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ARTICLE

Recombinase-Mediated Cassette Exchange to Rapidly and Efficiently Generate Mice With Human Cardiac Sodium Channels

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Summary: *SCN5A* encodes the predominant voltage-gated sodium channel isoform in human heart and nearly 100 variants have now been described and studied in vitro. However, development of animal models to analyze function of such large numbers of human gene variants represents a continuing challenge in translational medicine. Here, we describe the implementation of a two stage procedure, recombinase-mediated cassette exchange (RMCE), to efficiently and rapidly generate mice in which a full-length human cDNA replaces expression of the murine ortholog. In the first step of RMCE, conventional homologous recombination in mouse ES cells was used to replace *scn5a* exon 2 (that contains the translation start site) with a cassette acceptor that includes the thymidine kinase gene, flanked by loxP/inverted loxP sites. In the second step, the cassette acceptor site was replaced by the full-length wild-type human *SCN5A* cDNA by Cre/loxP-mediated recombination. The exchange event occurred in 7/29 (24%) colonies, and the time from electroporation to first homozygotes was only 8 months. PCR-restriction fragment length polymorphism (RFLP) showed that the murine isoform was replaced by the human one, and functional studies indicated that mice with human cardiac sodium channels have wild-type sodium current density, action potential durations, heart rates, and QRS durations. These data demonstrate that RMCE can be used to generate mice in which a targeted allele can be rapidly and efficiently replaced by variants of choice, and thereby can serve as an enabling approach for the functional characterization of ion channel and other DNA variants. genesis 44:556–564, 2006. **Published 2006 Wiley-Liss, Inc.**†

Key words: mouse; sodium channel; transgenesis; cassette exchange; heart

INTRODUCTION

Identification of large number of variants across human genomes has produced important new insights into variant protein expression and function in vitro. A limitation of such in vitro studies is their incomplete success in accurately predicting resultant phenotypes in whole organs. While generation of transgenic animal models is a logical next step in many settings, the resources required to generate dozens of such models by homologous recombination are generally prohibitive.

Over a hundred mutations in *SCN5A*, encoding the cardiac sodium channel, have been linked to multiple cardiac arrhythmia phenotypes (Bunch and Ackerman, 2006; Splawski *et al*., 2000; Tester *et al*., 2005). In some cases, functional changes revealed by in vitro assays correlate well with the clinical presentations. However, there are now multiple examples in which the in vitro genotype cannot be used to directly infer the clinical phenotype.

The gene is large (28 exons, >100 kb), so conventional gene targeting has been used infrequently (Nuyens *et al*., 2001; Papadatos *et al*., 2002; Remme *et al*., 2005). We show here that a recent advance in transgenic technology, Cre-recombinase mediated cassette exchange (RMCE) (Bouhassira *et al*., 1997; Cesari *et al*., 2004; Feng *et al*., 1999; Jones *et al*., 2005; Roebroek *et al*., 2006; Seibler *et al*., 1998), can be used as a platform to rapidly and efficiently generate ES cells in which the mouse gene has been deleted and the full-length human cDNA inserted in the same locus and expressed in its place. RMCE is a two step procedure; the first step is

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replacement of a targeted allele by a cassette acceptor (most often flanked by a pair of inverted loxP sites (loxP/loxP^{inv}) using conventional homologous recombination. In the second step, the cassette acceptor locus is targeted for Cre-mediated replacement by a DNA sequence of choice. The approach was first described in the late 1990's, and has been used to delete genes (Cesari *et al*., 2004), and to replace selected genomic regions. The recent development of a sequential positive–negative selection approach has been reported to markedly improve the efficiency of the second step, allowing ES cells with desired exchange events to be rapidly and reliably generated (Long *et al*., 2004).

Disrupting *scn5a* exon 2, which contains the ATG translation start codon, completely ablates sodium channel expression in mice, indicating that alternative start sites are not available (Papadatos *et al*., 2002). Accordingly, we hypothesized that RMCE could be used to replace mouse exon 2 with a cDNA encoding the full-length human *SCN5A* coding sequence; this sequence could encode wild-type channels, mutant channels, or other proteins. As proof of principle, we have substituted wild-type human *SCN5A* cDNA into the mouse exon 2 locus and show here that this substitution leads to viable animals with normal sodium channel function in which only transcripts of the human isoform are detected in heart.

Conventional homologous recombination was used to generate ES cell lines with *scn5a* exon 2 modified to include a cassette acceptor site. The targeted region was ~5 kb, with exon 2 roughly centered in the middle; thus the endogenous core promoter (Yang *et al*., 2004), located ~20 kb upstream, remained intact. The cassette acceptor included a neomycin resistance gene (*neo^{R}*) and a thymidine kinase gene (*tk*) flanked by loxP/loxP^{inv} (Fig. 1a). A recently-described potential alternate transcription start site in mice (Shang and Dudley, 2005) was included in the floxed region, and was retained in the final exchanged allele as described below.

Southern blotting of genomic DNA isolated from G418-resistant clones identified 7/~800 clones (0.87%) in which the expected recombination had occurred (Fig. 1b). Long range PCR reactions with external primers from both ends were used to further verify the correct recombination events (all primers used are presented in Table 1). Two primer sets were used. For one pair (176A and B), one primer was within the 5′ targeted arm and other was 3′ to the targeted region, and for the second pair (178A and B), one primer was within the 3′ targeted arm and the second was 5′ to the targeted region. Figure 1c shows that the two ES cell lines chosen for further experiments, designated A and E, both displayed PCR fragment sizes consistent with the presence of a wild-type and a recombinant allele. Fragments of 12 and 9.5 kb were amplified from the wild-type allele, while recombinant amplicons were 11.3 and 8.8 kb (Fig. 1c); junction regions across homologous elements and the cassette acceptor were sequenced to further confirm the targeting events to be correct.

Replacement of the cassette acceptor site with an *SCN5A* exchange cassette by RMCE was conducted in clones 1E10 and 3A10, which displayed the recombination event described above. The sequence between the loxP sites in the exchange vector included a segment of intron 1, the 5′ end of exon 2 (up to the ATG start site), a 6 kb fragment encoding the full-length human *SCN5A* coding sequence with the initial ATG positioned at the 3′ end of the noncoding exon 2 fragment, a bovine growth hormone polyA tail, and a hygromycin resistance cassette flanked by Frt sites. Inclusion of the Frt enables later excision of the hygromycin resistance cassette but this was not necessary in our experiments.

The ES cells were electroporated with the exchange cassette and a plasmid encoding Cre, and then underwent dual selection with hygromycin and ganciclovir in a staggered positive–negative fashion [see details in methods (Long *et al*., 2004)], 29 clones survived. Analysis of insert size and orientation (Fig. 2a,b) identified 7/29 with RMCE events; parental clone 1E10 generated 4 ganciclovir survivor clones from 120 hygromycin resistant, and for parental clone 3A10 there were 3/96. In 2/7 colonies, the *SCN5A* cDNA was oriented only in the correct forward orientation.

