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Complex Genomic Rearrangement in CCS-*LacZ* Transgenic Mice

Dina Myers Stroud¹, Bruce J. Darrow¹, Sang Do Kim², Jie Zhang¹, Monique R.M. Jongbloed¹, Stacey Rentschler¹, Ivan P.G. Moskowitz^{3,4}, Jonathan Seidman², and Glenn I. Fishman^{1,*}

¹Leon H. Charney Division of Cardiology, New York University School of Medicine, New York, New York

²Department of Genetics, Harvard Medical School, Howard Hughes Medical Institute, Boston, Massachusetts

³Department of Pediatrics, Institute for Molecular Pediatric Sciences, University of Chicago, Chicago, Illinois

⁴Department of Pathology, Pritzker School of Medicine, University of Chicago, Chicago, Illinois

Summary

The cardiac conduction system (CCS)-*lacZ* insertional mouse mutant strain genetically labels the developing and mature CCS. This pattern of expression is presumed to reflect the site of transgene integration rather than regulatory elements within the transgene proper. We sought to characterize the genomic structure of the integration locus and identify nearby gene(s) that might potentially confer the observed CCS-specific transcription. We found rearrangement of chromosome 7 between regions D1 and E1 with altered transcription of multiple genes in the D1 region. Several lines of evidence suggested that regulatory elements from at least one gene, *Slco3A1*, influenced CCS-restricted reporter gene expression. In embryonic hearts, *Slco3A1* was expressed in a spatial pattern similar to the CCS-*lacZ* transgene and was similarly neuregulin-responsive. At later stages, however, expression patterns of the transgene and *Slco3A1* diverged, suggesting that the *Slco3A1* locus may be necessary, but not sufficient to confer CCS-specific transgene expression in the CCS-*lacZ* line.

Keywords

transgenics; mouse; conduction system; CCS-*lacZ*; *Slco3A1*

The cardiac conduction system (CCS)-*lacZ* mouse strain is an insertional mutant in which reporter gene expression is observed in the CCS of the developing and adult heart. These mice have been used to clarify mechanisms of CCS formation and function (Kitajima *et al.*, 2006; Poelmann *et al.*, 2004; Rentschler *et al.*, 2001, 2002), visualize effects of various genetic mutations on CCS development (Morley *et al.*, 2005; Yang *et al.*, 2006), and identify the role of the CCS in cardiac rhythm disturbances (Gonzalez *et al.*, 2004; Jongbloed *et al.*,

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*Correspondence to: Glenn I. Fishman, MD, New York University School of Medicine, Smilow Research Center, 8th Floor, 522 First Avenue, New York, NY 10016. glenn.fishman@med.nyu.edu.

Current address for Bruce J. Darrow: Department of Medicine, Mount Sinai School of Medicine, New York, NY 10029.

Current address for Monique R.M. Jongbloed: Department of Cardiology, Leiden University Medical Center, The Netherlands.

Current address for Stacey Rentschler: Department of Medicine, University of Pennsylvania, Philadelphia, PA 19104.

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2004, 2005). Our initial studies suggested that the expression of *lacZ* in the CCS was dependent upon the site of integration and the flanking genomic sequences rather than *Engrailed-2* regulatory elements within the transgene proper (Rentschler *et al.*, 2001, 2002). Accordingly, we sought to characterize the site of integration and identify nearby gene(s) that might confer CCS-specific transcription upon the integrated transgene.

Fluorescent in situ hybridization (FISH) on chromosomal DNA from a homozygous CCS-*lacZ* mouse revealed fluorescent signal on both copies of chromosome 7 (Fig. 1). The intensity of staining on one chromosome from each pair was greater than on the second copy, suggesting that during breeding to homozygosity the transgene locus had undergone a recombination event, resulting in unequal copies of transgene array on each chromosome of the mouse studied. Supporting this possibility, Southern blots performed with a *lacZ* probe revealed two distinct banding patterns in offspring from homozygous $\times$ wild-type matings (data not shown).

