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Switch Transcripts in Immunoglobulin Class Switching

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B cells can exchange gene segments for the constant region of the immunoglobulin heavy chain, altering the class and effector function of the antibodies that they produce. Class switching is directed to distinct classes by cytokines, which induce transcription of the targeted DNA sequences. These transcripts are processed, resulting in spliced "switch" transcripts. Switch recombination can be directed to immunoglobulin G1 (IgG1) by the heterologous human metallothionein II_A promoter in mutant mice. Induction of the structurally conserved, spliced switch transcripts is sufficient to target switch recombination to IgG1, whereas transcription alone is not.

Switch recombination is induced in B cells upon activation. It occurs between highly repetitive DNA sequences, the switch (s) regions (located 5′ of the C_H genes), and is directed to distinct immunoglobulin (Ig) classes by cytokines (*1*). The mechanism of this control is not clear, but several molecular changes in the 5′ flanking regions of the switch regions correlate with targeting of switch recombination. These include specific cytokine-inducible protein binding, induction of deoxyribonuclease I–hypersensitive sites, and specific demethylation and transcription (*2*). Switch transcription starts upstream of the switch region, runs through the switch region, and terminates 3′ of the corresponding C_H gene. The transcripts are processed in a way that an exon located 5′ of the switch region (the I_H exon) is spliced to the C_H exons, generating a "switch transcript," also referred to as "germline transcript" or "sterile transcript" (*3*). Despite an almost perfect correlation of induction of switch transcription and switch recombination, the functional relation between the two events is still unclear.

Switch recombination to IgG1 or IgG2b is almost completely abolished in B cells in which the promoter and I exons of the IgG1 (*4*) or IgG2b (*5*) switch transcripts are deleted. Likewise, deletion of the Ig heavy chain gene (IgH) intron enhancer and part of the I exon of IgM disables recombination of the IgM switch region (*6*). However, switch transcription is apparently not sufficient to direct switch recombination. Replacement of the interleukin-4 (IL-4)–responsive promoter of IgE switch transcripts in the 18.81A20 pre-B cell line and in normal B cells by the promoter and enhancer of the Ig variable region genes (V_H promoter–IgH intron enhancer) results in only marginal switch recombination to IgE at about 1% of the frequency induced by IL-4 (*7*). Moreover, because they used V_H control elements, they could not exclude the possibility that even this marginal switch recombination is targeted to IgE by inherent V_H recombination control elements rather than by induction of transcription.

Here, we describe genomic replacement of the IL-4–responsive recombination control element for switch recombination to IgG1 by a heterologous promoter in normal B lymphocytes. The functional role of switch transcription and of structurally conserved, spliced switch transcripts in the targeting of switch recombination to IgG1 are assessed. We chose the human metallothionein II_A (hMT) promoter, which is not known to be involved in any recombination process and which responds to a variety of inductive stimuli like growth factors and differentiation factors [for example, lipopolysaccharide (LPS)] (*8*).

A 1.7-kb region of DNA directly upstream of the IgG1 switch region, containing all known IL-4–inducible molecular elements of the 5′s_γ1 region (*2*), was exchanged for an inversely oriented neomycin resistance gene and the hMT promoter by homologous recombination in murine embryonic stem (ES) cells. We used two different gene-targeting constructs (*9*) differing only in the presence of a 114-bp sequence (Fig. 1), which contains the splice donor site of the I_γ1 exon. Thus, IgG1 switch transcripts generated from the s-hMT–modified (s indicates the splice donor site), but not from the hMT-modified, allele contain the splice donor site of the I_γ1 exon (Fig. 1B). Mutant ES cells (*10*) were generated by homologous recombination of the hMT vector in E14-1 ES cells ($IgH^{a/a}$) and of the s-hMT vector in Δ5′s_γ1 ES cells ($IgH^{\Delta 5's_\gamma 1 a/a}$); the latter are E14-1 cells with a deletion of the control region for IgG1 switch recombination on one allele, as described (*4*). Cloned heterozygous homologous recombinant cells were detected by polymerase chain reaction

Table 2. IgG1-positive splenic lymphoblasts of heterozygous mutant mice upon LPS stimulation.

Culture	IgG1-positive lymphoblasts (%) of mice		
	Control	hMT/+	s-hMT/s-hMT
1	0.07	0.00	6.12
2	0.03	0.02	7.77
3	0.04	0.00	5.55
Mean	0.05	0.01	6.5

Table 1. IgH loci and expected fragment lengths (in kilobases). The wild-type IgH^a and the mutated $IgH^{\Delta 5's_\gamma 1}$ locus do not contain the neomycin gene.