Mice homozygous for exon 2 deletion display no *scn5a* expression, and die at embryonic day 10.5 (Papadatos *et al*., 2002). Accordingly, we anticipated that *SCN5A*-exchanged mice would also be embryonic lethal if the exchange did not result in *SCN5A* expression. Conversely, a viable homozygous exon2-*SCN5A* exchange mouse would indicate that the transgenic cDNA directed synthesis of human sodium channels and thus was supporting cardiac electrical behavior.

The mouse and human orthologs display >95% sequence homology at the nucleotide level. To establish that the hearts of homozygous knock-in animals indeed expressed only the human isoform, we used restriction fragment length polymorphisms unique to either mRNA isoform to analyze RT-PCR amplicons from the hearts of genotyped mice. As shown in Figure 2c–f, these experiments demonstrate that only the human sodium channel can be detected in hearts of homozygous knock-in animals.

Matings of heterozygous animals resulted in 4 wild-type homozygotes, 10 heterozygotes, and 4 homozygous human SCN5A/human SCN5A (H/H) animals. The H/H animals have no obvious morphological abnormalities, and reproduce normally. Comparisons of electrocardiograms (Fig. 3a,b), ventricular myocyte action potentials (Fig. 3c, Table 2), and ventricular myocyte sodium currents (Fig. 3d–g) recorded in wild-type and H/H animals were virtually identical. There was no difference in mouse ventricular cell size: 118.7 ± 3.6 pF (wild-type, $n = 15$) vs. 115.4 ± 3.2 pF (H/H, $n = 18$). Thus, the human channel provides a physiologic substitute for the mouse channel in H/H animals.

A key advantage of RMCE is that mice in which the targeted locus is replaced by a DNA sequence of choice can be generated rapidly (within months), and in paral-

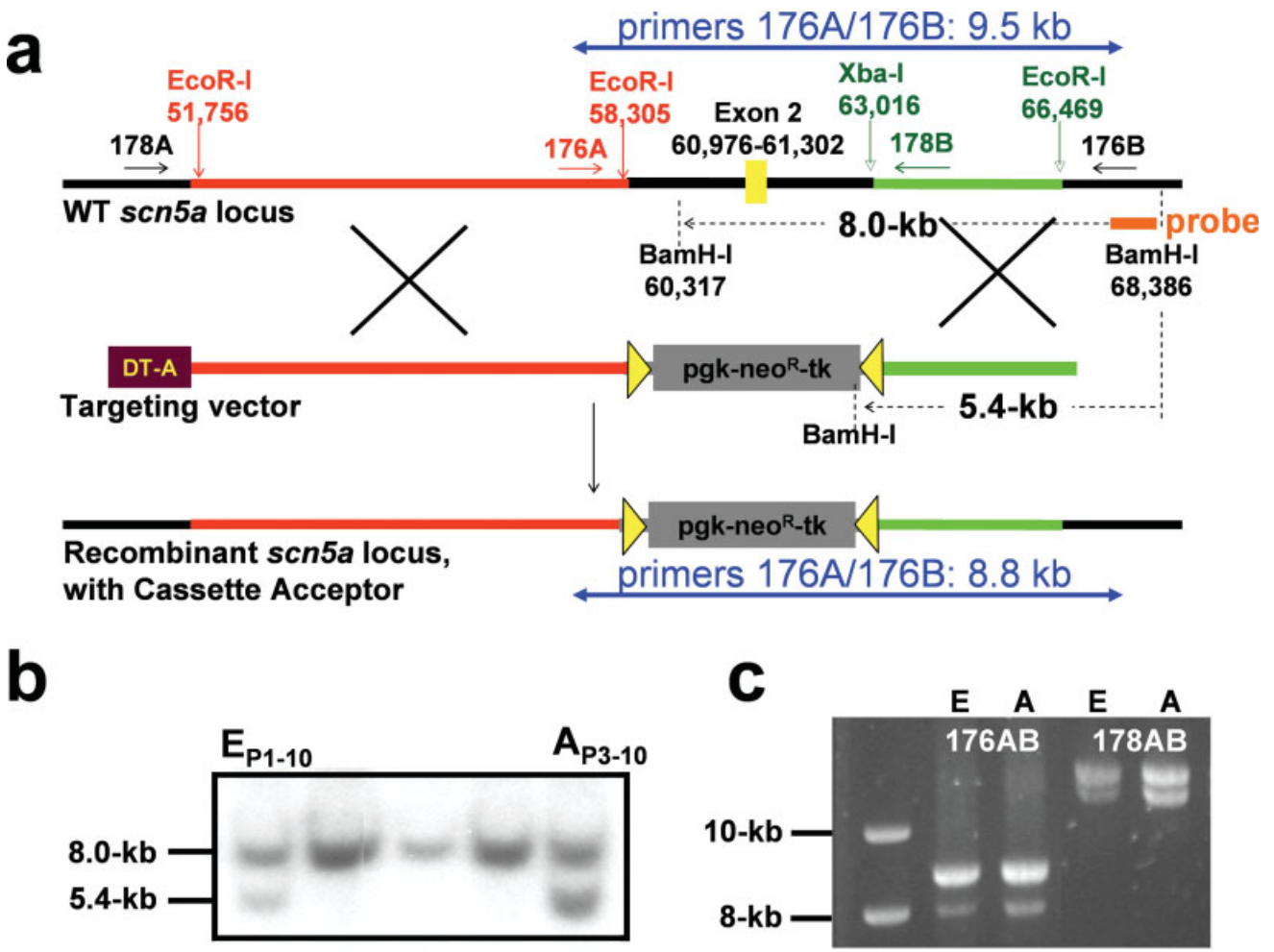

FIG. 1. Creation of the *scn5a* exon 2 cassette acceptor by gene targeting. (**a**) Schematic representation of the wild-type *scn5a* exon 2 locus and the targeting vector. After homologous recombination, a 4.7 kb exon 2 locus would be replaced by the selection cassette that includes pgk-*neo* and pgk-*tk*. The 5′ end DT-A indicates the diphtheria toxin encoding cDNA that is used for negative selection. *Bam*HI digestion was predicted to generate an 8.0 kb fragment from the wild-type allele and a 5.4 kb fragment from the recombinant allele using the 3′ external DNA probe denoted in orange. (**b**) Southern blot analysis of genomic DNA of selected ES clones using the probe shown in (a). The anticipated double crossing event was identified in clones E_{P1-10} and A_{P3-10} and appeared as a doublet of 8.0 and 5.4 kb bands. (**c**) Verification of the targeting events by long range PCR. The upper panel shows the schematic structure of the targeting locus and the external primer pairs. Primer 176A is within the targeted region and 176B is 3′ to the targeted region; the blue lines indicate the expected sizes of the product amplified from the wild-type allele (9.5 kb, top) and modified allele (8.8 kb, bottom). Similarly, the 178A/178B pair is designed to verify the 5′end reaction, with predicted fragment sizes of 12 kb (wild-type) and 11.3 kb (modified allele). The result shown in (c) demonstrates that both clones E and A include fragments consistent with the wild-type and the expected recombinant allele.

lel. This markedly lowers the barrier to developing mouse models to functionally study many mutant proteins in physiologic context. The experiment reported here represents a crucial proof of concept experiment to implementing this approach to study variant sodium channel function in the intact heart, since the H/H mice lack murine sodium channels, but are viable and display physiologic sodium channel function. Thus, we infer that expression of the human *SCN5A* cDNA is regulated by endogenous murine promoter and regulatory sequences that remain intact. These include not only the core promoter, but also other regulatory regions, including a recently described potential alternate transcription start site (Shang and Dudley, 2005).

