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9. For construction of the gene-targeting vectors, we created the Sal I–Neo-hMT cassette by subcloning the 574-bp Hind III–Pvu II hMT fragment from hMTIacO(−10)CAT [M. C.-T. Hu and N. Davidson, *Cell* **48**, 555 (1987)] and the 1.6-kb Xho I–Eco RV FRT-neomycin-FRT fragment from pFRT2neo (*4*) {modified from pMC1neo-poly(A) [K. R. Thomas and M. R. Cappecchi, *Cell* **51**, 503 (1987)]}. We replaced the 1.7-kb Acc I–Xmn I fragment for construction of the hMT vector [$s_\gamma 1$ sequence: GenBank accession number M12389: positions 310 (Acc I) to 2014 (Xmn I)] or the 1.6-kb Acc I–Sau I fragment for construction of the s-hMT vector [positions 310 (Acc I) to 1900 (Sau I)] by a Sal I linker in the plasmid, which carries the 3.6-kb Hind III–Sca I fragment of the 5′$s_\gamma 1$ region [from $p_\gamma 1$/EH10.0 (*18*)], and inserted the Sal I Neo-hMT cassette into the Sal I linker of the 5′$s_\gamma 1$ plasmid. Transcription of the *neo* gene was oriented away from the γ1 switch region. The Sca I site was exchanged for a Not I site. The Hind III–Sca I fragment of this mutated 5′$s_\gamma 1$ region was inserted into the vector pIC19R/MC1-TK [S. L. Mansour, K. R. Thomas, M. R. Capecchi, *Nature* **336**, 348 (1988)]. The 5′ long homologous arm was extended by insertion of an 8-kb Hind III fragment (5′5′$s_\gamma 1$ region) from Charon γ1-13 [A. Shimizu, N. Takahashi, Y. Yaoita, T. Honjo, *Cell* **28**, 499 (1982)].
10. Approximately 2.5×10^7 E14-1 ES cells [R. Kühn, K. Rajewsky, W. Müller, *Science* **254**, 707 (1991)] were transfected with 10 μg of Not I–linearized, gene-targeting vector by electroporation with a transfection efficiency of about 7.5×10^{-5} and selected with G418 (0.3 mg/ml) and 2 μM ganciclovir for stable transfectants. The ganciclovir-enrichment factor was 2.5 as determined by comparison to neomycin-only selection plates. The Δ5′$s_\gamma 1$ cells are described in (*4*).
11. Homologous recombinant clones were detected by PCR. The 5′ PCR primer (5′-TTCCACAACTGCACTCCACC-3′) was located at the hMT transcription start point in the vector, and the 3′ PCR primer (5′-CCTTCATTCTAACCTGCCCC-3′) was upstream of the endogenous Sca I site in the murine $s_\gamma 1$ region. Positive clones were confirmed by restriction analysis. One out of 21 hMT and one out of 8 s-hMT G418-ganciclovir double-resistant clones were homologous recombinants.
12. Splenic lymphocytes were activated with LPS or LPS and IL-4 in vitro (*4*). After 5 days, the cells were fixed in formaldehyde, permeabilized by saponin [M. Assenmacher *et al.*, *Eur. J. Immunol.* **24**, 1097 (1994)], and stained for cytoplasmic Ig with 10 μg/ml of biotinylated Ig(4a)10.9 (anti-$IgG1^a$) (*4*), 0.5 μg/ml of streptavidin-phycoerythrin, 2.5 μg/ml of digoxigenized anti-IgG1 (Miltenyi Biotec, Bergisch Gladbach, Germany), and 2 μg/ml of anti-digoxigenin–fluorescein isothiocyanate (Boehringer Mannheim, Germany). Cytometric analysis was performed in a FACScan (Becton Dickinson).
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20. We thank K. Rajewsky, A. Egert, R. Kühn, G. Siebenkotten, and R. Torres for discussion and advice; R. Kühn for the E14-1 ES cell line; S. Irlenbusch and G. von Hesberg for technical assistance; and U. Ringeisen for graphics. This work was supported by the Bundesministerium für Forschung und Technologie through Genzentrum Köln.

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Role of Steroidogenic Acute Regulatory Protein in Adrenal and Gonadal Steroidogenesis

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Congenital lipoid adrenal hyperplasia is an autosomal recessive disorder that is characterized by impaired synthesis of all adrenal and gonadal steroid hormones. In three unrelated individuals with this disorder, steroidogenic acute regulatory protein, which enhances the mitochondrial conversion of cholesterol into pregnenolone, was mutated and nonfunctional, providing genetic evidence that this protein is indispensable for normal adrenal and gonadal steroidogenesis.

