recombination between the two *loxP* sites, deleting the intervening DNA sequence and resulting in cells with one *loxP* site (1lox). This recombination reconstructs the *Hprt* minigene cassette and deletes the puromycin and neomycin resistance genes. The ES cells can then be directly selected for clones with the desired chromosomal recombination by selection with the drug aminopterin, which blocks the *de novo* pathway of purine synthesis. Aminopterin selection is accompanied by hypoxanthine and thymidine supplements in the media (HAT media), which provide the substrates on which the *Hprt* enzyme acts to synthesize purines. *Cre*-mediated chromosomal recombination also deletes the puromycin and neomycin resistance genes, which are positioned so they will be removed in deletions but retained in inversions or duplications, allowing for

drug sensitivity selection to determine the nature of the chromosomal recombination.

The high degree of linkage conservation between human chromosome band 7q11.23 and mouse chromosome 5G1-G2 makes an accurate and precise WBSCR deletion model highly feasible (Figure 5) (Valero et al. 2000). The orthologous region in mouse lacks the LCRs that flank the human WBS deletion region, but has the full complement of genes which is inverted compared to the most common human orientation.

The *Cre-loxP* chromosomal engineering method has been used to generate a mouse model of other diseases such as Velocardiofacial/DiGeorge syndrome (VCFS) and Down syndrome (DS). VCFS is the most common deletion syndrome in humans (Lindsay et al. 1999). Lindsay et al. generated mice carrying a deletion (*Df1*) that encompasses most of the *del22q11* region which causes VCFS. Because the *del22q11* region has a homolog on mouse chromosome 16 that is similar in size and in gene content, the authors were able to use *Cre-loxP* chromosome engineering to create heterozygously deleted mice. These mice had cardiovascular abnormalities that are of the same type as those associated with *del22q11*. The authors were able to rescue the phenotype by genetic complementation of the deletion using a chromosome duplicated for *Df1*, revealing the pathogenic basis of the most clinically severe aspect of VCFS. The method of genetic complementation used did not compensate for an effect of the deletion on neighboring genes, or for the deletion of anchor point genes, making

it possible to attribute the cause of the phenotypic rescue to reestablishment of dizygosity for the deleted genes.

A DS mouse model was created by Olson et al. (2004) using *Cre-loxP* chromosome engineering to test the hypothesis that trisomy for the genes in the Down syndrome critical region (DSCR) is sufficient to produce the dysmorphology of the craniofacial skeleton that results in facial features associated with DS. Studies of previously generated DS model mice (Ts65Dn), which contain a marker chromosome that results in segmental trisomy for orthologs of about half the genes that rare segmental trisomy 21 DS individuals have in triplicate, showed skull dimensions that correspond with the reduced facial dimensions in humans with DS. Olson et al. (2004) attempted to more accurately model DS, using the *Cre-loxP* strategy to generate mice that are trisomic for mouse orthologs of 33 conserved and minimally conserved genes in the DSCR. In contrast to the previous findings, they concluded that trisomy of only critical region genes is not sufficient to produce the facial dysmorphology seen in Ts65Dn mice. They further hypothesize that while the solitary effect of a triplicated gene may be inconspicuous, it could be that interactions among noncontiguous genes produce the observed phenotype.

The approaches taken in these studies are directly applicable to the development of a WBS mouse model. The VCFS and DS syndrome models represent ways to narrow the genetic causes of phenotypes associated with human deletion or duplication syndromes. The DS model exemplifies the utility

of chromosomally engineered mouse models in determining the genetic cause of distinct phenotypes, especially in identifying those that could not have been predicted from or observed in human studies. The approaches taken by the aforementioned authors can be used to functionally dissect the WBS deletion region and to determine which genes, interactions between genes, and/or interactions between genes outside the deletion region and the deletion itself are responsible for particular aspects of the WBS phenotype in humans.

The continually increasing genome database and sequence resources, combined with the development over the last decade of computer applications aimed at manipulating sequence information in a molecular biological context, have made computer-based experimental design and planning an accessible and valuable tool for the biological researcher. The online genomic resources available, including NCBI (the National Center for Biotechnology Information, <http://www.ncbi.nlm.nih.gov/>), the University of California Santa Cruz Genome Browser (<http://genome.ucsc.edu/>), Ensembl (<http://www.ensembl.org/>) and Mouse Genome Informatics (<http://www.informatics.jax.org/>) to name a few, have made navigating both the mouse and human genomes a relatively easy task. Computer applications such as VectorNTI, Sequencher, and Gene Construction Kit have made it possible to create and maintain a sequence database of the DNA, RNA, and protein resources available, and to manipulate them using both standard and custom molecular biology reagents on the computer (*in silico*) before attempting to perform a protocol in the laboratory. This array of resources

and techniques has made it possible to design protocols and complex molecular constructs that would otherwise require time-consuming trial-and-error or indirect approaches to accomplish.

Currently, the Francke laboratory has generated DNA constructs targeting three of the four endpoints needed to create the WBS compound heterozygote mouse model (Figure 6). The compound heterozygote WBS mouse model in the process of being developing will be hemizygous for all of the genes in the WBS deletion region except for *Limk1*, for which it will be null. This is the case because screening of the Hprt Δ 3' and Hprt Δ 5' libraries yielded only one clone near the center of the deletion region, targeting *Limk1* exons 3 and 4. The insert was released from the Hprt Δ 3' in which it was first isolated, and ligated into the Hprt Δ 5' construct to facilitate the generation of the two ES cell lines necessary to make the compound heterozygote mouse with the full WBS deletion. This thesis describes the production of three transgenic mouse embryonic stem cell lines to be used in the continued development of a mouse model of Williams-Beuren Syndrome.

MATERIALS & METHODS

Alan Bradley's Cre/*loxP* strategy for manipulating mouse ES cells was used for this study.

Targeting Vector Construction

A targeting vector was constructed using a plasmid containing the previously described chromosome engineering cassette Hprt Δ 3' (Ramirez-Solis et al. 1995) and an unknown insert (Hprt Δ 3'-?) (Figure 7A). The plasmid was obtained by screening the Hprt Δ 3' 129/SvEv genomic lambda library provided by the Bradley laboratory in 2000. The cassette includes a prokaryotic selectable marker gene (*amp*), a eukaryotic selectable marker gene (*puro*), a *loxP* site, and exons 3-9 of a human hypoxanthine phosphoribosyl transferase (*Hprt*) minigene cassette. To release the insert, 3 μ l of a 50 μ l miniprep of the bacterial clone containing the Hprt Δ 3'-? Plasmid was digested with 5 units of the restriction enzyme *Ascl* for 1 hour in a 37°C water bath. To digest the insert into at least two fragments, the *Ascl* digestion products were then digested with 5 units of the restriction enzyme *EcoRI* (which was shown to be a non-cutter in the vector by restriction mapping) for 1 hour in a 37°C water bath. After being gel-purified, 60 ng of the vector band was then dephosphorylated using 3 units of Shrimp Alkaline Phosphatase (Promega Catalog #M8201) by incubating in a 37°C water

bath for 15 minutes, and then the enzyme was deactivated by incubating in a 65°C water bath for 10 minutes.