Dozens of sodium channel mutants have been identified in patients, and associated with a range of electrophysiologic phenotypes from normal to high propensity for serious arrhythmias. However, elucidating in vitro channel function may not fully inform (and can even mislead) inference of the clinical phenotypes. For example, we have recently reported that the *SCN5A* mutant resulting in L1825P displays unstable inactivation, resulting in maintained inward current during the action potential plateau (Liu *et al.*, 2005). However, the predicted in vivo phenotype, prolongation of the QT interval on the surface ECG, was absent in the probands (Makita *et al.*, 2002), and our studies indicated that L1825P not only gates abnormally but is also retained in

Table 1
PCR Primers

Name	Sequence	Targeted gene	Primer start site	Use in this experiment
125A	ATTCCTGTGCAGTCACATTCAGAGACCTGG	*scn5a* (m)	67,057 (g)	Screening probe for mouse BAC genomic library
125B	TGCTGCTTGCTAGTTTCAGGCCAACATC	*scn5a* (m)	67,635 (g)	
168A	CGCCCTGGCATCCATTTGCA	*scn5a* (m)	58,283 (g)	Recombinant PCR to fuse mouse *scn5a* 5′UTR to human *SCN5A* cDNA
168B2	TGAACCTGCGGAAGCTGCTGGT	Fusion (m-H)	195 (c)	
169A3	AGAAGCAGGATGAGAAGATGGCAAACTTC	Fusion (m-H)	195 (c)	
169B	GCCATGATAATCACACTAAAGTCCAGCCAG	*SCN5A* (H)	805 (c)	
124A	CTCCATGGTTGTACACTCAGTCTCTGGTGGC	*scn5a* (m)	67,451 (g)	3′ external probe in the screening of homologous recombination
124B	CCTGGAGGAGGCATGTAAGTCCCAATGA	*scn5a* (m)	68,128 (g)	
178A	CTGGACAGTGTTGGGCCCTTGGAGA	*scn5a* (m)	51,339 (g)	5′ external screening primer
178B	TGTGCGTACCCCAGGACAGATGCAG	*scn5a* (m)	63,446 (g)	3′ internal screening primer
176A	GTCGACATATGGAGCAGCGATGTGGAG	*scn5a* (m)	58,011 (g)	5′ internal screening primer
176B	CACATACAACGCTGCACATGGAGAGACC	*scn5a* (m)	66,788 (g)	3′ external screening primer
F1	GTCGACATATGGAGCAGCGATGTGGAG	*scn5a* (m)	58,011 (g)	RMCE screening primer, forward 1
R1	TGAGCATGTTGAAGAGCGAGTGAACCAG	*SCN5A* (H)	603 (c)	RMCE screening primer, reverse 1
F2	CAGCCGTGGGCATCCTCTTCTTCA	*SCN5A* (H)	5,425 (c)	RMCE screening primer, forward 2
R2	TGTGCGTACCCCAGGACAGATGCAG	*scn5a* (m)	63,446 (g)	RMCE screening primer, reverse 2
R11	CGCACATGCATCTGCCTCAGGAAAG	*scn5a* (m)	58,363 (g)	RMCE screening primer, reverse 11
F22	CCGTCTGGACCGATGGCTGTGTAGAA	hyg^R	1,586 (c)	RMCE screening primer, forward 22
289F	CCTCACCAGCGCACTGGAAGAGTT	*SCN5A* (H)	2,198 (c)	RFLP at *Eco*RI 2,425 (H); forward primer
288R	ATGATTTGGCCAGCTTGAAGACCCG	*SCN5A* (H)	2,658 (c)	RFLP at *Eco*RI 2,425 (H); reverse primer
325F	AGGAGGCCATGGAAATGCTCAAGAA	*SCN5A* (H)	1,498 (c)	RFLP at *Sac*II 1,773 (H); forward primer
327R	AACTCTTCCAGTGCGCTGGTGAGGAC	*SCN5A* (H)	2,221 (c)	RFLP at *Sac*II 1,773 (H); reverse primer
315F	GAATTTGTGGACCTGGGCAAT	*SCN5A* (H)	816 (c)	RFLP at *Sac*I 911 (m); forward primer
314R	GACAAGCATGAAGAAGATCATGTAGATC	*SCN5A* (H)	1,382 (c)	RFLP at *Sac*I 911 (m); reverse primer
318F	AGAGTGGATCGAGACCATGTGGGACTG	*SCN5A* (H)	2,885 (c)	RFLP at *Sac*II 3,035 (m); forward primer
320R	CTCCGTGCCCAGGCTGTTCTCCTCAT	*SCN5A* (H)	3,404 (c)	RFLP at *Sac*II 3,035 (m); reverse primer

m, mouse; H, Human; g, genomic DNA (mouse *scn5a*), c, cDNA (Human *SCN5A*).

the endoplasmic reticulum. A logical next step is to determine how these diverse in vitro characteristics act to generate a whole heart phenotype, and how these may vary, or be manipulated to therapeutic advantage, in the in vivo setting. The implementation of the RCME-mediated approach will allow this experiment to proceed. Other channel mutations displaying such in vitro/in vivo phenotype disparities include A1924T in Brugada syndrome (Rook *et al*., 1999); D1275N which has been associated with diverse phenotypes including dilated cardiomyopathy and atrial fibrillation (McNair *et al*., 2004; Olson *et al*., 2005) or atrial standstill (Groenewegen *et al*., 2003); R1193Q, which has been associated with channel dysfunction (Wang *et al*., 2004) or dismissed as a common polymorphism in Chinese subjects (Hwang *et al*., 2005); and ΔK1500 which has been associated with multiple arrhythmia subtypes (Grant *et al*., 2002).

RMCE offers major advantages over the conventional gene targeting. First, the time between generation of an exchange cassette and actual development of a first-generation heterozygote mouse can be as short as 4 months. Second, since Cre-mediated recombination is enzymatic (in contrast to homologous recombination), it is more efficient, and allows multiple exchange experiments to be executed in parallel: while the recombination event in the initial exon 2 targeting was very rare (7/∼800 screened colonies), the second step generated 7/29 colonies with the desired recombination event and two were suitable for mouse generation. Third, a single targeted sequence is placed in its appropriate genomic location, allowing physiologic regulation of gene expression. In the case of *SCN5A*, the core promoter is intact ∼20 kb upstream (as in the wild-type animals), and other regulatory sequences, such as those reported in intronic regions (Shang and Dudley, 2005), are also intact in the H/H mouse. Fourth, the present study demonstrates that it is feasible to insert a >6 kb full-length coding sequence into a targeted locus, thereby providing an efficient approach to the in vivo study of other large genes.