Fragment	Length for allele				
	IgH^a	$IgH^{neo\Delta 5's_\gamma 1}$	$IgH^{\Delta 5's_\gamma 1}$	$IgH^{s\text{-}hMT}$	IgH^{hMT}
neo–Kpn I	–	2.9	–	3.6	3.5
s_γ1–Eco RI	16.5	10.0	10.0	10.7	10.6

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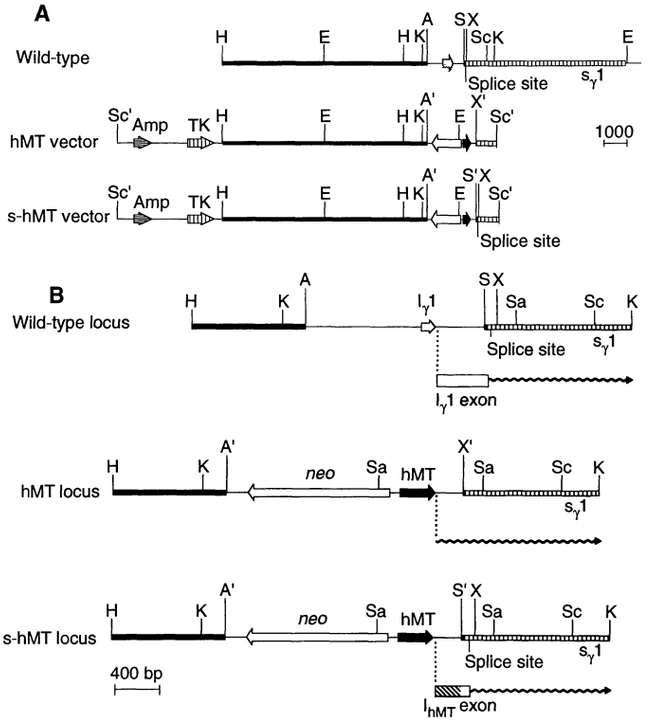


Fig. 1. Replacement of the murine 5′ $s_\gamma 1$ flanking region by the human metallothionein II_A promoter. (**A**) Genomic structure of the murine wild-type $s_\gamma 1$ region (hatched bar) with 5′ flanking sequences without (black line) or with (black bar) homology to the gene-targeting constructs, the hMT vector, and the s-hMT vector. A, Acc I; H, Hind III; E, Eco RI; K, Kpn I; Sc, Sca I; S, Sau I; and X, Xmn I (only relevant sites are indicated). Amp, ampicillin resistance gene; TK, herpes simplex virus thymidine kinase gene. The genomic Sca I site was changed to a Not I site (Sc′) in the vectors; the genomic Acc I, Xmn I, and Sau I sites were changed into Sal I sites (A′, X′, and S′). The Sal I replacement cassette for the 5′$s_\gamma 1$ region consisting of a neomycin resistance gene (*neo*, large white arrow) and a human metallothionein II_A promoter (hMT, black arrow) is located between both homologous arms. (**B**) Structure of the mutated IgH alleles. The genomic 1.7-kb Acc I–Xmn I fragment (hMT) or the genomic 1.6-kb Acc I–Sau I fragment (s-hMT) with the IL-4–dependent $I_\gamma 1$ promoter (small white arrow) of the 5′$s_\gamma 1$ region were replaced by an hMT promoter (black arrow). Although the $I_\gamma 1$ and hMT transcripts (squiggled line) of the wild type and the s-hMT locus contain an endogenous $I_\gamma 1$ exon (white bar) or an artificial I_{hMT} exon (diagonal hatched bar), both of which are spliced to the first $C_\gamma 1$ exon, the hMT locus contains no $I_\gamma 1$ splice donor site. The s-hMT allele contains 239 bp of bacterial vector backbone nucleotides and 114 endogenous nucleotides with the $I_\gamma 1$ splice donor site [$s_\gamma 1$ sequence: GenBank accession number M12389: positions 1900 (Sau I) to 2014 (Xmn I)].