The mouse bacterial artificial chromosome (BAC) RPCI 24-230L19, which contains mouse genomic sequence 5' to and including the *Fkbp6* gene (Figure 7B), was obtained from the BACPAC Resource Center at the Children's Hospital Oakland Research Institute (<http://bacpac.chori.org/>). The BAC (4.8 µg) was digested with 20 units of *Mlu*I, a restriction enzyme that shares compatible sticky ends with *Asc*I, by incubating overnight in a 37°C water bath.

The resulting fragments were ligated together with the dephosphorylated vector cassette using the Fermentas Rapid DNA Ligation Kit (Catalog #K1421) to make the *Hprt*Δ3'-*Fkbp6* targeting construct. The product was electroporated into Invitrogen ElectroMAX Stbl4 Competent Cells (Catalog# 11635-018) using a 0.2 cm gap cuvette in a BioRad Gene Pulser at 200 Ω, 25 µF and 2.5 volts. The transformed bacteria were then plated onto standard ampicillin-containing LB-agarose plates and incubated overnight at 30°C. Ten ampicillin-resistant bacterial colonies were picked off of a re-streaked plate because the original plate produced a lawn of bacterial colonies.

Insert size and orientation were tested by digesting a 4 µl of 50 µl miniprep of each *Hprt*Δ3'-*Fkbp6* clone with 5 units of *Eco*RV for 1 hour in a 37°C water bath. In preparation for transformation into ES cells, 38 µg of the *Hprt*Δ3'-*Fkbp6* clone with the insert in the correct orientation (clone A6) was linearized by

digestion with 25 units of KpnI for 2 hours in a 37°C water bath; KpnI cuts once in the construct, toward the center of the genomic insert.

2lox ES Cells

The linearized *Hprt*Δ3'-*Fkbp6* targeting construct DNA was electroporated into ES cell clone 4D4, a line of AB2.2 ES cells derived from *Hprt*-deficient 129SvEvBrd mice that had been previously targeted in the Francke laboratory with the *Hprt*Δ5'-*Limk1* construct and selected for Neomycin resistance. The AB2.2 cell line was originally obtained from Stratagene in The Mouse Kit (Catalog #745002). The BioRad Gene Pulser was used to electroporate the ES cells, using a 0.4 cm gap cuvette, 250 V and 250 μF.

Twenty-four hours after transformation, Puromycin selection was begun to test for insertion of the *Hprt*Δ3'-*Fkbp6* targeting vector into genomic DNA. Selection was accomplished by supplementing ES cell media with 5 μg/ml Puromycin for 7 days. Two hundred forty-eight Puromycin-resistant colonies were isolated and screened by Southern blotting (Figure 8). Southern blotting was performed by digesting 5 μg of ES cell genomic DNA with 50 units each of *SpeI* and *SalI* overnight in a 37°C water bath, which in the wild-type genome cut outside the genomic sequence used for homologous recombination and yield a 13.3 kb fragment, and in targeted ES cell DNA also cut within the construct sequence and yield a 11.5 kb fragment and a 16.1 kb fragment. Two probes were developed to hybridize with the genomic DNA restriction fragments, Probe

A and Probe B (Appendix 2). Probe A hybridizes with the wild-type fragment, and with the 11.5 kb targeted fragment. Probe A was generated by PCR using the RPCI 24-230L19 BAC as a template that amplified a 542 bp sequence. Probe B hybridizes with the wild-type fragment and with the 16.1 kb targeted fragment. Probe B was generated by PCR using the RPCI 24-230L19 BAC as a template that amplified a 753 bp sequence.

1lox ES cells

To generate 1lox ES cells and to determine whether the *Fkbp6* targeting event occurred on the same chromosome as the previous *Limk1* targeting event, 4 doubly-targeted ES cell clones (1A6, 1D4, 8C4 and 10B2) were transfected by electroporation with plasmid pOG231 (O'Gorman et al. 1997), which contains and expresses the *Cre* recombinase gene. Clones 1A6 and 1D4 were chosen for their positive Southern blot results and for their desirable growth characteristics and colony morphology (discussed in the Results section of this thesis), and clones 8C4 and 10B2 were chosen specifically because they had suspicious Southern blot results but good growth characteristics and colony morphology. Clones with suspicious Southern results still included cells that were doubly-targeted, and Cre-mediated recombination might successfully result in 1lox cells; these two less-desirable clones were chosen in order to perform the ES cell culture techniques used in this thesis for the first time, and for this first round of Cre deletion it was advisable to be conservative and to use cells that

were less than perfect in case a mistake in technique was made, so as not to waste the most optimal cells. For each clone, between 3.44×10^6 and 6.64×10^6 cells were transfected. Transformation was performed using the same equipment and parameters used previously in this study and described in the 2lox ES Cells section above. Cre-mediated chromosomal recombination occurs at a frequency of approximately 10% between *loxP* sites on the same chromosome (*in cis*), and that efficiency does not alter appreciably the distance between the two targeted *loxP* sites is changed from a few kb to up to 10 Mb (Yu and Bradley 2001). Cre-mediated recombination between *loxP* sites on different chromosomes (*in trans*) occurs with an efficiency that is 2 to 3 orders of magnitude less than that when the *loxP* sites are *in cis*. Therefore ES cell clones with 2 *loxP* sites *in cis* are expected to generate 100 to 1000 times more HAT-resistant clonal colonies than clones with *loxP* sites *in trans*.

To select for ES cell clones in which the Cre recombinase had successfully recombined *loxP* sites and reconstructed the *Hprt* minigene, HAT selection was begun twenty four hours after transfection by supplementing ES cell media with HAT Media Supplement (Sigma Catalog# H0262) at 1x final concentration. For each of the four clones transfected, cells were plated onto four 6 cm tissue culture treated plates at three different concentrations (two plates with 2.5×10^6 cells, one plate with 10^6 cells, one plate with 10^5 cells). After 10 days, five colonies were isolated from clone 1D4 (from 2 different plates) and six clones were isolated from clone 10B2 (from 3 different plates). The

eleven HAT-resistant colonies were plated onto individual wells on 24-well tissue culture treated plates, and later expanded.

Puromycin and Neomycin sensitivity was tested for by supplementing ES cell media with Puromycin (5 μ g/ml final concentration) and G418 (400 μ g/ml final concentration), respectively. HAT-resistant and Puromycin- and G418-sensitive ES cell clones were confirmed by Southern blotting (Figure 9).