While the technique was first described in the late 1990's, it is only with recent methodological advances (such as positive–negative selection sequencing) that we have been able to implement it for our studies. Further advances, such as use of loxP sequences incorporating mutations in the region's 8-bp linker to avoid unwanted inverse recombination (Langer *et al*., 2002), or flanking the insert with loxP and Frt sites (Lauth *et al*., 2002), may additionally improve the efficiency of the exchanges. However, the approach we describe may not apply to genes with multiple start sites, or to genes that serve very different functions in mice and humans (like some cardiac potassium channels).

The demonstration that the human isoform can physiologically substitute for the murine one is perhaps not unexpected given the high degree of sequence homology between the two. Nevertheless, the demonstration that the 6 kb human cardiac sodium channel cDNA can be expressed at the mouse *scn5a* locus (while disrupting expression of the murine isoform) represents an important milestone in development of advanced animal

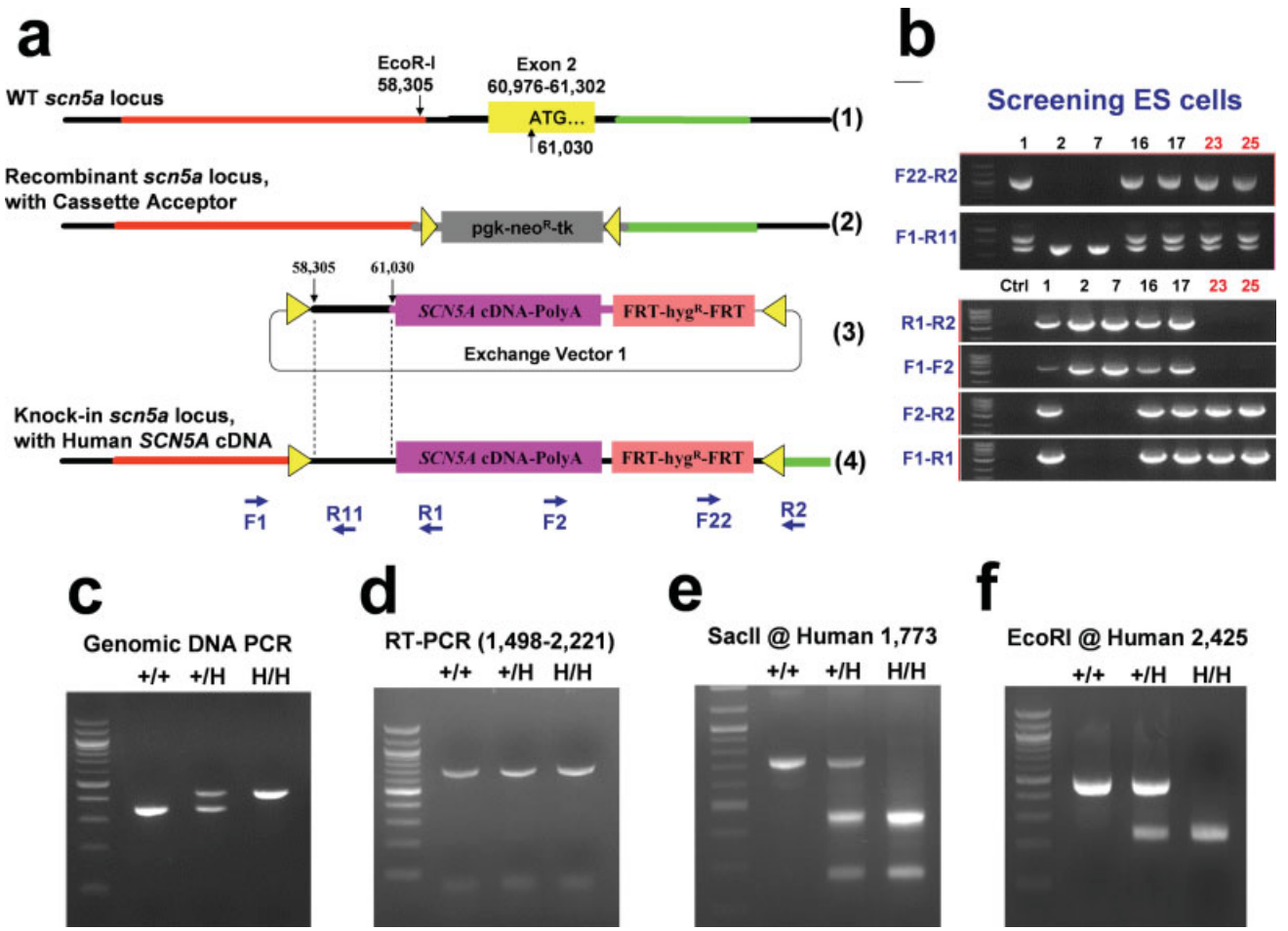

FIG. 2. RMCE to generate mice with a humanized cardiac sodium channel. (**a**) Line 1: the wild-type mouse scn5a exon 2 locus. Line 2: the floxed allele in which the mouse scn5a exon 2 is replaced by the Cassette Acceptor. Line 3: The exchange vector that consists of four structural elements: (1) a pair of inverted loxP sites (loxP/loxP^{inv}, yellow triangles) flanking the exchange components; (2) exon 2 upstream sequence (black) from position 58,305–61,030 (the last nucleotide before the ATG start site), which may include an important transcription regulatory region (Shang and Dudley, 2005); (3) the entire coding sequence of human *SCN5A* followed by an artificial polyA signal (pink); (4) a hygromycin resistance gene cassette (orange) for positive selection in RMCE. Line 4: expected exchanged allele; arrows in blue represent PCR primer pairs used to screen ES cell colonies after exchange. (**b**) Results of screening ES cell colonies with the primer sets indicated in (a). The F1-R11 pair detects correctly-oriented mouse or human alleles; the different size bands reflect the presence or absence of the 34 bp loxP region; thus, for example, colonies two and seven appear to lack the loxP insert. The F1-R11 primer pair was later used in primary screening of mouse pups. Pairs R1-R2 and F1-F2 should only generate products if an incorrectly-oriented insert is present, and F1-R1, F2-R2, and F22-R2 should only generate products if a correctly-exchanged allele is present. Thus, among the colonies screened here, No. 23 and No. 25 have predominantly the forwardly-oriented exchange event. (**c**) Genotyping transgenic mice using F1/R11 primers and tail genomic DNA. Lane 1 shows the wild-type *scn5a* locus with no exchange (+/+); Lane 2 shows a heterozygous mouse with a larger fragment due to the presence of a loxP site, designated H; lane 3 shows only the larger size band, representing a homozygous knock-in (H/H). (**d**) RT-PCR using heart RNA from wild-type (+/+), heterozygote for *SCN5A* (+/H) and homozygote (H/H) shows products with identical length using species consensus primers to amplify the 1,498–2,221 region. (**e**) Digestion of these fragments by *Sac*II (human *SCN5A* 1,773) yielded the expected digestion patterns unique to each genotype, verifying that in the (H/H) mouse heart, only the human knock-in *SCN5A* cDNA is being expressed. (**f**) A second PCR-RFLP experiment, using primers 288/289 and an *Eco*RI site at human *SCN5A* 2,425 showed the same result. Analogous results were obtained when mouse *scn5a* specific sites at *Sac*I 911 and *Sac*II 3,035 were tested.

models of human disease. The technique not only will allow study of *SCN5A* mutations, but also will allow us to insert reporters or tagged channels to enable studies of protein–protein interactions and processing in a physiologic context. More generally, the technology should be widely applicable to the study of human or engineered mutations in other genes in a physiologic context.