Steroid hormone synthesis is increased in response to trophic hormone stimulation. Although increased transcription of genes that encode steroidogenic enzymes is important in the chronic hormonal response (*1*), the rate-limiting step in the acute response is the transport of cholesterol into mitochondria (*2*). Several molecules have been proposed to participate in this transport step, but their roles have not been definitively established (*3*). However, a defect in such a molecule would be predicted to impair steroidogenesis.

Congenital lipoid adrenal hyperplasia (lipoid CAH) is an autosomal recessive disorder that is characterized by a deficiency of adrenal and gonadal steroid hormones (*4*, *5*). Affected infants die from salt loss, hyperkalemic acidosis, and dehydration unless treated with steroid hormone replacement therapy; genetic XY males are born with female external genitalia as a result of the absence of testicular testosterone synthesis. Because mitochondria from affected adrenal glands and gonads fail to convert cholesterol to pregnenolone, the disease had been thought to be caused by a defect in the cholesterol side chain cleavage enzyme, cytochrome P450scc (*4*–*6*). However, a role for P450scc was subsequently eliminated by molecular genetic analysis of affected individuals (*6*, *7*). We have suggested that the defect may involve the transport of cholesterol into mitochondria (*3*, *6*). Thus, elucidation of the molecular defect for lipoid CAH may identify a crucial component in this important process.

Placental progesterone synthesis is necessary for the maintenance of pregnancy (*8*). Because pregnancies with a lipoid CAH fetus progress normally to term and the placenta produces progesterone (*9*), the affected factor appears to be required for adrenal and gonadal, but not placental, steroidogenesis. A 30-kD phosphorylated protein is thought to mediate the rapid, cycloheximide-sensitive response of steroidogenesis to trophic stimulation (*10*). This protein, termed steroidogenic acute regulatory protein (StAR), was purified from MA-10 murine Leydig tumor cells, and mouse (*11*) and human (*12*) StAR complementary DNAs (cDNAs) have

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been cloned. Transient expression of StAR cDNA in MA-10 cells and COS-1 cells results in enhanced steroidogenesis (*11*, *12*). The mRNA that encodes StAR is abundant in adrenal glands and gonads, but not in placenta (*12*). Because StAR appeared to be a candidate for the factor affected in lipoid CAH, we examined the StAR gene in three unrelated patients (*13*).

We prepared StAR cDNA from patients 1 and 2 by reverse transcription and the polymerase chain reaction (RT-PCR) with testicular mRNA as template. With PCR primers based on the sequences of the 5′ and 3′ untranslated regions, the principal product was StAR cDNA, but related species that contained a large number of sequence differences were also generated. These species led to the identification of a StAR pseudogene (*12*). With a primer based on a sequence termed S1 in the 5′ untranslated region that distinguishes the authentic StAR gene from its pseudogene, we amplified the expected 974–base pair (bp) StAR cDNA from normal controls and the two patients (Fig. 1). The RT-PCR products were subcloned into the pCRII vector and sequenced.

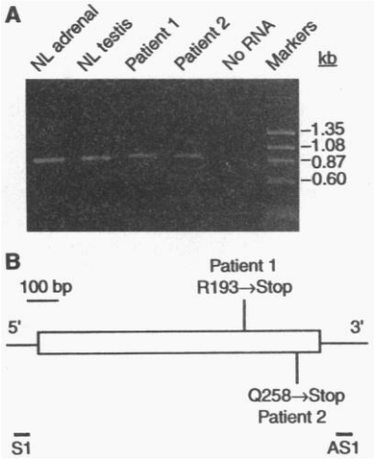

Fig. 1. Detection of nonsense mutations in StAR cDNA from patients with lipoid CAH. (**A**) StAR RT-PCR products from normal (NL) human fetal adrenal and testicular RNA, testicular RNA from patients 1 and 2, and a no-RNA control were separated on a 1% agarose gel and stained with ethidium bromide. The molecular size markers (shown in kilobases) are Hind III–cleaved bacteriophage λ. (**B**) Map of StAR cDNA. R193 → Stop represents the substitution of a Stop codon (TGA) for the Arg^{193} codon (CGA), and Q258 → Stop the substitution of a Stop codon (TAG) for the Gln^{258} codon (CAG). The open box represents the coding region of StAR cDNA. The small bars below the map indicate the PCR primers. Sense primer S1, 5′-GCAGCAGCAGCGGCGGCAGCAG-3′ [nucleotides (nt) 66 to 84 in the cDNA] (*12*); antisense primer AS1, 5′-ATGAGCGTGTGTACCAGTGCAG-3′ (nt 1016 to 1037). The PCR program was 30 cycles of 94°C for 45 s, 64°C for 30 s, and 72°C for 60 s.