Southern blotting was performed by digestion of 5 μ g of ES cell genomic DNA with 50 units of HindIII overnight in a 37°C water bath, which in the 2lox genome yields a 2.7 kb fragment, and in 1lox ES cell DNA yields a 4.9 kb fragment.

Probe L1A was developed to hybridize with the both genomic DNA restriction fragments (Table 1). Probe L1A was generated by PCR using the *Hprt* Δ 3'-*Fkbp6* construct as a template that amplified a 560 bp sequence including exons 3 – 6 of the *Hprt* minigene cassette.

RESULTS

Targeting Vector Construction

Serial *Ascl* and *EcoRI* digestion released the insert from the *Hprt* Δ 3'-? plasmid and digested it into two fragments (Figure 10). Digestion products run on an 0.8% agarose gel and stained with ethidium bromide showed one band at >10 kb (predicted vector size), and two bands at approximately 3.4 and 3.5 kb, which is consistent with the estimated 7 kb size of the unknown insert. Undigested plasmid DNA ran at approximately 18 kb, confirming these estimations.

Digestion of BAC RPCI 24-230L19 with *MluI* resulted in three DNA fragments of 9.9 kb, 63.3 kb and 117.4 kb in length, as predicted by *in silico* restriction enzyme digestion and confirmed by agarose gel electrophoresis on an 0.8% agarose gel stained with ethidium bromide. The 9.9 kb fragment was not gel purified since the larger digestion fragments were too large to be cloned into the *Hprt* Δ 3' vector and the 9.9 kb fragment would be the only one to successfully ligate into the vector; therefore the mixture of digestion products was used in the ligation process.

Ligation of the digested BAC and dephosphorylated plasmid generated products in which the 9.9 kb BAC fragment was inserted into the *Hprt* Δ 3' plasmid in both possible orientations. After transforming the ligation into DH5 α competent bacterial cells, one bacterial clone (clone A6) was identified in which

the recombined plasmid had the insert in the desired orientation by restriction enzyme digestion with EcoRV (Figure 11). Digestion with EcoRV was predicted to generate 16.2 and 4.2 kb bands in plasmids in which the insert was in the desired orientation, and 12.7 and 7.8 kb bands in plasmids in which the insert was in the opposite orientation. Gel electrophoresis using a 0.8% agarose gel showed that clone A5 contained the insert in the incorrect orientation, and clone A6 contained the insert in the correct orientation.

Clone A6 was successfully linearized by digestion with KpnI (Figure 12). The restriction enzyme KpnI cuts once in the Hprt Δ 3'-*Fkbp6* construct, toward the middle of the genomic insert. Digestion with KpnI yields a linearized piece of DNA with large (3 kb and 6 kb) genomic sequence arms flanking the Hprt Δ 3' construct sequence, facilitating insertion into genomic DNA by providing long sequences which align with endogenous genomic DNA in the process of homologous recombination.

2lox ES Cells

Insertion of the linearized Hprt Δ 3'-*Fkbp6* plasmid into ES cell genomic DNA previously targeted with the Hprt Δ 5'-*Limk1* construct established the boundaries for the deletion to include the *Fkbp6* gene and exons 1 – 4 of the *Limk1* gene (Figure 13). When the *Cre* recombinase gene is expressed in doubly-targeted cells, the recombination reaction will bring together *loxP* sites that are in the same orientation and delete the intervening sequence, so in this

case deleted cells will be missing the entire *Fkbp6* gene and exons 1 - 4 of *Limk1* on one copy of chromosome 5.

Initial screening of the 248 Puromycin-resistant colonies by Southern blotting using Probe A yielded 12 positive clones, 7 of which were reconfirmed by Southern blotting with Probe B (Figure 14). The initial Southern screen using Probe A showed 12 clones that were positive for the targeting construct, with both wild-type bands at 13.3 kb and targeted bands at 11.5 kb. The results from the reconfirming Southern in which Probe B was used show that three clones, 13B5, 9C1 and 4B3, appear to be mosaic because their wild-type 13.3 kb bands are twice as strong as their targeted 16.1 kb bands, indicating that only some cells in the colony are heterozygous for being double-targeted as desired, and these clones were therefore ruled out for further use. Clones 4D6 and 3B3 show no bands, indicating that either the DNA was not in solution and therefore none was digested, that the restriction enzyme digestion did not occur correctly or that transfer was somehow blocked. Seven clones (1A6, 1D4, 3A2, 3D6, 8C4, 9C3, and 10B2) were concluded to be successfully doubly-targeted and clonal.

ES cell colony growth characteristics and morphology are indicative of the extent to which the cells are undifferentiated versus differentiated, and are useful in determining the best clone for use in blastocyst injection. Colonies that grow vertically into a mound (as opposed to spreading out in a flat mass), and have small nuclei and clear cytoplasm are indicative of undifferentiated ES cells that are best for injecting into mouse blastocysts for the purpose of generating

chimeric mice. The 2lox clones that were positive for the *Fkbp6* targeting event as determined by both Southern blots using both Probes A and B, and had the most desirable morphology and growth characteristics, were clones 1D4, 3A2, 3D6, 9C1, and 9C3.

1lox ES cells

Transfection of 2lox ES cell clones 1D4 and 10B2 with the pOG231 plasmid followed by 10 days of HAT yielded 5 and 6 colonies, respectively. This number of colonies is very low compared to the expected number based on transfection efficiency. If the *loxP* sites are *in cis*, 10,000 – 250,000 colonies per plate were expected, and if they are *in trans*, 10 – 2,500 colonies per plate were expected. Even if there were high levels of cell death during the electroporation, decreasing the number of live cells by up to 2/3, many more colonies per plate would have been expected (3,333 – 83,333) if the *loxP* sites are *in trans*. This low number of HAT-resistant colonies could therefore be due to the *loxP* sites being *in trans* in ES clones 1D4 and 10B2. The colonies from each clone came from 2 separate plates, indicating that they were independently derived. Four of the five HAT-resistant colonies from clone 1D4 grew when isolated and expanded in HAT media; none of the colonies from clone 10B3 grew when isolated and expanded in HAT media. None of the other six 2lox clones yielded HAT-resistant colonies after transfection with the pOG231 plasmid.