METHODS

Isolation of an *scn5a* Genomic Clone

PCR primers 125A-B (all primers used are listed in Table 1) derived from mouse intron 3 sequences were used to amplify a 579-bp probe from wide-type 129 S6/SvEv DNA. The probe was ^{32}P-labeled and used to screen the RPCI-22 (CHORI, Oakland, CA) BAC mouse genomic

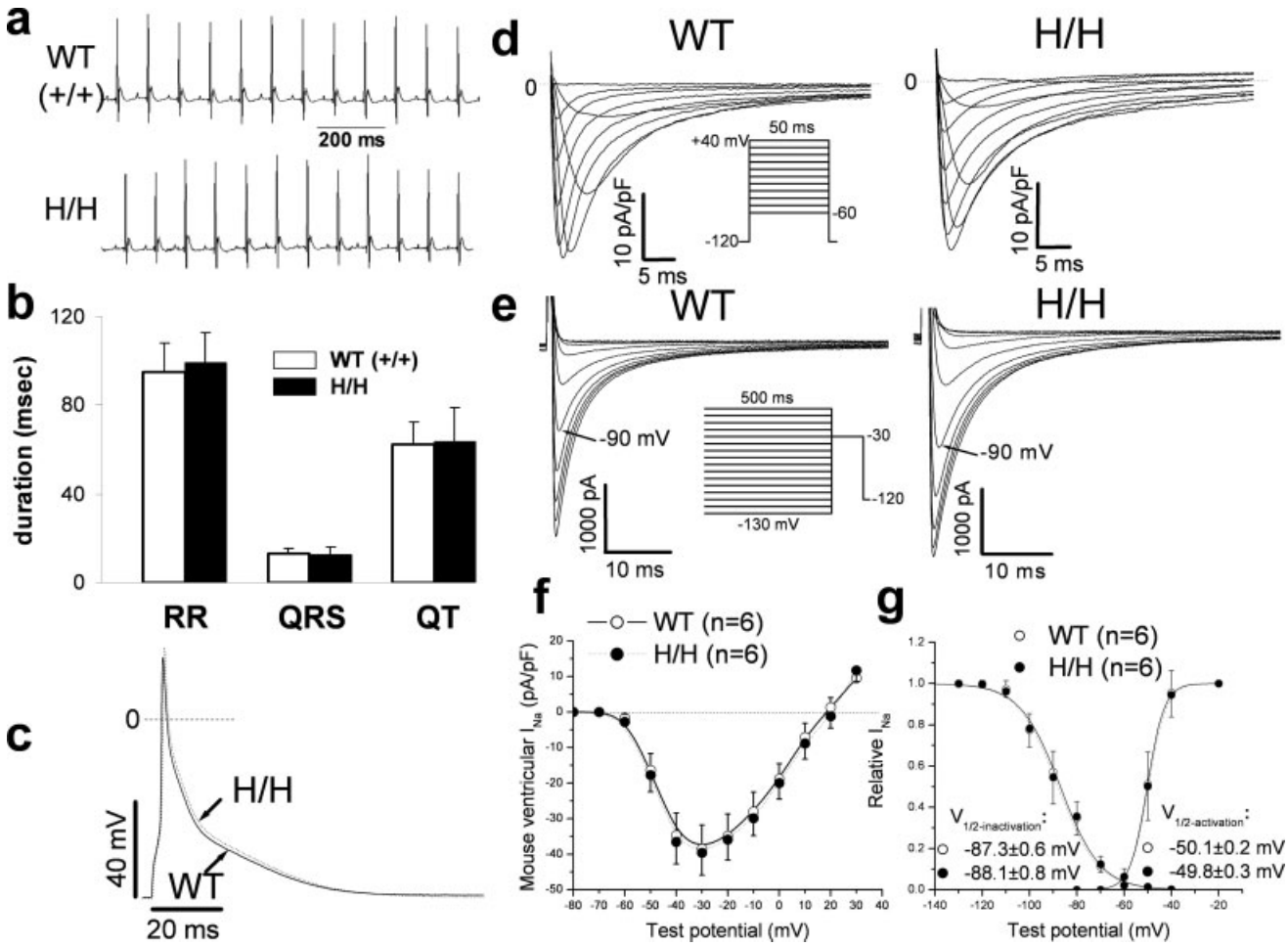

FIG. 3. Sodium channel function in wild-type vs H/H mice (**a**) ECGs (**b**) Summary ECG data. There was no difference in RR, QRS, or QT durations. RR is the inverse of heart rate, and QRS duration is a measure of sodium channel dependent conduction. $n = 4$ (WT), $n = 13$ (H/H) (**c**) ventricular myocyte action potentials. The records are indistinguishable. Summary data are presented in Table 2. There was no difference in resting potential, action potential amplitude, maximum phase 0 upstroke slope (an index of sodium current amplitude), or in action potential duration (APD) measured at 20, 50, and 90% repolarization. (**d**) Activating sodium current. Currents elicited by 50 ms depolarizations from −120 mV to a range of positive potentials are shown for a wild-type (left) and an H/H (right) ventricular myocyte. (**e**) Voltage-dependence of sodium channel inactivation. Currents elicited at −30 mV following 500 ms prepulses to a range of potentials are shown for a wild-type (left) and an H/H (right) ventricular myocyte. (**f**) Activating sodium current amplitude as a function of voltage. There was no difference between data obtained in wild-type (○) and H/H (●) myocytes. Data are shown normalized to cell size. (**g**) Voltage-dependence of activation and of inactivation. Neither the shape nor the mid-points of the curves differed between wild-type (○) and H/H (●) myocytes.

Table 2
Mouse Ventricular Action Potential Properties

	WT ($n = 7$)	H/H ($n = 7$)
MRP (mV)	−70.9 ± 1.2	−69.8 ± 1.1
APA (mV)	113.2 ± 4.1	116.4 ± 5.1
V_{max} (V/s)	140.7 ± 9.2	144.2 ± 8.9
APD_{20} (ms)	3.7 ± 0.2	3.8 ± 0.3
APD_{50} (ms)	7.1 ± 0.1	7.3 ± 0.2
APD_{90} (ms)	37.8 ± 1.2	38.5 ± 0.9

1 Hz (±SE, 22–23°C, current-clamp mode).

MRP, membrane resting potential; APA, action potential amplitude; V_{max}, maximal rising rate of action potential 0 phase; $APD_{20,50,90}$, action potential duration at 20, 50, and 90% repolarization to baseline, respectively.