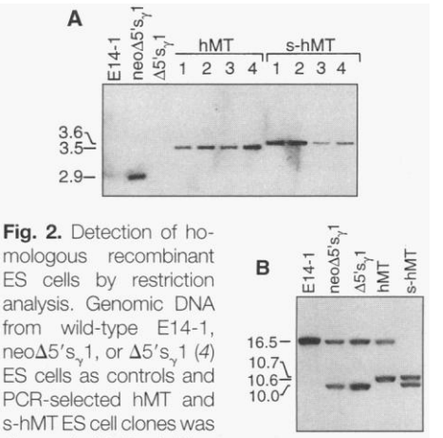


Fig. 2. Detection of homologous recombinant ES cells by restriction analysis. Genomic DNA from wild-type E14-1, neoΔ5′$s_\gamma 1$, or Δ5′$s_\gamma 1$ (*4*) ES cells as controls and PCR-selected hMT and s-hMT ES cell clones was digested with Kpn I (**A**) or Eco RI (**B**) and hybridized to a neomycin (A) or $s_\gamma 1$ probe (B) (*14*). The 16.5-kb $s_\gamma 1$ Eco RI fragment of one of the wild-type IgH^a alleles is disrupted in the hMT ES cells and in the s-hMT ES cells of the remaining wild-type allele. The s-hMT ES cells show a *neo*–Kpn I fragment that is approximately 0.1 kb longer than that in hMT cells, indicating the presence of the 114-bp additional endogenous sequence. The expected restriction fragments of the various IgH alleles are indicated in Table 1.

(PCR) and confirmed by restriction analysis (*11*) (Fig. 2 and Table 1). We injected hMT and s-hMT ES cells into C57BL/6 ($IgH^{b/b}$) blastocysts to generate chimeric mice, which were bred with C57BL/6 mice to obtain heterozygous mutant animals. Homozygous animals were obtained by heterozygous intercross. ES cell–derived B lymphocytes express Ig of the *a* haplotype, whereas blastocyst-derived B lymphocytes express Ig of the *b* haplotype.

For analysis of IgG1 class switching, splenic B lymphocytes of BALB/c × C57BL/6 ($IgH^{a/b}$) control mice, heterozygous hMT mice ($IgH^{hMTa/b}$), and homozygous s-hMT mice ($IgH^{s\text{-}hMTa/s\text{-}hMTa}$) were stimulated polyclonally in vitro with bacterial LPS and LPS plus IL-4 for 5 days and then analyzed for expression of IgG1 by intracellular immunofluorescence and flow cytometry (*12*) (Fig. 3). Immunoglobin class switching to IgG1 was dependent on IL-4 in wild-type B cells, with IL-4 raising the frequency of $IgG1^a$-expressing cells from 0.05% to 15.4% of total lymphoblasts (Tables 2 and 3). B cells homozygous for the s-hMT mutation were induced to switch to IgG1 also in the absence of IL-4; after 5 days of culture, 6.5% of total lymphoblasts were $IgG1^a$-expressing cells, and in the presence of IL-4, $IgG1^a$ cells were 20.5% of total lymphoblasts.

Thus, the s-hMT promoter can direct switch recombination to IgG1. However, it can do so only with the s-hMT replacement, containing the endogenous splice donor site of the $I_\gamma 1$ exon. No switching to $IgG1^a$ was induced in $IgH^{hMTa/b}$ B cells neither in the presence nor absence of IL-4 (Tables 2 and 3), although switch recombination to wild-type IgG1b was not affected in these cells. Nevertheless, the frequency of switched cells cultured with LPS plus IL-4 (20%) was higher than that of cultures with LPS alone (6.5%). The reason for this may be the pleiotropic effect of IL-4. It not only induces IgG1 switch recombination, but it also enhances proliferation and clonal size of IgG1 cells (*13*).

In vivo, the phenotypic difference between hMT and s-hMT replacement mutations is as evident as in vitro. Quantitation of serum IgG1 in heterozygous mutant hMT, s-hMT, Δ5′$s_\gamma 1$, and control (BALB/c × C57BL/6) mice showed that s-hMT mice have serum levels of $IgG1^a$ (Fig. 4) (mean: 311 μg/ml) comparable to those found in control mice (mean: 271 μg/ml), whereas hMT and Δ5′$s_\gamma 1$ mice produce no detectable serum $IgG1^a$ (≤80 ng/ml). Heterozygous hMT mice had lower levels of serum $IgG1^b$ (by a factor of 5.5) than those found in control, s-hMT, and Δ5′$s_\gamma 1$ mice. The reason for this is not clear.