All patient clones from independent RT-PCR reactions were identical to the wild-type sequence except for a C → T transition in codon 193 (Arg) in patient 1 and a C → T transition in codon 258 (Gln) in patient 2. These mutations generated premature stop codons, resulting in encoded proteins that lack 93 or 28 COOH-terminal amino acid residues, respectively.

To confirm the identity of these mutations, we analyzed StAR genes from patient genomic DNA. Because the structure of the StAR gene was unknown, we first obtained by PCR a genomic clone that contained the exons harboring the mutations. Various combinations of sense and antisense primers derived from the cDNA sequence were used to amplify normal genomic DNA. The primer pair S2-AS2 yielded two specific products of 437 and 290 bp (Fig. 2A). The sequence of the 437-bp fragment matched the cDNA sequence at both ends perfectly and contained a 141-bp intron in the middle, and thus was derived from the StAR gene. The 290-bp fragment was derived from the StAR pseudogene and lacked the intron. Subsequently, the combination of an intronic primer, S3, and primer AS1 yielded a 2.1-kb PCR product (Fig. 2B). Mapping and sequencing of this DNA fragment revealed that the sequences of the exons matched the cDNA perfectly. Thus, the 2.1-kb fragment was identified as the 3′ half of the StAR gene. The sequence information obtained from the 2.1-kb clone enabled us to construct intronic primers to amplify the exons by PCR (Fig. 2C).

The presence of the nonsense mutations at codons 193 and 258 was confirmed by direct sequencing of PCR products of genomic DNA. Whereas patient 1 has a C → T transition at codon 193, her father and mother each have both C and T at the corresponding position (Fig. 3A). Thus, we concluded that patient 1 is homozygous for the Arg^{193} → Stop mutation and that both her parents are carriers of this mutation. Similarly, patient 2 was homozygous for the Gln^{258} → Stop mutation (Fig. 3B). As expected, the mother of patient 2 was heterozygous for this mutation and a normal sibling had no mutation (*14*). In addition, patient 3 was homozygous for the same mutation as patient 2 (Fig. 3B); her mother was also a carrier (*14*). Because patient 2 is an ethnic Korean and patient 3 an ethnic Japanese, this observation suggests a common origin for this mutation in these two related ethnic groups.

To investigate the functional consequences of these premature stop codons in the StAR gene, we analyzed the wild-type and mutant proteins for their ability to enhance steroidogenesis. Coexpression of wild-type StAR with the cholesterol side chain cleavage system in COS-1 cells resulted in an approximately eightfold increase in pregnenolone production with cholesterol as a substrate (Table 1). Both mutant StAR proteins were inactive, indicating that each of the two nonsense mutations caused lipoid CAH. Unlike cholesterol, 20α-hydroxycholesterol readily diffuses into mitochondria and thereby bypasses the mitochondrial cholesterol transport system (*15*). With 20α-hydroxycholesterol as a substrate, there were no significant differences ($P > 0.05$ by analysis of variance) in pregnenolone production among wild-type and mutant StAR proteins (Table 1). The differential effects of StAR on pregnenolone production with cholesterol and 20α-hydroxycholesterol as substrates suggest that StAR mediates the transport of cholesterol into mitochondria.