There were no statistical differences between WT and H/H mice.

library. One BAC clone (7P2) covering the *scn5a* locus was isolated and DNA was extracted using a large-construct kit (QIAGEN, Valencia, CA).

Construction of the Targeting Vector

Scn5a exon 2 flanking elements, a 6.5-kb *Eco*RI fragment at the 5′ end and a 3.4-kb *Xba*I/*Eco*RI fragment at the 3′ end, were subcloned into the pBK-CMV vector (Stratagene, La Jolla, CA) as the homologous arms of the targeting vector (Fig. 1a). A cassette acceptor was engineered to include the neomycin-resistance gene (neo^R) and *Herpes Simplex Virus* thymidine kinase (*tk*) gene with both driven by the pgk promoter and flanked by loxP/inverted loxP sites. The cassette acceptor was then

inserted between the homologous arms to replace the exon 2 target locus (4.7-kb). The diphtheria toxin A-fragment gene (DT-A) was subcloned upstream of the long arm for negative selection against random genomic integration (Yagi *et al.*, 1990).

Gene Targeting

One hundred fifty micrograms of the purified and *Not*I-linearized targeting vector was electroporated into 50×10^8 ES cells at 800 V and a capacitance of 3 μF. The TL1 ES cell line was selected due to its high efficiency of germline transmission in previous homologous recombination experiments (Kupershmidt *et al.*, 1999). Cells were plated onto irradiated neomycin-resistant feeder cells and exposed to G418 for positive selection. Surviving colonies were harvested and screened by Southern blotting. The results were further confirmed by DNA PCR analysis and direct sequencing.

Genomic DNA isolated from ES cells after homologous recombination was digested with *Bam*HI, Southern blotted and probed. The 3′ external probe was amplified by primers (124A-B) and targets an 8-kb *Bam*HI fragment from the wide-type allele, and a 5.4 kb fragment from the recombinant exon2^{loxP} allele (Fig. 1a).

To further verify the targeting events, long range PCR was performed using Expand Long Template PCR System (Roche Applied Science, Indianapolis, IN). External primers (5′, 178A; 3′, 176B) from both ends were paired with internal primers (3′, 178B; 5′, 176A) and all products were sequenced to confirm the recombination events.

Construction of the Exchange Vector

The exchange vector (Fig. 2a, line 3) was assembled in five sequential steps. First, a backbone vector derived from pBK-CMV was engineered to have loxP and inverted loxP sites flanking the reorganized multiple cloning site. Second, exon 2 upstream sequence was fused in frame to the wild-type human *SCN5A* cDNA using BAC genomic DNA and *SCN5A* cDNA construct (a gift from Dr. Al George) as templates for recombinant PCR with 4 primers (168A, 168B2, 169A3, and 169B). The resultant product was subcloned into the pPCR-Script Amp SK vector (Stratagene), and then the fusion fragment was excised with *Not*I/*Xho*I double digestion and ligated to the backbone vector. The *Not*I site is located at 5′ end to the exon 2 upstream sequence in the pPCR-Script vector and the *Xho*I site is at nucleotide position 728 in human *SCN5A* cDNA sequence (Gen Bank accession number NM000335). Third, an *Xho*I segment containing the rest of the *SCN5A* cDNA and a bovine growth hormone (bGH) gene polyA signal was excised from the *SCN5A* cDNA construct and assembled to the backbone. Fourth, the 5′ *Not*I site was destroyed by Klenow fill-in (Stratagene) and a new *Not*I site was introduced into the 3′ end of the *SCN5A* cDNA as the acceptor site for the positive selection cassette hygromycin-resistance gene (*hyg*^{R}). Finally, pgk-*hyg*^{R} flanked by two FRT sites was excised from the plasmid pFRT2. Hygro by *Not*I digestion and inserted into the backbone for completion of the exchange vector (Long *et al.*, 2004).

Recombinase-Mediated Cassette Exchange

While the concept of recombinase-mediated cassette exchange (RMCE) has considerable appeal, the detailed methodology has only recently matured (Long *et al.*, 2004). A problem in early experiments was that exposure of ES cells for prolonged periods of time to both negative and positive selection makers resulted in uniform cell death; the mechanism is not established, but persistent thymidine kinase activity (expression is driven by a strong promoter) may be responsible. To allow *tk* activity to decay, but to avoid ES cell differentiation, a staggered positive/negative selection strategy has been adopted (Long *et al.*, 2004). ES cells containing the exon2^{loxP} allele (clone numbers 1E10 and 3A10) were first electroporated with a Cre-expressing plasmid and the exchange vector (100 μg each), that includes a hygromycin fusion gene (Fig. 2a). Cells were placed under hygromycin B (Invitrogen, Carlsbad, CA) at 200 μg/ml for positive selection (~7 days). The surviving clones were picked, transferred to 96 well plates, allowed to grow for an additional 5 days, and then split into 3 plates. One plate was used to identify resistance to ganciclovir (Sigma, St. Louis, MO) at 8 μM for another 5 days, and the other two served as master plates for subsequent DNA isolation and for stocking frozen cells. Ganciclovir-resistant clones were identified by inspection, and genomic DNA from the corresponding wells screened for RMCE events by PCR (Fig. 2b). The primer combinations were designed to amplify junction regions across either loxP or loxP^{inv} sites to give directionality; products of the F1-R1, F2-R2, F1-R11, and F22-R2 reactions specified a forward signal, while the F1-F2 and R1-R2 pairs were used to analyze the reverse direction (primer pairs are presented in Table 1). Those demonstrating correctly-oriented RMCE events were expanded from the frozen master plate for blastocyst injection.

ES Cell Microinjection and Mouse Genotyping

ES cells with the correctly-orientated wild-type *SCN5A* cDNA (clones 23 and 25) were microinjected into the blastocoel cavity of embryos derived from natural matings of C57BL/6 mice. The resulting male chimeras were bred to 129/Sv females (Taconic, Hudson, NY). Genomic DNA was prepared from tail snips using standard methods (Hogan *et al.*, 1994). Genotyping was accomplished by PCR-based methods.

RT-PCR and RFLP Analyses

Total RNA from wild-type, heterozygous, and homozygous *SCN5A* cDNA knock-in mouse hearts was isolated using TRIZOL (GIBCOBRL, Gaithersburg, MD) and RT-PCR was carried out using Titan One Tube RT-PCR System (Roche Applied Science). An *SCN5A* primer pair complementary to a region of identity in human and mouse channels (325F-327R) was employed to amplify a 724-bp piece corresponding to human *SCN5A* cDNA nu-

cleotide position from 1,498 to 2,221. A unique *Sac*II site, present at human nucleotide 1,773 but absent in the mouse *scn5a*, was used in a restriction fragment length polymorphism (RFLP) analysis to differentiate the expression of human *SCN5A* mRNA from that of the mouse isoform. Similarly, three more primer pairs 289F-288R, 315F-314R, and 318F-320R were designed to test RFLP sites at *Eco*RI (2,425; human), *Sac*I (911; mouse) and *Sac*II (3,035; mouse), respectively.