To identify sequences flanking the transgene, we prepared a phage library from genomic DNA from a homozygous CCS-*lacZ* mouse. The library was screened for clones including transgene elements using a radiolabeled *lacZ* DNA probe. Ten out of 12 independent phage clones contained portions of *lacZ* sequence and flanking genomic sequence from chromosome 7. However, given the size of the transgene array (over 14 kB per copy), none of the positive clones included flanking genomic sequence at both ends of insert. Surprisingly, analysis of genomic DNA sequences from the isolated phage clones implicated three distinct regions of chromosome 7 as flanking portions of the transgene. These regions (chromosomal regions D1, E1, and F2) span over 30 million bases. The discrete localization of the FISH signal at a single locus of chromosome 7 suggested that portions of these three disparate regions were brought into proximity during transgene integration through a complex recombination event. The presence of more than two unique flanking sequence elements also indicated a complex genomic structure and suggested that there must be (a) two different integration sites on at least one copy of chromosome 7, separated from one another at a distance below the level of resolution by FISH analysis, and/or (b) different integration sites on each chromosome. Because all hemizygous offspring have the CCS phenotype, the first possibility appeared more likely.

The size and complexity of the integration site led us to create a bacterial artificial chromosome (BAC) library from CCS-*lacZ* genomic DNA. Pooled samples of BACs were screened by PCR using primers for the *Engrailed-2/lacZ* junction of the transgene. Ten independent positive BAC clones were purified and end sequenced. Of these 10 clones, six had genomic sequence from chromosome 7 regions E1 or D1, including three with E1 at both ends and three with a combination of E1 and D1, confirming our hypothesis that the chromosome had undergone rearrangement during integration of the transgene. Raw sequence data from phage and BAC libraries are available in an online data supplement.

Using RT-PCR, we characterized the expression patterns of 10 genes in the C-E1 region in wild type and homozygous CCS-*lacZ* mice (Fig. 2). Three tissues were examined, including the brain, the superior portion of the heart (atria, appendages and AV junction), and the inferior portion (ventricle). Six of the 10 genes exhibited a reduction in transcript levels between wild type and mutant (Fig. 2). Furthermore, we saw tissue specific differences in the extent of altered expression (Fig. 2).

The RT-PCR data suggested the D1 region was the most influenced by the transgene integration. To narrow our search for candidate genes, we examined a list of sequence tags from a SAGE library generated from microdissected left bundle branch (LBB) tissue that met two criteria. First, they were enriched in LBB preparations compared to whole

ventricular muscle, and second, they mapped to the D1 region of chromosome 7. A single transcript from the *Slco3A1* gene met both these criteria. RT-PCR (Fig. 2) and Northern blot analysis (not shown) demonstrated marked downregulation of *Slco3A1* expression in the hearts of homozygous CCS-*lacZ* mice compared to wild-type controls. *Slco3a1* is a member of a large protein superfamily (Hagenbuch and Meier, 2003, 2004), and it has been cloned from mouse as well as rat and human (Adachi *et al.*, 2003; Melia *et al.*, 1998; Tamai *et al.*, 2000). Members of the *Slco* family, each having 12 transmembrane domains, have been implicated in the transport of both natural substances (prostaglandins, bile salts, thyroid, and steroid hormones) and exogenous drugs (including but not limited to digoxin, angiotensin converting enzyme inhibitors, HMG-coenzyme A reductase inhibitors, methotrexate, and rifampin) across the cell membrane (Hagenbuch and Meier, 2003, 2004). To date, there are no data indicating an association of *Slco3A1* with the CCS. Interestingly, our initial sequence analysis placed the SAGE library sequence tag ~9 kB downstream of the last exon (exon 10) of *Slco3A1*, i.e., beyond the putative transcribed region. However, detailed examination of mouse EST databases and a comparison to ESTs from the human *Slco3A1* orthologue predicted an alternatively spliced transcript joining a portion of exon 10 with a previously unrecognized mouse exon 11, within which the sequence tag resided (Fig. 3a,b). RT-PCR with isoform specific primers confirmed expression in the heart (Fig. 4a).