To correlate switch recombination on the s-hMT– and hMT-*IgH* alleles to induction of transcription and switch transcripts, we analyzed s-hMT and hMT B cells of homozygous mutant mice, activat-

Table 3. Same as in Table 2, but with LPS and IL-4 stimulation.

Culture	IgG1-positive lymphoblasts (%) of mice				
	Control		hMT/+		s-hMT/s-hMT
	$IgG1^a$	$IgG1^b$	$IgG1^a$	$IgG1^b$	$IgG1^a$
1	15.0	15.1	0.08	13.3	25.0
2	14.9	16.0	0.05	17.3	17.8
3	16.3	14.3	0.04	16.9	18.8
Mean	15.4	15.2	0.06	15.8	20.5

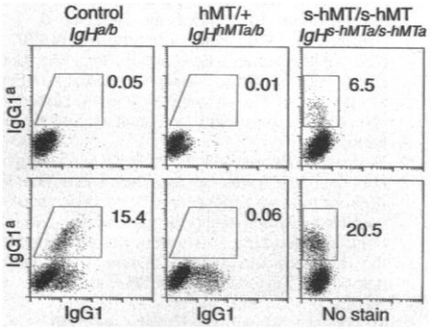


Fig. 3. In vitro analysis of Ig class switch. Flow cytometric analysis of LPS- (top panels) and LPS–IL-4–stimulated (bottom panels) splenic lymphocytes from BALB/c × C57BL/6 control mice ($IgH^{a/b}$), heterozygous hMT mice ($IgH^{hMTa/b}$), and homozygous s-hMT mice ($IgH^{s\text{-}hMTa/s\text{-}hMTa}$). The lymphocytes were fixed on day 5 of culture and stained by two-color cytoplasmic immunofluorescence for total IgG1 (allotypes *a* and *b*) and $IgG1^a$. s-hMT cells were stained only for $IgG1^a$. Each plot represents 5000 cells. The frequencies of $IgG1^a$ lymphoblasts are indicated.

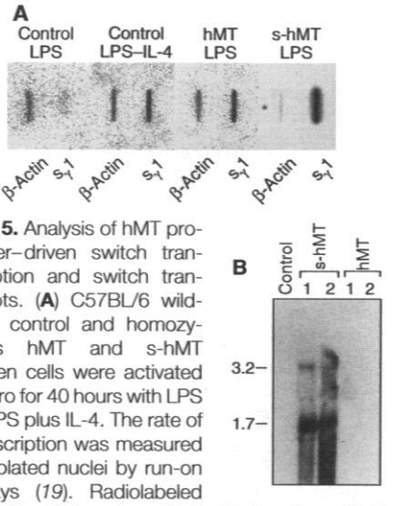


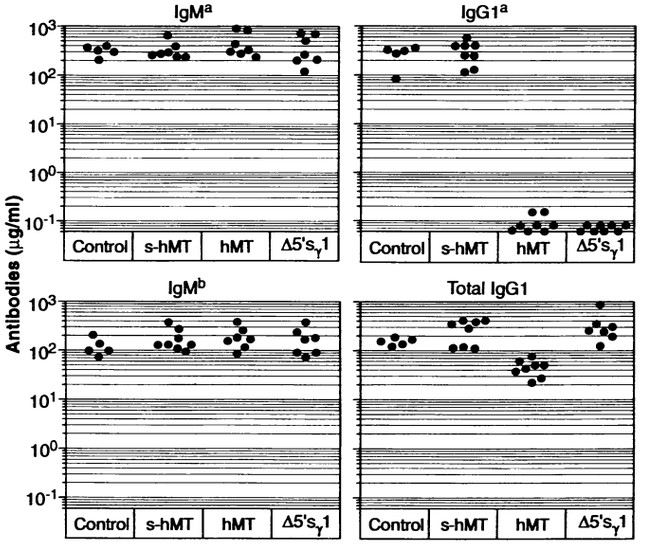
Fig. 5. Analysis of hMT promoter–driven switch transcription and switch transcripts. (**A**) C57BL/6 wild-type control and homozygous hMT and s-hMT spleen cells were activated in vitro for 40 hours with LPS or LPS plus IL-4. The rate of transcription was measured in isolated nuclei by run-on assays (*19*). Radiolabeled RNA (9×10^5 cpm) was blotted to β-actin and $s_\gamma 1$ slots. (**B**) Northern blot analysis. Equal amounts of total RNA from spleen cells of wild-type C57BL/6 control mice and more than 90% chimeric hMT and s-hMT mice were activated in vitro for 3 days with LPS or LPS plus IL-4, then were separated according to size by electrophoresis in agarose, blotted onto nitrocellulose, and hybridized to an hMT probe (*14*). Molecular size markers are shown on the left in kilobases.