In Vivo Telemetry ECGs in Unrestrained Mice

Adult mice (>23 g) were anesthetized with pentobarbital and ketamine (33 μg/g, intraperitoneally [i.p.] each) and implantable transmitters (Data Sciences International, St. Paul, MN) were positioned into the abdominal cavity by a midline incision in the mid-abdominal wall (Temple *et al*., 2005). The leads were run subcutaneously from the abdomen to the right and left shoulders in a standard lead I configuration. Continuous monitoring was performed at the time of lead placement to verify adequate signal before closing the incision. ECGs were then continuously recorded in awake and unrestrained mice after at least 24 h recovery interval. ECG intervals were determined by signal averaging 10 s epochs. Data collection was performed using Dataquest software, and off-line data analyses were performed using Physiostat ECG analysis software, version 3.1 (Data Sciences International). Variables for analysis were QRS, QT, and mean RR intervals. The individual performing this analysis was blinded to genotype.

Isolation of Healthy Mouse Cardiac Ventricular Myocytes

Adult wild-type and H/H mouse ventricular myocytes were isolated by a modified collagenase/protease method (Mitra and Morad, 1985). After intraperitoneal injection of 500 IU of heparin, adult mice were anesthetized using inhaled isoflurane/oxygen mixture, hearts excised, and their aortae rapidly cannulated and perfused with modified Tyrode's solution (MTS) for 3 min followed by MTS containing collagenase (Liberase Blendzyme-4, Roche, 0.04 mg/ml) for 5–7 min at a constant pressure of 80 mmHg and temperature of 34°C. The MTS contained (in mmol/l) NaCl 130, HEPES 10, glucose 10, KCl 5.4, $MgCl_2$ 1.2, NaH_2PO_4, 2,3-butanedione monoxime 10, pH of 7.2. The digested ventricles were minced in MTS containing 1 mg/ml bovine serum albumin and 0.2 mmol/l $CaCl_2$ and triturated by gently pipetting. The resulting solution was strained and the myocytes allowed to sediment in MTS of increasingly higher Ca^{2+} concentrations (0.2, 0.5, and 1 mmol/l). This procedure routinely yielded 60–80% rod-shaped, Ca^{2+}-tolerant myocytes that were used for the electrophysiology studies.

Action Potential Recordings

Action potentials from isolated myocytes were elicited in current clamp mode (Axopatch 200B amplifier, Molecular Diagnostics, Sunnyvale, CA), at 1 Hz with stimulus current (1–2 nA, 2–4 ms). The bath (extracellular) solution contained (in mmol/l): NaCl 140, HEPES 5, glucose 10, KCl 4, $CaCl_2$ 1.8, and $MgCl_2$ 1, with a pH of 7.4 (adjusted by NaOH). The pipette (intracellular) solution had (in mmol/L): Aspirate-K 120, HEPES 5, KCl 25, Na_2ATP 4, $MgCl_2$ 1, Na_2Phosphocreatine 2, NaGTP 2, $CaCl_2$ 1, and EGTA 10, with a pH of 7.2 (adjusted by KOH). Junction potentials between pipette and bath solutions were compensated electronically. Data acquisition was carried out at a sampling frequency of 50 kHz, and analyzed using Clampex 9.2 and pClampfit 9.2.

Sodium Current Recordings

To allow recording of native cardiac Na^+ current (I_{Na}), external Na^+ concentration was lowered to 20 mM, electrodes with tip resistance <1 MΩ were used, and experiments were conducted at room temperature. I_{Na} was recorded using whole-cell voltage clamp. The pipette-filling (intracellular) solution contained (in mmol/L): NaF 10, CsF 110, CsCl 20, EGTA 10, HEPES 10, with a pH of 7.4 adjusted with CsOH. The bath (extracellular) solution had (in mmol/L): NaCl 20, CsCl 110, TEA-Cl 5, $CaCl_2$ 0.1, $MgCl_2$ 1, HEPES 10, and glucose 10, with a pH of 7.4 adjusted by CsOH. To eliminate L- and T-type inward calcium currents, 1 μM nisoldipine and 200 μM NiCl were added to the bath solution. Data acquisition was carried out using an Axopatch 200B patch-clamp amplifier and pCLAMP version 9.2 software (Molecular Diagnostics). Currents were filtered at 5 kHz (−3 dB, four-pole Bessel filter) and digitized using an analog-to-digital interface (Digidata 1322A, Molecular Diagnostics). To minimize capacitive transients, capacitance, and series resistance were adjusted to 70–85%. The holding potential was −120 mV for all experiments, and details of the pulse protocols are presented schematically in the figures. I_{Na} densities in wild-type and H/H mice were compared as pA/pF after normalization to cell sizes which were generated from the cell capacitance calculated by Membrane Test (OUT 0) in pClamp 9.2.

Electrophysiologic data were analyzed using Clampfit version9.2 (Axon Instruments), and the figures were prepared by using Origin 7.0 (OriginLab). Current-voltage relations for steady-state activation and inactivation were fit with the Boltzmann equation $I/I_{max} = (1 + \exp[(V - V_{1/2})/k])^{-1}$ to determine the membrane potential for half-maximal activation ($V_{1/2\text{-activation}}$) and inactivation ($V_{1/2\text{-inactivation}}$). The time course of inactivation of macroscopic current was fit with a monoexponential Chebyshev equation: $I = A_1{*}\exp[-(t-K)/r_1] + C$. Results are presented as mean ± SE, and statistical comparisons were made using the unpaired Student's *t* test. A value of $P < 0.05$ was considered as statistically significance.

LITERATURE CITED

Bouhassira EE, Westerman K, Leboulch P. 1997. Transcriptional behavior of LCR enhancer elements integrated at the same chromosomal locus by recombinase-mediated cassette exchange. Blood 90:3332–3344.

Bunch TJ, Ackerman MJ. 2006. Promoting arrhythmia susceptibility. Circulation 113:330–332.

Cesari F, Rennekampff V, Vintersten K, Vuong LG, Seibler J, Bode J, Wiebel FF, Nordheim A. 2004. Elk-1 knock-out mice engineered by Flp recombinase-mediated cassette exchange. Genesis 38:87–92.

Feng YQ, Seibler J, Alami R, Eisen A, Westerman KA, Leboulch P, Fiering S, Bouhassira EE. 1999. Site-specific chromosomal integration in mammalian cells: Highly efficient CRE recombinase-mediated cassette exchange. J Mol Biol 292:779–785.

Grant AO, Carboni MP, Neplioueva V, Starmer CF, Memmi M, Napolitano C, Priori S. 2002. Long QT syndrome, Brugada syndrome, and conduction system disease are linked to a single sodium channel mutation. J Clin Invest 110:1201–1209.