We next examined whether *Slco3A1* was preferentially expressed in microdissected tissue enriched in Purkinje fibers. RNA was prepared from subendocardial Purkinje fibers isolated from adult murine hearts and working ventricular muscle from the epi- and mid-myocardium. RT-PCR was performed using primers that amplified both *Slco3A1* transcripts. To confirm the specificity of the harvesting procedure, we also examined expression of the CCS marker *Connexin 40* (*Cx40*). Both *Cx40* and *Slco3A1* are enriched in the Purkinje fiber preparations compared to working myocardium (Fig. 4b).

LacZ expression in cultured CCS-*lacZ* hearts is markedly induced by neuregulin only between E9.5 and E12.5 (Rentschler *et al.*, 2002). To determine whether the endogenous *Slco3A1* gene was similarly neuregulin-responsive, we treated pools of 10 isolated hearts from E10.5 or E12.5 mice with neuregulin or vehicle alone. After 48 h in culture, expression of the *Slco3A1* transcript was detectable in control hearts and markedly induced in the neuregulin-treated E10.5 but not E12.5 samples (Fig. 4c), paralleling the behavior of the CCS-*lacZ* reporter gene.

RNA in situ hybridization was performed on sections from E12.5 and neonatal hearts to determine the endogenous *Slco3A1* expression pattern. Although weakly expressed, *Slco3A1* was observed in the right atria, AV junction, along the interventricular septum, and ventricular trabeculae in a pattern quite similar to CCS-*lacZ* reporter gene expression at this stage (Fig. 5a-c). Higher magnification images revealed *Slco3A1* expression to be primarily myocardial, rather than endocardial (Fig. 5d,e). In neonates, expression appeared modestly enriched in the AV node (Fig. 6a-d) and His bundle (Fig. 6e-h). However, at this latter stage, no specific expression was observed in the bundle branches or Purkinje fibers as suggested by SAGE and RT-PCR, conceivably due to a difference in the sensitivity of the assays and the very low abundance of the *Slco3A1* transcript.

We examined this issue further by investigating the expression of other candidate genes. *Nr2f2* (*Coup-TFII*) is required in the embryo for many processes including heart development and angiogenesis, but its expression is not associated with the CCS (Lee *et al.*, 2004; Pereira *et al.*, 1999). ST8SiaII (STX) is a polysialic acid transferase whose target is the neural cell adhesion molecule (NCAM) (Angata and Fukuda, 2003). This was intriguing, as polysialylated-NCAM and NCAM itself have been used as markers of conduction system in chick and human respectively (Soler *et al.*, 2001; Watanabe *et al.*, 1992).

Immunohistochemical studies with PSA-NCAM antibodies on E13.5 mice revealed neural but not cardiac expression (data not shown). Furthermore, *ST8SiaII* was neuregulin inducible at E12.5, a stage when we no longer see significant upregulation of *CCS-lacZ* transgene expression (data not shown). Neurotrophin 3, (TrkC; Ntrk3), is expressed in the developing CCS of the rat from E11 onwards (Hiltunen *et al.*, 1996). There was no expression of Ntrk3 within or adjacent to the CCS in adult mouse hearts (data not shown). Hapln3 is expressed within smooth muscle cells but not cardiac myocytes (Ogawa *et al.*, 2004). A bioinformatics analysis of all known microRNAs from chromosome 7 was performed, but none were associated with the D1 region. Therefore, of the various candidate genes in the D1 region, *Slco3A1* most faithfully, albeit incompletely, reflected the expression pattern of the *CCS-lacZ* reporter gene.

Taken together, these data suggest that regulatory elements from the *Slco3A1* gene may influence, but are not solely responsible for, conduction system specific transgene expression in the *CCS-lacZ* line. The extensive chromosomal rearrangement may have resulted in the serendipitous assembly of requisite *cis*-acting sequences. Alternatively, a yet uncharacterized gene within this locus exists, whose pattern of expression more closely parallels that of the *CCS-lacZ* transgene. Regardless, it appears unlikely that discrete regulatory elements from this strain that can be used to heterologously express other genes of interest specifically in the CCS will be identified. Nonetheless, the *CCS-lacZ* mouse remains an important tool for studies of conduction system biology. In addition to our initial studies visualizing CCS maturation and functional activity (Rentschler *et al.*, 2001, 2002), the mice have recently been used in lineage tracing experiments, studies of the role of the neural crest in CCS formation, as well as studies of arrhythmogenic foci in CCS remnants (Jongbloed *et al.*, 2005; Kitajima *et al.*, 2006; Poelmann *et al.*, 2004). Thus, the *CCS-lacZ* line has been and will continue to be a valuable resource for analysis of mutants with potential morphologic or functional conduction system abnormalities (Morley *et al.*, 2005; Yang *et al.*, 2006).