ed by LPS, for their transcription rate by nuclear run-on assays and for the presence of processed transcripts by Northern (RNA) blotting (Fig. 5). In the run-on assays, the LPS-induced $I_\gamma 1$ promoter showed no transcription of the $s_\gamma 1$ region. The hMT promoter and the IL-4–induced $I_\gamma 1$ promoter showed equal rates of transcription, despite their drastic difference in switch recombination. The transcription rate of the s-hMT promoter is higher than that of the IL-4–induced $I_\gamma 1$ promoter, although s-hMT B cells switch at a lower frequency. Thus, the rate of switch transcription in normal and mutant B cells is not correlated with the frequency of switch recombination. However, hMT and s-hMT transcription differs in quality, as shown by Northern blot analysis (Fig. 5B) and probing for processed switch transcripts (*14*). The s-hMT B cells showed two transcripts (of about 1.7 kb and 3.2 kb) with both the hMT and $C_\gamma 1$ probes. The 1.7-kb transcript corresponds to the processed $I_\gamma 1$ transcript with the secretory exon of $C_\gamma 1$, whereas the 3.2-kb transcript corresponds to the $I_\gamma 1$ switch transcript with the membrane exons of $C_\gamma 1$ (*15*). The hMT B cells did not express detectable amounts of processed transcripts hybridizing to the hMT probe (Fig. 5B).

The perfect correlation between hMT promoter–driven induction of artificial IgG1 switch transcripts and switch recombination to IgG1 shows that the induction of processed switch transcripts is sufficient to target switch recombination to a particular switch region and that the endogenous IL-4–driven promoter of IgG1 switch transcripts can be replaced by an entirely different heterologous promoter. Moreover, it strongly suggests that spliced switch transcripts, or the process of splicing, have a functional role in switch recombination. Alternatively, the additional 114 bp of endogenous sequence of the s-hMT replacement, which is essential for switch induction, might contain in addition to the splice donor site a necessary recombination control element. The latter seems less likely, because apart from the splice signal sequence, the 114-bp sequence contains no detectable sequence homology to any other I exon, in particular human $5's_\gamma 4$, as would be expected for a general or IL-4–specific recombination control element.

The fact that artificial hMT switch transcripts are sufficient to direct switch recombination shows that the replaced wild-type $I_\gamma 1$ exon (*16*) sequence per se is not required for the control of recombination. Because the hMT-induced IgG1 switch transcripts do not contain the first 20 nucleotides of the open reading frame of the wild-type IgG1 switch transcripts (*17*), "switch peptides" encoded by the wild-type switch transcripts are not involved in the targeting of recombination. Although our results show that artificial induction of structurally conserved, spliced switch transcripts can target switch recombination, they also show that switch transcription as such cannot. This finding is in accordance with previous work (*7*), where the IL-4–inducible promoter of IgE switch transcripts was replaced by a V_H promoter–IgH intron enhancer and the I_ϵ splice donor site was removed (*7*). In their experiments, a residual frequency of IgE switch recombination was observed, which might be explained by the aberrantly processed IgE switch transcripts reported (*7*). The functional role of switch transcripts in switch recombination remains an object of speculation. The most intriguing speculation is that switch transcripts are part of the switch recombinase, providing the specificity to target distinct switch regions.

Fig. 4. In vivo analysis of Ig class switch. The serum concentrations of IgM^a, IgM^b, $IgG1^a$, and total IgG1 ($IgG1^a$ and $IgG1^b$) of 7-week-old control mice (BALB/c × C57BL/6) and heterozygous mutant s-hMT, hMT, and $\Delta 5's_\gamma 1$ mice were determined by enzyme-linked immunosorbent assay (*4*).



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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 hMTlacO(−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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14. The neomycin probe was a 1550-bp Xho I–Eco RV FRT-neomycin-FRT probe from pFRT2neo (*4*); the $s_\gamma 1$ probe was a 9-kb Ssp I–Eco RI fragment of the γ1 switch region (*18*); and the hMT probe was a 307-bp Xho I–Sal I fragment of the hMT gene-targeting vector and contained only bacterial DNA.
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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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