Two colonies (1D4-1, 1D4-3) were both resistant to HAT media and sensitive to Puromycin and G418 selection, and they were confirmed by Southern blotting (Figure 15). Untargeted AB2.2 ES cells showed a wild-type background band larger than 5 kb. This background band may be the result of Probe L1A hybridizing with endogenous *Hprt* pseudogene exons since the AB2.2 ES cells do not express *Hprt* but probably have *Hprt* exons in their genome in the form of pseudogenes. When wild-type B6 mouse genomic DNA is digested with HindIII, exons 3 – 6 of the endogenous *Hprt* gene will generate bands of 11.6, 6.6 and 1.0 kb when hybridized with Probe L1A; pseudogenes should produce similar bands since they are the result of genomic duplications, therefore the observed background band is not unexpected. Control 2lox undeleted ES cell genomic DNA showed the background band and the expected 2lox 2.7 kb band, and the two ES cell clones that were HAT-resistant and Puromycin- and G418-sensitive, 1D4-1 and 1D4-3, showed the expected 1lox 4.9 kb band. Two other clones, 1D4-2 and 1D4-4 were also resistant to HAT supplemented media, but not sensitive to Puromycin or G418 selection, indicating that they still contain the genes that were introduced in the two targeting events that confer resistance to those drugs.

DISCUSSION

This study shows that it is possible to generate transgenic mouse ES cells using *in silico*-designed restriction digestion of a BAC in conjunction with the Cre-*loxP* strategy for chromosomal rearrangement to precisely target a specific genomic locus. Historically such targeting efforts have been limited by the results of library screening, in which a probe is used to screen for clones which contain genomic sequence including the sequence that is to be targeted. The approach used in this thesis is a more precise one since known sequence data can be used to minimize the impact on nearby genes and regulatory sequences, restricting the deletion event to sequence that is determined by the researcher and minimizing unwanted effects on normal genomic functioning. With the ever-increasing availability of both mouse genomic sequence and mouse BACs, the use of BACs to precisely target genomic loci is becoming a valuable tool in the effort to functionally dissect the mammalian genome by generating knockout mice.

The ES cells produced in this research project were tested and confirmed similarly to the ES cells used to make mouse models of Down syndrome and VCFS mouse models (Lindsay et al. 1999; Olson et al. 2004). They were shown to have been successfully targeted at the desired loci by Southern blotting, and Cre-mediated chromosomal rearrangement resulted in HAT-resistant and Puromycin and G418-sensitive ES cells. The deletion event was confirmed by

Southern blotting, but in order to determine whether the two targeting events occurred on the same chromosome, it will be necessary to perform further assays such as Fluorescence In Situ Hybridization (FISH), and to test whether a 2lox mouse made from the doubly targeted ES cells can produce 1lox offspring when crossed with a mouse in which the *Cre* gene is expressed in the gametes.

Previous studies have implicated the telomeric region of the WBS syndrome in generating the cognitive phenotype profile characteristic to the syndrome. This project has generated 1lox ES cells which can be used to generate mice deleted for the centromeric portion of the deletion region; these mice will constitute a model that can to confirm that hypothesis the mice are found not to have the typical WBS cognitive phenotype. The mice generated using the ES cells from this project will also be useful in making the compound heterozygote WBS mouse model that can demonstrate any additive phenotypic effects of the complete deletion.

The *Cre-loxP* strategy of chromosome engineering has been used to make a number of deletion mouse models including the model of VCFS (Lindsay et al. 1999). The *loxP* targeting constructs used in the VCFS mouse model were designed to replace a 200 bp fragment containing exon 4 of *Es2/Dsgi* on one end of the deletion and a 4.5 kb fragment containing exons 2-3 of the *Ufd1l* gene (Lindsay et al. 1999). In contrast to that approach, the targeting construct designed in this study was designed not to replace exons in genes, but to

duplicate genomic sequence in the process of homologous recombination that was utilized to insert the *loxP* sites and selection genes into the mouse genome.

Using the 1lox and 2lox ES cells

This research has resulted in three mouse ES cell lines from ES cell clones 1D4 (2lox) and 1D4-1 and 1D4-3 (1lox) which will be used in the development of a mouse model of WBS. To make a transgenic mouse from ES cells, the ES cells are injected into mouse blastocysts, and the resulting embryos are transferred to pseudo-pregnant foster mothers (Yu and Bradley 2001). The chimeras generated from this blastocyst injection are then crossed with wild-type mice to establish germline transmission of the modified genomic region. The progeny that are confirmed to carry the engineered chromosome region are then used to establish a mutant mouse line.

The phenotypic characterization of compound heterozygote WBS mice will clarify the extent to which mice and humans are similarly affected by hemizyosity for the genes in the deletion region. Mice heterozygous for each half deletion or for both deletions will also be useful in the study of WBS and visuo-spatial brain development. Mice that are generated from ES cells that are targeted but not deleted (2lox) for each half deletion will be useful as controls and in studying the effects of tissue-specific deletion of the genes between the two targeted *loxP* sites. These studies can be accomplished by crossing the 2lox mouse with a *Cre* transgenic mouse in which the *Cre* gene is driven by a tissue-

specific promoter. Such a study would allow *Cre* expression to be limited to the central nervous system (CNS), for instance, to see whether it is the neuron or accessory cells that require the deleted genes to be normally expressed to ensure normal cognitive development. CNS-specific *Cre*-deletion of genes in the WBS deletion region could also decrease the deleterious effects of hemizyosity for genes like *Eln* which can have phenotypic effects that decrease mouse viability and life span.

Phenotypic characterization using standard behavioral tests and careful observation combined with thorough morphological and biochemical analysis of both 1lox and 2lox WBS region deletion mice will be important steps toward evaluating the mouse model. The 2lox mice are expected to have no phenotype compared to normal controls, as their genome has only been minimally disturbed and no genes are deleted. The *Fkbp6* targeting event occurred outside the gene and should not affect its transcription. The *Limk1* targeting event resulted in duplicated exons 3 and 4, and RT-PCR on mouse genomic DNA from transgenic mice targeted at the *Limk1* locus showed that both abnormal RNA transcripts with the duplicated exons and normal RNA transcripts are being produced, but no abnormally long *Limk1* protein was found by Western blotting (data not shown). Mice heterozygous for the *Limk1* targeting event were phenotypically indistinguishable from normal littermates, were fertile and had a normal lifespan. Mice heterozygous for one half of the WBS deletion region can be phenotypically characterized to determine which half of the deletion is responsible for a subset

of the WBS phenotype. Any phenotypes present in the compound heterozygotes that are lacking in both of the half-deletion mice could indicate an interaction between genetic elements in the two halves to produce the phenotypic character. These studies will allow researchers to efficiently focus their efforts on genes in one of the sub regions, and on genes regulated or otherwise affected by genes or gene products coded for in one of the two halves of the deletion region.

Access to tissues from WBS mice will allow researchers to conduct expression studies on both the genes in the deletion region and on any gene that has been cloned, especially those found to associate with a gene product coded for in the deletion region. Expression studies on WBS mouse tissues will allow for the discovery of genes whose expression is regulated by transcription factors coded for in the deletion region. Such information will make it possible to focus research in the human to candidate genes and gene products, reducing the need to do extensive preliminary research using rare human WBS tissue samples. WBS mouse tissues will also provide the samples necessary to conduct research aimed at discovering the proteins that interact with gene products coded for in the deletion region.