Groenewegen WA, Firouzi M, Bezzina CR, Vliex S, van Langen IM, Sandkuijl L, Smits JP, Hulsbeek M, Rook MB, Jongsma HJ, Wilde AA. 2003. A cardiac sodium channel mutation cosegregates with a rare connexin40 genotype in familial atrial standstill. Circ Res 92:14–22.

Hogan B, Beddington R, Constantini F, Lacy E. 1994. Manipulating the mouse embryo: A laboratory manual. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Hwang HW, Chen JJ, Lin YJ, Shieh RC, Lee MT, Hung SI, Wu JY, Chen YT, Niu DM, Hwang BT. 2005. R1193Q of SCN5A, a Brugada and long QT mutation, is a common polymorphism in Han Chinese. J Med Genet 42:e7.

Jones JR, Shelton KD, Magnuson MA. 2005. Strategies for the use of site-specific recombinases in genome engineering. Methods Mol Med 103:245–257.

Kupershmidt S, Yang T, Anderson ME, Wessels A, Niswender KD, Magnuson MA, Roden DM. 1999. Replacement by homologous recombination of the *minK* gene with *lacZ* reveals restriction of *minK* expression to the mouse cardiac conduction system. Circ Res 84:146–152.

Langer SJ, Ghafoori AP, Byrd M, Leinwand L. 2002. A genetic screen identifies novel non-compatible loxP sites. Nucleic Acids Res 30:3067–3077.

Lauth M, Spreafico F, Dethleffsen K, Meyer M. 2002. Stable and efficient cassette exchange under non-selectable conditions by combined use of two site-specific recombinases. Nucleic Acids Res 30:e115.

Liu K, Yang T, Viswanathan PC, Roden DM. 2005. New mechanism contributing to drug-induced arrhythmia: Rescue of a misprocessed LQT3 mutant. Circulation 112:3239–3246.

Long Q, Shelton KD, Lindner J, Jones JR, Magnuson MA. 2004. Efficient DNA cassette exchange in mouse embryonic stem cells by staggered positive-negative selection. Genesis 39:256–262.

Makita N, Horie M, Nakamura T, Ai T, Sasaki K, Yokoi H, Sakurai M, Sakuma I, Otani H, Sawa H, Kitabatake A. 2002. Drug-induced long-QT syndrome associated with a subclinical SCN5A mutation. Circulation 106:1269–1274.

McNair WP, Ku L, Taylor MR, Fain PR, Dao D, Wolfel E, Mestroni L. 2004. SCN5A mutation associated with dilated cardiomyopathy, conduction disorder, and arrhythmia. Circulation 110:2163–2167.

Mitra R, Morad M. 1985. A uniform enzymatic method for dissociation of myocytes from hearts and stomachs of vertebrates. Am J Physiol 249:H1056–H1060.

Nuyens D, Stengl M, Dugarmaa S, Rossenbacker T, Compernolle V, Rudy Y, Smits JF, Flameng W, Clancy CE, Moons L, Vos MA, Dewerchin M, Benndorf K, Collen D, Carmeliet E, Carmeliet P. 2001. Abrupt rate accelerations or premature beats cause life-threatening arrhythmias in mice with long-QT3 syndrome. Nat Med 7:1021–1027.

Olson TM, Michels VV, Ballew JD, Reyna SP, Karst ML, Herron KJ, Horton SC, Rodeheffer RJ, Anderson JL. 2005. Sodium channel mutations and susceptibility to heart failure and atrial fibrillation. JAMA 293:447–454.

Papadatos GA, Wallerstein PM, Head CE, Ratcliff R, Brady PA, Benndorf K, Saumarez RC, Trezise AE, Huang CL, Vandenberg JI, Colledge WH, Grace AA. 2002. Slowed conduction and ventricular tachycardia after targeted disruption of the cardiac sodium channel gene Scn5a. Proc Natl Acad Sci USA 99:6210–6215.

Remme CA, Veldkamp MW, Ginneken AC, Verkerk AO, Brunschot S, Belterman CN, Wilde AA, Bakker JM, Bezzina CR. 2005. Mice carrying the SCN5A Mutation 1798insD, equivalent to human 1795insD, display bradycardia, conduction delay and QT-prolongation. Circulation 112:II–220.

Roebroek AJ, Reekmans S, Lauwers A, Feyaerts N, Smeijers L, Hartmann D. 2006. Mutant Lrp1 knock-in mice generated by recombinase-mediated cassette exchange reveal differential importance of the NPXY motifs in the intracellular domain of LRP1 for normal fetal development. Mol Cell Biol 26:605–616.

Rook MB, Alshinawi CB, Groenewegen WA, Van Gelder IC, van Ginneken AC, Jongsma HJ, Mannens MM, Wilde AA. 1999. Human SCN5A gene mutations alter cardiac sodium channel kinetics and are associated with the Brugada syndrome. Cardiovasc Res 44:507–517.

Seibler J, Schubeler D, Fiering S, Groudine M, Bode J. 1998. DNA cassette exchange in ES cells mediated by Flp recombinase: An efficient strategy for repeated modification of tagged loci by marker-free constructs. Biochemistry 37:6229–6234.

Shang LL, Dudley SC Jr. 2005. Tandem promoters and developmentally regulated 5′- and 3′-mRNA untranslated regions of the mouse Scn5a cardiac sodium channel. J Biol Chem 280:933–940.

Splawski I, Shen J, Timothy KW, Lehmann MH, Priori S, Robinson JL, Moss AJ, Schwartz PJ, Towbin JA, Vincent GM, Keating MT. 2000. Spectrum of mutations in long-QT syndrome genes. KVLQT1, HERG, SCN5A, KCNE1, and KCNE2. Circulation 102:1178–1185.

Temple J, Frias P, Rottman J, Yang T, Wu Y, Verheijck EE, Zhang W, Siprachanh C, Kanki H, Atkinson JB, King P, Anderson ME, Kupershmidt S, Roden DM. 2005. Atrial fibrillation in KCNE1-null mice. Circ Res 97:62–69.

Tester DJ, Will ML, Haglund CM, Ackerman MJ. 2005. Compendium of cardiac channel mutations in 541 consecutive unrelated patients referred for long QT syndrome genetic testing. Heart Rhythm 2:507–517.

Wang Q, Chen S, Chen Q, Wan X, Shen J, Hoeltge GA, Timur AA, Keating MT, Kirsch GE. 2004. The common SCN5A mutation R1193Q causes LQTS-type electrophysiological alterations of the cardiac sodium channel. J Med Genet 41:e66.

Yagi T, Ikawa Y, Yoshida K, Shigetani Y, Takeda N, Mabuchi I, Yamamoto T, Aizawa S. 1990. Homologous recombination at c-fyn locus of mouse embryonic stem cells with use of diphtheria toxin A-fragment gene in negative selection. Proc Natl Acad Sci 87:9918–9922.

Yang P, Kupershmidt S, Roden DM. 2004. Cloning and initial characterization of the human cardiac sodium channel (SCN5A) promoter. Cardiovasc Res 61:56–65.