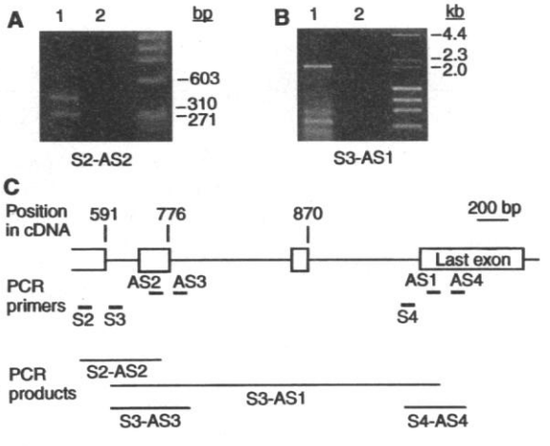

Fig. 2. PCR mapping of the StAR gene. (**A**) Genomic PCR products amplified with primers S2 and AS2 were separated on a 2% agarose gel and stained with ethidium bromide. The molecular size markers (base pairs) are Hae III–cleaved bacteriophage Φ×174. (**B**) Genomic PCR products amplified with primers S3 and AS1 were separated on a 1% agarose gel. Molecular size markers (kilobases) are Hind III–cleaved bacteriophage λ. Smaller fragments are unrelated PCR products. In both (A) and (B), PCR was performed in the presence (lanes 1) or absence (lanes 2) of genomic DNA. (**C**) Map of the 3′ half of the StAR gene. Open boxes represent exons, and numbers at the end of each exon are the corresponding nucleotide positions in the cDNA sequence (*12*). Locations of the various PCR primers and products are shown below the map. Sense primer S2, 5′-GACAAAGTGATGAGTAAAGTG-3′ (nt 442 to 462); antisense primer AS2, 5′-TGTGGCCATGCCAGCCAGCA-3′ (nt 717 to 738). The PCR program for S2-AS2 was 35 cycles of 94°C for 45 s, 58°C for 30 s, and 72°C for 60 s. Sense primer S3, 5′-GTGAGCAAAGTCCAGGTGCG-3′. The PCR program for S3-AS1 was 35 cycles of 94°C for 50 s, 64°C for 30 s, and 72°C for 90 s.

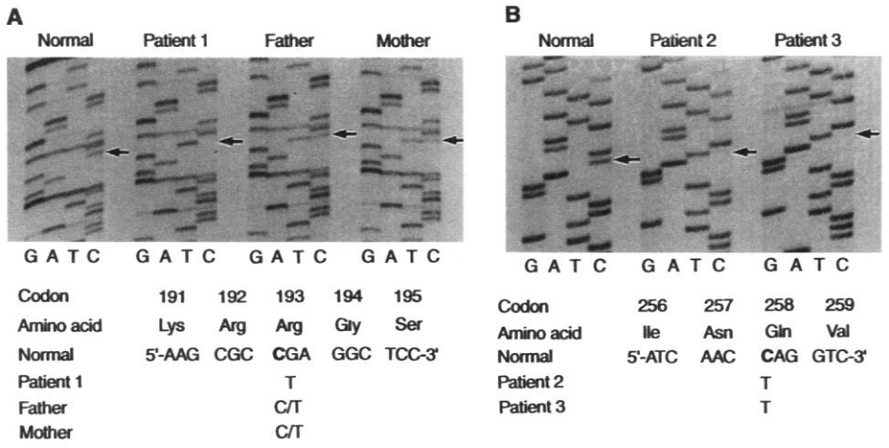

Fig. 3. Direct sequencing of PCR products. (**A**) Direct sequencing (Dynal, Lake Success, New York) of PCR products from a normal control, patient 1, and parents of patient 1. Arrows indicate the affected nucleotide in the nonsense mutation: C in control, T in patient 1, C and T in both parents. DNA and amino acid sequences are shown below. (**B**) Direct PCR sequencing of a normal control, patient 2, and patient 3. Arrows indicate a C in the control and a T in both patients 2 and 3. In (A), the sense PCR primer (S3) was described in the legend to Fig. 2 and the biotinylated antisense primer (AS3) was 5′-GGATGCAGTC-CACATGCTTGG-3′. The PCR program was 35 cycles of 94°C for 45 s, 64°C for 30 s, and 72°C for 45 s. A sense primer, 5′-GATACATTCATTACTCAC-3′ (nt 613 to 630), was used for sequencing. In (B), the sense biotinylated primer (S4) was 5′-CCTGGCAGCCTGTTTGTGATAG-3′ and the antisense primer (AS4) was 5′-CCTCATGTCATAGCTAATCAGTG-3′ (nt 1201 to 1223). The PCR program was 35 cycles of 94°C for 45 s, 63°C for 30 s, and 72°C for 45 s. Antisense primer AS1 was used for direct sequencing.