Research on patients that implicates certain genes in the WBSCR as being responsible for an aspect of the WBS phenotype can be confirmed by using a WBS mouse. Expression studies can determine whether the gene of interest has altered expression patterns in the WBS mouse, and whether expression is altered in other genes that may be regulated by the gene of

interest. Restoration of function of a deleted gene can be accomplished by mating a WBS mouse with a mouse transgenic for a single gene in the WBS deletion region, potentially rescuing or ameliorating any phenotype associated with it and providing concrete evidence for the relationship between the gene and the phenotype.

Problems/Challenges Faced

The genomic DNA used to target the 129SvEvBrd ES cells was derived from wild-type C57BL/6 ("B6") mice, which have a different genome compared to the highly inbred 129SvEvBrd ("129") mice. Using B6 mouse sequence information and genomic DNA to design and execute this research project was risky, since the 129 mouse strain has a genome that is up to 10% deleted compared to the B6 genome, and gene sequences are not always identical between the two strains, making the probability of successful homologous recombination smaller than if I had used 129SvEvBrd DNA to target the ES cells. Genome sequencing efforts directed at the mouse strains used to derive ES cells will make such gambles unnecessary and will increase the success of targeting efforts in the creation of transgenic mice.

The fact that the compound heterozygote WBS deletion mouse will be null for *Limk1* presents some challenges. WBS patients are hemizygous for *Limk1*, and some human cases have implicated hemizygosity for the gene in a cognitive phenotype, making it necessary to either subtract the phenotype of the *Limk*^{-/-}

mouse from the WBS mouse model, or to compensate for the loss of the gene. One option for compensating for the loss of *Limk1* would be to cross the WBS mouse with a *Limk1* transgenic mouse so that the resulting offspring will have one copy of *Limk1*. It would be necessary to use the endogenous *Limk1* promoter in the transgenic mouse to ensure normal gene expression, which should alleviate some if not all of the confounding phenotypic characters caused by absence of the gene product.

Applying the Techniques used in this Thesis

The experimental techniques used in this research will be a useful model for the generation of mouse models in which the deletion of precise sections of genomic DNA is required, such as mouse models of human microdeletion syndromes or human genetic diseases caused by the mutation or deletion of a single gene or section of DNA. Using the available mouse genomic sequence and mouse BAC libraries, it will be possible to more precisely design and generate a mouse model of a single-gene or microdeletion disorder.

In the design of the WBS compound heterozygote deletion model, the same genomic insert was used to target *Limk1* in each half-deletion, which will result in a compound heterozygote mouse null for *Limk1*. The accurate design of such a compound heterozygote mouse models will be made feasible by the ability to target genes next to each other in a deletion region, instead of having to rely on the sometimes limiting products of library screening.

Comment

At the beginning of this research project the sequences for the two targeting vectors were not yet published, and only their restriction maps were available. It was therefore necessary that sequencing be performed on them in order to facilitate the design of PCR primers and to confirm restriction enzyme site location, etc. Midway through the research project the full sequence for both constructs became available, saving time and resources and making the rapid progress of the project possible. With sequencing becoming easier, and with the increasing availability of sequences for a variety of constructs and genomes, researchers will be able to save time and resources by planning their experiments to a very detailed level ahead of time. They will also be able to use databases to find the best molecular tools for the job without having to resort to trial and error or constructing their own versions of a construct in order to accomplish their goal. With the growing repertoire of molecular tools in use in laboratories across the world, collaboration has never been so important.

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APPENDIX 1

Genes in the WBS Critical Region

Genes in the WBS critical region, in order of physical map location according to the UCSC Genome Bioinformatics Genome Browser, Human May 2004 (hg17) assembly. Adapted from Tassabehji 2003, RefSeq Gene Summary (NCBI).

POM121 (NM_172020) nuclear pore membrane protein 121

Position: chr7:71794586-71863488

Function: a member of the FG-repeat-containing nucleoporins; an integral membrane protein that localizes to the central spoke ring complex and participates in anchoring the nuclear pore complex to the nuclear envelope

NOL1R (NM_145414) NOL1R protein

Position: chr7:72162105-72167438

Other Names: Hypothetical protein WBSCR20A, Hypothetical protein FLJ10267

Function: has a NOL1/NOP2/sun domain and may play a role in the regulation of the cell cycle; a 120-kDa proliferation-associated nucleolar antigen, with similarity to p120 (NOL1)

Variants Identified: alternative splicing results in two transcript variants encoding different isoforms

FKBP6 (NM_003602) FK506 binding protein 6

Position: chr7:71884933-72217283

Function: a cis-trans prolyl isomerase that binds to the immunosuppressants FK506 and rapamycin; a member of the immunophilin protein family, which plays a role in immunoregulation and basic cellular processes involving protein folding and trafficking

FZD9 (NM_003508) Frizzled, *Drosophila* homolog, 9

Position: chr7:72292759-72295100

Other Names: FZD3

Function: possible involvement in tissue polarity and development, expressed predominantly in brain, testis, eye, skeletal muscle, and kidney; members of the 'frizzled' gene family encode 7-transmembrane domain proteins that are receptors for Wnt signaling proteins

BAZ1B (NM_023005) bromodomain adjacent to zinc finger domain 1B

Position: chr7:72299383-72381266

Other Names: WSTF

Function: encodes a member of the bromodomain protein family (the bromodomain is a structural motif characteristic of proteins involved in chromatin-dependent regulation of transcription)

Variants Identified: alternative splicing of this gene generates 2 transcript variants

BCL7B (NM_138707) B-cell CLL/lymphoma 7B

Position: chr7:72395336-72416675

Function: unknown; member of BCL7 protein family, contains a region that is highly similar to the N-terminal segment of BCL7A protein

Variants Identified: alternative splicing generates 2 transcript variants encoding distinct isoforms

TBL2 (NM_012453) transducin (beta)-like gene-2

Position: chr7:72428652-72437623

Function: possible role in intracellular signaling pathways or cytoskeletal organization; encodes a member of the beta-transducin protein family (most members are involved in regulatory functions) with four putative WD40 repeats

Variants Identified: alternative splicing generates 2 transcript variants

WBSCR18 (NM_032317) Williams Beuren syndrome chromosome region 18

Position: chr7:72539898-72542432

Function: encodes a member of the DNAJ molecular chaperone homology domain-containing protein family; intronless

WBSCR14 (NM_032954) Williams Beuren syndrome chromosome region 14

Position: chr7:72452174-72483521

Function: possible role in cell proliferation and/or differentiation; basic helix-loop-helix leucine zipper transcription factor of the Myc/Max/Mad superfamily