Methods

Mouse Strains

The *CCS-lacZ* line is a result of a fortuitous insertion of an *Engrailed-2/lacZ* fusion construct previously described (Logan *et al.*, 1993; Rentschler *et al.*, 2001). It is maintained in a CD-1 background. The *minK-lacZ* line was generated by knockin of *lacZ* into the endogenous *minK* locus (Kupersmidt *et al.*, 1999).

Fluorescent In Situ Hybridization

Fluorescent in situ hybridization was performed by SeeDNA (Windsor, Ontario) according to standard techniques using spleen lymphocytes (Feng *et al.*, 1994; Heng *et al.*, 1992). Biotinylated *lacZ* DNA was hybridized with the chromosomes, and fluorescein isothiocyanate conjugated with avidin was used to detect adherent probe. Comparison with samples stained with 4,6-diamidino-2-phenylindole (DAPI) was used to identify each chromosome (Heng *et al.*, 1992).

Construction and Screening of Genomic Libraries

Genomic DNA from homozygous *CCS-lacZ* mice was partially digested with *Sau3A1* and cloned into λ FIX II phage DNA (Stratagene, La Jolla, CA). Genomic DNA was also used to prepare a BAC library (Bio S&T, Montreal, Quebec). BACs were screened by PCR using oligonucleotide primers for the *Engrailed-2/lacZ* junction of the transgene (Logan *et al.*, 1993). NCBI and the UCSC genome browser (<http://genome.ucsc.edu/>) were used for

sequence analysis (Kent, 2002; Kent *et al.*, 2002). See online data supplement for raw sequence data from both phage and BAC libraries.

RNA Preparation and RT-PCR from Adult and Embryonic Mouse Tissues

RNA was prepared from adult and embryonic mouse tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA) or the PicoPure RNA isolation kit (Arcturus, Mountain View, CA) according to the manufacturer's instructions. "Purkinje fiber" fractions (P) were prepared by microdissection of the fiber network away from the exposed myocardial surface of the ventricle. This population will therefore contain both endocardial and subendocardial cells. "Working myocyte" fractions (M) were prepared from epicardial and mid-myocardial tissue, primarily from the left ventricle. Enrichment of the PF fraction for conduction tissue was analyzed by reverse transcriptase (RT)-PCR for the *Connexin40* transcript relative to the working myocyte fraction. RT-PCR was performed using a PTC-100 (MJ Research, Waltham, MA).

Preparation and Analysis of SAGE Library

Comparisons were made between SAGE libraries constructed from left-bundle branch conduction tissue and entire left ventricles from 19-day-old SvEv mice. The SAGE library was constructed by the micro sage technique (St. Croix *et al.*, 2000). The SAGE 3.0.1 program (courtesy of Victor Velculescu and Ken Kinzler, Johns Hopkins University School of Medicine, Baltimore, Maryland, United States) was used to extract SAGE tags and eliminate duplicate ditags. Identity of SAGE tags was obtained from the National Center for Biotechnology Information (NCBI) "reliable" tag map set for UniGene (available at <http://www.ncbi.nlm.nih.gov/SAGE>). The tag (CATGATATAAAGTA) corresponding to the *Slco3A1* gene was present in six copies in the conduction system library and one copy from the left ventricular library, corresponding to a *P*-value <0.10 (*P*-values determined by the monte carlo method using SAGE 2000 version 4.12, available at www.sagenet.org). Although these tag counts do not demonstrate conduction system enrichment by statistical criteria due to low tag count, the presence of the tag corresponding to *Slco3A1* in the conduction system library made it a candidate gene in the chromosomal region of interest.