Table 1. Effect of nonsense mutations on StAR activity. With the use of lipofectamine, nonsteroidogenic COS-1 monkey kidney cells were transfected with the vector pSPORT or with pSPORT containing normal human StAR cDNA (pStAR) or mutant StAR cDNA from patients 1 or 2 (or 3) (*12*). The cells were also transfected with either vectors encoding bovine P450scc and bovine adrenodoxin (scc-Adx) or a pECE vector encoding a fusion protein termed F2 that consists of the human cholesterol side chain cleavage system (H_2N–P450scc–adrenodoxin reductase–adrenodoxin–COOH) (*17*). The substrate was either cellular and serum cholesterol or added 20α-hydroxycholesterol (5 μg/ml) (20α). After incubation of cells for 48 hours, the medium was collected and assayed for pregnenolone by immunoassay. Values are means ± SD from four separate transfections.

Vector	Pregnenolone (nanograms per dish)			
	scc-Adx		F2	
	Cholesterol	20α	Cholesterol	20α
pSPORT	20 ± 1	158 ± 21	17 ± 3	60 ± 7
pStAR	175 ± 19	138 ± 15	131 ± 23	60 ± 11
Patient 1	19 ± 2	99 ± 23	18 ± 5	56 ± 7
Patient 2 or 3	25 ± 4	168 ± 35	22 ± 4	75 ± 7

StAR is synthesized as a 285–amino acid protein with a mitochondrial targeting sequence of 25 residues, which is cleaved from the NH_2-terminus after transport into mitochondria (*11*, *12*). The precursor and mature StAR proteins have half-lives in the range of minutes and hours, respectively (*10*, *11*). Quantitation of immunoblots with a BioImage Visage 2000 image analysis system (*11*) revealed that ~70% of StAR in COS-1 cells transfected with the wild-type plasmid was in the mature form. However, no mature form was apparent for the mutant protein from patient 1 and only ~10% of the mutant protein from patient 2 was in the mature form (Fig. 4), suggesting that lack of processing might impair StAR activity.

Lipoid CAH is the only inborn disorder of steroid hormone synthesis known that is not caused by a defective steroidogenic enzyme. The identification of mutant StAR proteins as the cause of lipoid CAH will now permit prenatal molecular diagnosis for this devastating disease. Lipoid CAH due to nonfunctional StAR is comparable to the effect of a StAR gene knockout, demonstrating that StAR is indispensable for adrenal and gonadal steroidogenesis. Thus, StAR is the first protein identified that plays an essential role for cholesterol access to P450scc. The sparing of placental steroidogenesis in fetuses with lipoid CAH and the absence of StAR expression in placenta

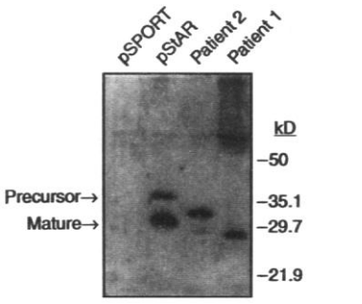

Fig. 4. Expressed mutant StAR proteins. COS-1 cells were cotransfected as described in Table 1 with the pECE vector encoding the fusion protein F2 and either pSPORT or pSPORT containing normal StAR cDNA (pStAR) or StAR cDNA from patients 1 or 2. Cell homogenates were prepared 48 hours after transfection. Proteins were solubilized in sample buffer, resolved on a 12.5% SDS-polyacrylamide gel, and transferred to a polyvinylidene difluoride membrane. Immunoblot analysis was performed with antibodies to mouse StAR (*11*). The positions of precursor and mature StAR as well as molecular size standards (kilodaltons) are indicated.

and other steroidogenic tissues (*12*), such as brain (*16*), suggest that different mechanisms may exist to facilitate cholesterol transport into mitochondria in these tissues. The critical role of StAR in lipoid CAH provides genetic evidence for the hypothesis that StAR is the molecule that mediates the acute trophic regulation of steroid hormone synthesis (*10*).

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Cardiac Malformation in Neonatal Mice Lacking Connexin43

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Gap junctions are made up of connexin proteins, which comprise a multigene family in mammals. Targeted mutagenesis of connexin43 (Cx43), one of the most prevalent connexin proteins, showed that its absence was compatible with survival of mouse embryos to term, even though mutant cell lines showed reduced dye coupling in vitro. However, mutant embryos died at birth, as a result of a failure in pulmonary gas exchange caused by a swelling and blockage of the right ventricular outflow tract from the heart. This finding suggests that Cx43 plays an essential role in heart development but that there is functional compensation among connexins in other parts of the developing fetus.