Variants Identified: alternative splicing generates 5 transcript variants

WBSCR22 (NM_017528) Williams Beuren syndrome chromosome region 22

Position: chr7:72542548-72557192

Function: unknown; contains a nuclear localization signal and an S-adenosyl-L-methionine binding motif typical of methyltransferases, suggesting that the encoded protein may act on DNA methylation

STX1A (NM_004603) Syntaxin 1A

Position: chr7:72558190-72578613

Function: plays a key role in intracellular transport and neurotransmitter release; syntaxins functions in the vesicle fusion process used during neurotransmitter release

WBSCR21 (NM_145215) Williams Beuren syndrome chromosome region 21

Position: chr7:72595536-72597446

Other Names: abhydrolase domain containing 11

Function: unknown; encodes a protein containing an alpha/beta hydrolase fold domain

Variants Identified: alternative splicing of this gene results in six transcript variants, two of which encode the same isoform

CLDN3 (NM_001306) claudin 3

Position: chr7:72627978-72629227

Function: Role in maintaining cellular polarity; protein component of tight junction strands in liver epithelial cells; intronless; low-affinity receptor for Clostridium perfringens enterotoxin

CLDN4 (NM_001305) claudin 4

Position: chr7:72689843-72691665

Other Names: CPER, CPETR, CPETR1, WBSCR8

Function: Role in maintaining cellular polarity; possible role in internal organ development and function during pre- and postnatal life; protein component of tight junction strands in kidney epithelial cells

ELN (NM_000501) elastin

Position: chr7:72887168-72927681

Function: Structural protein, component of elastic fibers; molecular determinant of the late arterial morphogenesis, stabilizing arterial structure by regulating

LIMK1 (NM_002314) LIM kinase-1

Position: chr7:72942806-72981505

Function: role in actin cytoskeletal reorganization essential for directional movement of neurons; Serine/threonine kinase with LIM domains, is a major ADF/cofilin inhibitor through phosphorylation kinase, thus regulating actin dynamics; likely to be a component of an intracellular signaling pathway and may be involved in brain development

Variants Identified: alternative splicing generates 2 transcript variants

EIF4H (BC010021) Eukaryotic translation factor 4H

Position: chr7:73033358-73056080

Other Names: WBSCR1

Function: encodes one of the translation initiation factors, which functions to stimulate the initiation of protein synthesis at the level of mRNA utilization, contains an RNA recognition motif

Variants Identified: alternative splicing generates 2 transcript variants

WBSCR5 (NM_173840) Williams-Beuren syndrome chromosome region 5

Position: chr7:73074995-73083717

Other Names: HSPC046, LAB (linker for activation of B cell proteins), WBSCR15

Function: role in normal B cell development

Variants Identified: alternative splicing generates 4 transcript variants which encode 2 isoforms with different sizes

RFC2 (NM_181471) Replication factor C, subunit 2

Position: chr7:73090484-73113389

Function: 40 kD subunit of replication factor C complex (an activator of DNA polymerases during replication), responsible for binding ATP

Variants Identified: alternative splicing generates 2 transcript variants

CYLN2 (NM_003388) cytoplasmic linker protein

Position: chr7:73148455-73264915

Other Names: WBSCR4, CLIP-115

Function: associates with both microtubules and the dendritic lamellar body; the protein encoded by this gene belongs to the family of cytoplasmic linker proteins, which have been proposed to mediate the interaction between specific membranous organelles and microtubules

Variants Identified: alternative splicing generates 2 transcript variants

GTF2IRD1 (NM_016328) TFII repeat domain-containing transcriptional regulator
1

Position: chr7:73312770-73461561

Other Names: WBSCR11

Function: may play a role in activating/repressing gene transcription; member of GTF2I transcription factor family with leucine-zipper; contains five GTF2I-like repeats, each of which possesses a potential HLH motif

Variants Identified: alternative splicing generates at least 2 transcript variants

GTF2I (NM_032999) General transcription factor II-I

Position: chr7:73516680-73619673

Other Names: BAP135, SPIN, TFII-I

Function: roles in transcription and signal transduction; Multifunctional phosphoprotein, containing leucine-zipper and six HLH-like domains (I-repeats); functions both as a basal factor and as an activator

Variants Identified: 4 transcript variants.

NCF1 (NM_000265) neutrophil cytosolic factor 1

Position: chr7:74017096-74032410

Function: 47-kilodalton cytosolic subunit of NADPH oxidase found in the neutrophil

GTF2IRD2 (NM_173537) GTF2I repeat domain containing 2

Position: chr7:73655134-73712492

Function: unknown, but inferred to be a transcription factor based on the presence of GTF2I-like repeats; contains both I-repeats and a Charlie8-like transposase motif; mRNA and EST evidence suggest that this gene is transcribed, and the encoded protein has a homolog in mouse, with which it shares 78% sequence identity

APPENDIX 2

Southern Probes

Southern probes used in this thesis, their PCR primers, templates, length, and sequence.

Probe A

Forward Primer: (UF7395) 5' CAACACAGGTGGCAGTCAGTCCTC 3'

Reverse Primer: (UF7396 5') CTTTGTATGGTTGGGGGGGG 3'

Template: mouse bacterial artificial chromosome (BAC) RPCI 24-230L19

PCR Product: (542 bp)

5'CAACACAGGTGGCAGTCAGTCCTCTATCCTGCCGCCCCCTCCCTCT
GTCATATTTTCCCTTCCCCCAGGGCTGCTGAAAGCCTAGGGAGAAAA
GGATGCCAGCCCTGGATTCTGATCCTAGCGCCTACTATGACTCTGAG
CCTCAGTTTCCCCGTCTGGGAAAAAAGGACTTAAGTGGAAAACAATG
GCCACTATGAGTCTGGATATATAGATCAACTGCAAGTCTAATTCAGAG
TTTTTTCTCCATACATGAGGTCTGGGTTCAATCTCTAGCATCACAAA
CGTCACATTGAGCTAGGACCTTCCCCCATGTAACCCCAGCATCTGGG
GGGGATTGAACCCAGAGCCTTGCGCATGAACAGCCACTCATGAAATT
GTCCTGAAGCAATGATTCTCAGTGTGTGGATTGCAACCCTTTTGAGG
TTCCGACAACCCTTTCAAGGTGGTTGCCTAAGACCACTGGGAAAATA
CAGATGTTTATAACAGTAGCAAAATTAGTTATGAGGTACCAACAAAAA
TACTTTGTTATGGTTGGGGGGGG 3'

Probe B

Forward Primer: (UF7398) 5' ACAGTGTCTTTACAACCGCCAGGG 3'

Reverse Primer: (UF7400) 5' GGTTAGAGCACACGAAGGAAGCACA 3'