RNA In Situ Hybridization

Three riboprobes were made using RT-PCR against a mouse cDNA heart library. The PCR primers had both T7 and T3 promoter sequences for transcription (T7 for antisense and T3 for sense probes). The riboprobes were transcribed using ³⁵S-UTP for labeling. In situ hybridizations were carried out on hearts or embryos fixed with 4% paraformaldehyde. Tissues were paraffin embedded and cut into 7 μm sections. Slides were dewaxed and rehydrated with (30, 50, 70, 95, 100% × 3) EtOH and DEPC mixture. Slides were prepared for hybridization with 0.85% Tampon Saline Buffer, microwaved in citrate buffer, bleached with hydrogen peroxide, and proteinase K treated for 20 min. Probes were hybridized overnight at 50°C in a moist chamber. After washing the slides with several washes in 5×, 2× and 1 × SSC, the slides were emulsed in Kodak NTB2 autoradiography emulsion. Slides were exposed for 4 weeks and then developed using Kodak developer and fixer. The slides were counter-stained with toluidine blue and mounted. Images were viewed with Fiber-Lite red light illuminator.

LacZ Staining

Hearts were whole-mount *lacZ* stained, paraffin embedded and sectioned as previously described (Moskowitz *et al.*, 2004).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIG. 1. CCS-*lacZ* transgene is on chromosome 7. **(a)** Fluorescent in situ hybridization (FISH) performed on CCS-*lacZ* mouse chromatin with tagged nucleotide probe for *lacZ*. Arrows indicate probe binding at region of transgene integration. **(b)** DAPI staining with chromosome 7 labeled.

FIG. 2.

RT-PCR analysis of integration region. Ten genes (listed on left) from the C–E1 (position listed on right) region of chromosome 7 were tested for alterations in transcription. RNA from the brain, atria, appendages, and AV junction (A-AVJ), and ventricle (Vent) of wild type (WT) or homozygous *CCS-lacZ* (CCS) mice was used as template. *Cyclophilin* (*cyc*) was employed as a loading control for each reaction. A representative set of control reactions is shown.

FIG. 3. *Slco3A1* locus **(a)** Diagram of *Slco3A1* gene with previously described exons 1–10 in red. Exon 11 is shown in blue. **(b)** Detail of exons 8– 11 (box outline in A) showing the common region of exon10 (E10a) and the two splice variants, *Slco3A1a* (red) and *Slco3A1b* (blue). Primers to detect the different forms were made specifically to these areas.

FIG. 4.

RT-PCR analysis of *Slco3A1* (a) Both *Slco3A1b* and *Slco3A1a* transcripts are expressed in the heart. (b) RT-PCR for *Cx40*, *cyclophilin* (*cyc*), and *Slco3A1* using RNA from working ventricular muscle (M) and Purkinje-fiber (P) enriched tissue. *Cyclophilin* serves as a loading control. (c) Analysis of *Slco3A1* and *cyclophilin* (*cyc*) expression in hearts from E10.5 and E12.5 embryos either untreated (-) or treated (+) with neuregulin for 48 h.

FIG. 5.

In situ hybridization of E12.5 embryos. **(a)** Whole-mount *lacZ* staining of an E12.5 CCS-*lacZ* heart. Reproduced with permission from Rentschler *et al.* (2001). **(b)** Radioactive RNA in situ hybridization of E12.5 embryonic heart performed with a cocktail of three *Slco3A1* antisense probes. **(c)** Hybridization of serial section with control *Slco3A1* sense probes. **(d)** *LacZ* staining of an E12.5 *minK-lacZ* heart shows primarily subendocardial staining in the septum where the forming His bundle and bundle branches are located. **(e)** *Slco3A1* expression does not appear to be in the endocardium (arrowheads).

FIG. 6.

Central conduction system expression of *Slco3A1* in neonatal hearts. **(a)** Section of the atrioventricular junction at the level of the AV node in a neonatal mouse heart hybridized with *Slco3A1* sense control probes. **(b)** Serial section probed with antisense *Slco3A1* showing expression in the node. **(c, d)** Close up views of the AV node regions from panels a and b. **(e)** Control section at the level of the His bundle. **(f)** *Slco3A1* expression can be clearly seen in the His bundle in this serial section. **(g, h)** High magnification views of panels e and f.