The connexins are a family of at least 12 proteins, each the product of a distinct gene, that make up the intercellular membrane channels of gap junctions (*1*). Gap junction channels composed of hexamers of connexins provide conduits for the direct passage of ions and other low molecular weight molecules, including second messengers, between cells (*2*), a property that has been postulated to be important for many physiological and developmental processes (*3*).

All of the connexins have four membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic NH_2- and COOH-termini. Sequence similarity is concentrated in the transmembrane domains and extracellular loops. In contrast, most of the sequence diversity and length variation among the connexins resides in the cytoplasmic loop and COOH-terminal tail, which are assumed to account for the distinct biophysical and regulatory properties that are exhibited by gap junction channels composed of different connexins (*1*). In addition, individual gap junctions may be composed of more than one type of connexin (*4*, *5*). Given the distinct developmental expression patterns of the different connexins, this could result in complex regulation of intercellular coupling. The biological significance of connexin diversity is, however, uncertain. Antibody and antisense studies in vitro show the general significance of gap junctional coupling (*6*), but mutational analysis is required to test the role of the different connexins in vivo. The first genetic evidence for a specific developmental role for a member of the connexin family was the demonstration that X-linked Charcot-Marie-Tooth disease, a neuropathological condition involving peripheral demyelination in humans, is associated with mutations in the gene encoding connexin32 (*7*).

We have created a null mutation in mice in the gene (*Gja1*) encoding another member of the connexin family, connexin43 (Cx43). The gene is expressed from the onset of zygotic transcription in early cleavage and supplies subunits for the first gap junctions to form in mammalian development (*8*–*10*). It is also widely expressed in postimplantation embryos and adult organs, often in conjunction with other connexins (*11*). A mutation was generated in the Cx43 gene by homologous recombination in 129 strain R1 (*12*) embryonic stem (ES) cells with a construct that replaced almost the entire coding sequence with the *neo*^{r} gene and that lacked a promoter (Fig. 1). This strategy was made feasible by the high level of Cx43 expression in ES cells (*13*). One in three *neo*^{r} clones was correctly targeted. Several ES cell lines that were homozygous for the Cx43 deletion were obtained by an increase in the G418 concen-

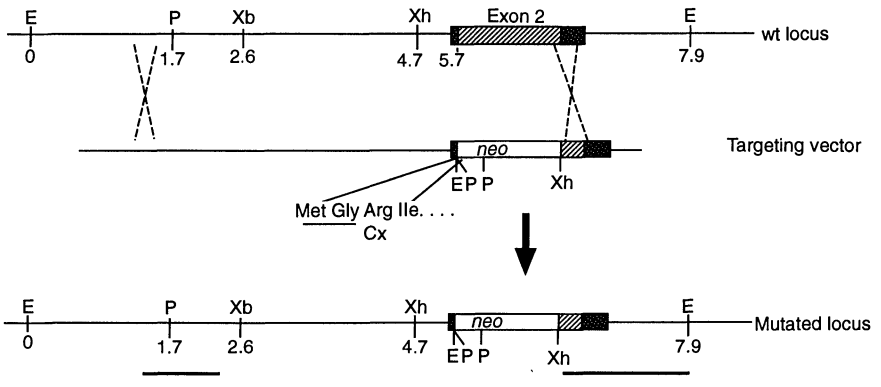

Fig. 1. Genomic structure of Cx43 and targeting strategy. A rat Cx43 complementary DNA probe (pG2A) was used to screen a 129Sv mouse genomic library. Six overlapping clones were isolated and the genomic structure determined. The coding sequence (striped box) is contained entirely within one exon, and there is an untranslated 5′ exon approximately 11 kilobases upstream (not shown). A targeting vector was designed to place the promoterless *neo* gene after the second amino acid of Cx43 and delete all the transmembrane regions of the Cx43 gene. This vector was introduced into R1 ES cells and *neo*^{r} clones isolated in the standard way (*39*) except that ES cells were grown in leukemia inhibitory factor (1000 U/ml) without feeders throughout. Homologously recombined clones were recognized by distinctive bands on Southern (DNA) blots after Eco RI digestion and probing with 5′ and 3′ probes, shown as bars under the genomic structure. E, Eco RI; P, Pst I; Xb, Xba I; and Xh, Xho I.

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