Template: mouse bacterial artificial chromosome (BAC) RPCI 24-230L19

PCR Product: (753 bp)

5'ACAGTGTCTTTACAACCGCCAGGGCTGGCTTCTGGCCTCCACACCC
ATAAATGTGCATGCACACCAACACCTAAAGAAAAGTAGAGAGGTGGC
TGGCTAAGTGGTTAAGAACACTGTCTGGTCTTCTAGAGGACCCAGGT
TCGATAGGCAGCACCCACATGGCAGCTTACAACCATCTGTAACCTCA
ATTCCTGGGAATCCAATGCTGTCTTCGGCTTCTGTGGGCTCTTCACA
CTGACATATATGCAGGCAAAACACTCATACACATAAAATAAGAATCAG
GAAGAAAGAAAACAAGGAGCCACACATGGACAGGATGGGCTCAGGA
GCTGTTGAGTTTTCCAGCGCTCACTAACTTTCCCCTTCCGAGGTCTG
AAGAGTATACGTCAAGGTCCCTCATTCTGGTTGTCCCTGCCTGGGAC
ATCTCCTTCTCACACGTAATAGTTGACTCCACAGATGAACTTCTCTG
CACCTGTGCTCTGCACACATGTCACTCTCATTTCCTGCCTGCCTG
CACACCTCGGCCTTCTCGGCTGTCACCCCAGTCCCTGGCCATCACCT

TCCTCCACAGCGTTGTCATAGCCTGAGGCAATTATCTGTTGCTATAGC
CACCACAGGGTTGGGACCAGACTGCACGACTGGTCTGTGAGAACCC
CAATGGCAGACACTTGACTGCCATTATTGTCCTTGTGCTCAGTGACA
GGCACACATGGAGAGCAGCTTAGGGTTAGAGCACACGAAGGAAGCA
CA 3'

Probe L1-A

Forward Primer: (UF7712) 5' GCA CAA TCA CAG TTC ACT CCA GCC 3'

Reverse Primer: (UF7714) 5' GCC TGA CCA AGG AAA GCA AAG CT 3'

Template: *Hprt*Δ3'-*Fkbp6* plasmid, linearized with KpnI

PCR Product: (560 bp)

5'GCACAATCACAGTTCACCTCCAGCCTCAACATCCTGCACTAAAGTGA
TTTTCCACCTCACCTCTCAAGTAGCTGGGACTACAGGTACATGCTA
CCATGCCTGGCTAATTTTTTTTTTTTTTGCAGGCATGGGGTCTCACTAT
ATTGCCCAGGTTGGTGTGGAAGTTTAATGACTAAGAGGTGTTTGTAT
AAAGTTTAATGTATGAACTTTCTATTAAATTCCTGATTTTATTTCTGTA
GGACTGAACGTCTTGCTCGAGATGTGATGAAGGAGATGGGAGGCCA
TCACATTGTAGCCCTCTGTGTGCTCAAGGGGGGCTATAAATTCTTTG
CTGACCTGCTGGATTACATCAAAGCACTGAATAGAAATAGTGATAGAT
CCATTCCTATGACTGTAGATTTTATCAGACTGAAGAGCTATTGTAATG
ACCAGTCAACAGGGGACATAAAAGTAATTGGTGGAGATGATCTCTCA
ACTTTAACTGGAAAGAATGTCTTGATTGTGGAAGATATAATTGACACT
GGCAAAACAATGCAGACTTTGCTTTCCTTGGTCAGGC 3'

FIGURES

Figure 1

Transcript map of the Williams-Beuren syndrome critical region at human chromosome band 7q11.23. Duplicons (blocks A, B and C) within the Low-Copy Repeat regions are highlighted flanking the deletion. Regions encompassing the common centromeric and telomeric deletion breakpoints are labeled. Diagram not to scale. Adapted from Tassabejhi et al. (2003).

Figure 2

Schematic depiction of inter- and intrachromosomal recombination.

A) Interchromosomal recombination between two chromosomes resulting in a deletion (top result) and a duplication (bottom result). B) Intrachromosomal recombination within one chromosome resulting in a deletion; the deleted sequence is not shown. Black circles represent the centromere, red rectangles represent a duplicated sequence, blue and purple rectangles represent unique sequences, black "x" represents the site of non-homologous recombination. Figure not to scale.

Figure 3

Physical map of the two deletion regions for the proposed compound heterozygote Williams-Beuren syndrome deletion mouse model. One mouse chromosome 5 will be deleted for the proximal group of genes indicated by the "DEL PROX" bracket, including *Gtf2i* and *Limk1*. The other mouse chromosome 5 will be deleted for the distal group of genes indicated by the "DEL DIS" bracket, including *Limk1* and *Fkbp6*. The compound heterozygote would be functionally null for the *Limk1* gene. Adapted from Valero et al (2000).

Figure 4

Alan Bradley's Cre-*loxP* strategy for mouse chromosome rearrangement as used in this thesis. In step 1, an initial targeting construct, consisting of a 5' part of the *hprt* minigene cassette (blue rectangle), a *loxP* site (red arrow) and a neomycin resistance gene (green rectangle), is targeted to a specific mouse locus (in this case *Limk1*) using homologous recombination. In step 2, a second targeting construct, consisting of a puromycin resistance gene (yellow rectangle), *loxP* site, and the 3' portion of the *hprt* minigene cassette (purple rectangle), is targeted in the same cells to a second locus (in this case, *Fkbp6*) using homologous recombination and with the *loxP* sites in the same orientation. In step 3, transient expression of the Cre protein leads to chromosomal rearrangement resulting in the 5' and 3' sections of the *hprt* minigene cassette being brought together to form a functional minigene and confer HAT resistance to the cells; the puromycin and neomycin resistance genes along with all the mouse genes between them are lost from the chromosome as a circular piece of DNA. Adapted from Zheng et al. (2000).

Figure 5

Comparison of the genomic organization of human chromosome 7q11.23 (top) and the conserved homologous region on mouse chromosome 5G1 (bottom). Centromeric end of the region is denoted by "CEN", telomeric by "TEL". The entire region is inverted in human with respect to mouse, with the gene order maintained but reversed with respect to centromeric and telomeric markers between the Williams-Beuren breakpoints (outside the genomic sections depicted). Genes deleted in Williams-Beuren syndrome include FKBP6 and GTF2I. Adapted from Valero et al. (2000).

Figure 6

Diagram of the current status of the Francke laboratory's efforts in creating the compound heterozygote Williams-Beuren syndrome mouse model. Black lines represent two copies of mouse chromosome 5, with centromeric and telomeric directionality indicated. Directionality of *loxP* sequences at targeting sites is indicated by red triangles.

Figure 7A

Diagram of *Hprt* Δ 3' chromosome engineering cassette with no insert generated in the application Gene Construction Kit. *Ascl* restriction enzyme cloning site is labeled; puromycin gene with PGK promoter is represented by green arrows; ampicillin resistance gene and promoter represented by blue arrow; *loxP* site location and directionality indicated by red arrow; intron and *Hprt* minigene exons 3 – 9 are indicated by purple arrows; K14 promoter and agouti cDNA are represented by black arrow and line. Figure to scale.

Figure 7B

Schematic representation of the mouse chromosomal insert in BAC RPCI 24-230L19 generated in Sequencher. Top diagram shows 9.9 kb fragment generated by *Mlu*I digestion in relation to exons of surrounding genes; green arrows represent exons for the genes *Pom121* and *Fkbp6*; *Mlu*I digestion product (9.9 kb) occurs between the two genes and is labeled. Bottom diagram shows the mouse genomic insert in the pTARBAC bacterial artificial chromosome construct. Vector sequence is marked with a blue bracket; 9.9 kb *Mlu*I digestion fragment is marked with a red bracket; *Mlu*I sites in the construct are marked. Figure is to scale.

Depiction of the Southern Blot strategy for 2lox ES cells. Genomic DNA is represented by a thick red horizontal line. Thin red and green lines represent genomic sequence used in the targeting vector. The point where the red and green lines meet is where the targeting vector is linearized by digestion with KpnI and inserts into genomic DNA via homologous recombination. Untargeted wild type genomic DNA (top) produces a 13294 bp fragment when digested with Swal and SpeI. Two probes (A and B) were developed using PCR on AB2.2 ES cell DNA to the genomic sequence outside that used to target the locus. Targeted genomic DNA (bottom) yields a 11549 bp fragment that hybridizes with Probe A, and a 16100 bp fragment that hybridizes with Probe B when digested with Swal and SpeI. In the bottom diagram, targeting vector sequence is represented by flanking black lines (genomic sequence), and black and colored arrows that correspond to the plasmid map depicted in figure 7A in this thesis.

Figure 9

Depiction of the Southern Blot strategy for 1lox ES cells. Genomic DNA is represented by a thick red horizontal line. Genomic DNA from 2lox undeleted cells (top) produces a 2709 bp fragment when digested with HindIII. Probe L1A (560 bp) was developed using PCR on the targeting vector to include exons 3 – 6 of the *hprt* minigene, and is represented by a red bar above the genomic DNA. Genomic DNA from 1lox deleted cells (bottom) yields a 4977 bp fragment when digested with HindIII that hybridizes with Probe L1A. Genomic DNA in 1lox cells in this region includes targeting vector sequence including the 5' part of the *hprt* minigene from the *Hprt*Δ5'-*Limk1* construct, and the 3' part of the *hprt* minigene from the *Hprt*Δ3'-*Fkbp6* construct, joined at the *loxP* site (red arrow).

2lox DNA:

1lox DNA:

Figure 10

Picture of an ethidium-bromide stained agarose gel under UV light showing the results of digestion of Hprt Δ 3' with unknown insert (HPRT Δ 3'-?) with Ascl and EcoRI.

Figure 11

Restriction enzyme digestion to test orientation of 9.9kb *Fkbp6* genomic insert in *HprtΔ3'* targeting vector. Top diagrams show the two possible insert orientations, and the locations in those plasmids of restriction sites for *EcoRV*. Plasmid with the insert in the correct orientation will yield digestion fragments of 4223 and 16295 bp (left). Plasmid with the insert in the incorrect orientation will yield digestion fragments of 7823 and 12695 bp (right). Picture of an ethidium-bromide stained agarose gel visualized under UV light showing the results of plasmid DNA digested with *EcoRV* shown below; clone A6 (middle lane) has the insert in the correct orientation.

Figure 12A

Picture of an agarose gel stained with ethidium bromide and visualized under UV light showing the results of linearization of Hprt Δ 3'-*Fkbp6* plasmid with KpnI. Linearized plasmid (middle lane) runs as a discrete band, while unlinearized plasmid (right lane) runs as a larger band with a streak, as is characteristic of large, circular plasmids.

Figure 12B

Plasmid map of *Hprt* Δ 3'-*Fkbp6* (19731 bp) showing *KpnI* linearization site. Genomic insert is labeled and represented by internal black line. Elements of the *Hprt* Δ 3' construct are highlighted and represented by green, red, blue and black arrows. The single *KpnI* restriction site is highlighted in the genomic insert.

Figure 13

Schematic diagram of mouse chromosome 5 targeted at *Fkbp6* (blue rectangle representing the 12 exons of the gene) and *Limk1* (green squares representing each of the first 6 exons of the gene) with *loxP* sites (red arrows). *Limk1* was targeted in such a way as to result in duplication of exons 3 and 4. *LoxP* sites are in the same orientation, allowing Cre-mediated recombination to occur, resulting in one remaining *loxP* site and a chromosome deleted for the sequence between the *loxP* sites.

Figure 14

Genomic Southern blot results for 2lox ES cell clones. A) Sample Southern blot results from the initial screen of 248 clones digested with *SpeI* and *SwaI* and probed with Probe A; clones were analyzed on a total of 6 different Southern blots. Probe A hybridizes with wild-type, untargeted bands at 13.3 kb, and with targeted bands at 11.5 kb. In this blot, ES cell clones 3A2 and 3B3 show the targeted band at 11.5 kb and are therefore positive for being targeted at the *Fkbp6* locus. B) Results from the confirming Southern blot of the 12 clones that were positive in the initial Probe A screen. Seven clones positive with Probe A, digested with *SpeI* and *SwaI* and probed with Probe B (10B2, 9C3, 8C4, 3D6, 3A2, 1D4 and 1A6) show the predicted banding pattern for 2lox cells, with wild-type bands at 13.3 kb and targeted bands at 16.1 kb. Clones 13B5, 9C1 and 4B3 show 13.3 kb bands that are twice as strong as their 16.1 kb bands, indicating that these colonies are mosaic and contain both singly-targeted and doubly-targeted cells. Clones 4D6 and 3B3 show no bands.

A

Clones positive with Probe A:

1A6, 1D4, 3A2, 3B3, 4B3, 4D6, 8C4, 9C1, 9C3, 10B2, 13B5

B

Clones positive with Probe B:

1A6, 1D4, 3A2, 3D6, 8C4, 9C3, 10B2

Figure 15

Genomic southern results on 1lox and 2lox ES cells digested with HindIII and probed with Probe L1A. Lanes 1 and 2 show the background band and the expected 4.9 kb band in DNA from 1lox deleted clones 1D4-1 and 1D4-3. Lane 4 shows a background band at >5kb in untargeted AB 2.2 ES cell DNA. Lane 3 shows the background band and the expected 2.7 kb band in 2lox undeleted control cell DNA.