

I_α exon-replacement mice synthesize a spliced HPRT–C_α transcript which may explain their ability to switch to IgA. Inhibition of switching to IgG in these mice

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Keywords: Ig class switching, gene targeting, germline transcripts

Abstract

Antibody class switching is regulated by transcription of unrearranged C_H genes to produce germline (GL) transcripts which direct the choice of isotype and are required for switching. However, their role is unknown. GL transcripts are initiated at the I exons located upstream of each switch region. Although deletion of the I exon by gene targeting prevents switch recombination to that C_H gene, the I_α exon can be replaced by an entirely different DNA segment, a minigene driven by the phosphoglycerate kinase (PGK) promoter and encoding hypoxanthine phosphoribosyl transferase (HPRT), oriented in the sense direction, without reducing antibody class switching to IgA. To understand why HPRT substitution of the I_α exon does not disrupt switch recombination, we have analyzed the structure of the transcript from the targeted allele in these mice. We identify a spliced transcript in which the HPRT exons are spliced to the C_α gene segments, resulting in a structure similar to normal GL transcripts. The abundance of this transcript is similar to that of the normal α GL RNA. We also demonstrate that switching to the four IgG subclasses in B cells from these mice is reduced in comparison to wild-type mice. We discuss the possibility that the strong PGK promoter inserted at the Ig α locus may interfere with interaction of the promoters for γ GL transcripts with the 3' IgH enhancer.

Introduction

When mature B lymphocytes expressing IgM and IgD on their surface are activated by antigen and accessory signals, they switch to express downstream Ig heavy chain constant region (C_H) genes while maintaining the same expressed variable region. Since the C_H region determines the antibody effector function, class switching allows the humoral immune response to adaptively respond to a variety of different infectious organisms. Antibody class switching is caused by an intra-chromosomal DNA recombination event called class switch recombination (CSR), which occurs within or near 1–10 kb switch regions containing tandemly repeated sequences located 5' of each C_H gene, except C_δ.

Numerous studies have established that transcription of unrearranged C_H genes occurs prior to CSR, producing what are termed germline (GL), or switch, transcripts (1–3). The role(s) of GL transcripts in class switching is unknown. GL

transcripts are initiated at the so-called I exons located upstream of each switch region, transcribed through the switch region and C_H region and polyadenylated near the normal poly(A) site for mature IgH mRNAs (4–6) (Fig. 1A). The I exons are spliced to the C_H exons and the spliced RNAs are exported to the cytoplasm. Although some of the GL transcripts have small open reading frames (5,7), no convincing detection of a translation product from these RNAs *in vivo* has been reported.

Two types of data indicate GL transcripts are essential for CSR. First, there is an excellent correlation between the induction of specific GL transcripts by cytokines and induction of switching to the same antibody class, or isotype. IL-4 induces GL γ1 and ε transcripts and directs switching to IgG1 and IgE. IFN-γ induces GL γ2α transcripts and switching to IgG2a, and transforming growth factor (TGF)-β1 induces GL

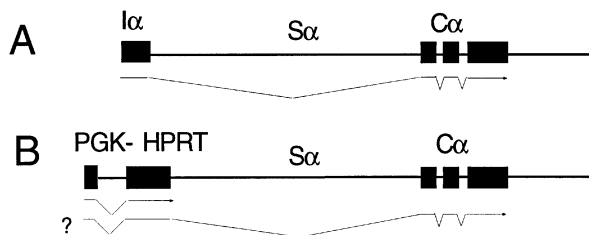


Fig. 1. Diagrams of wild-type and I_{α} knockout Ig C_{α} genes. (A) Wild-type GL α locus, and a simplified transcription map and splicing diagram for GL α transcripts (5,6). (B) $I_{\alpha}^{-/-}$ allele in which a HPRT minigene driven by the PGK promoter and having a SV40 poly(A) signal replaces the I_{α} exon and proximal promoter region (16). Below are illustrated one transcript known to be synthesized from this allele (16) and one potential transcript indicated by ?.

$\gamma 2b$ and α transcripts and switching to IgG2b and IgA. Although cytokines direct switch recombination to particular C_H genes, cytokines are not sufficient to induce switch recombination, which requires the addition of B cell mitogens/activators (reviewed in 8–11).

The second, and stronger, type of evidence is provided by a series of experiments testing the effects of targeted deletions and replacements of the I exons and their upstream regulatory elements. Deletion or replacement of I exons with a neomycin-resistance (*neo^r*) gene placed in the antisense orientation suggested that transcription of GL RNA is required for switch recombination (12,13). Additional experiments indicated that the act of transcription is not sufficient for directing CSR, since replacement of the I_{ϵ} exon by an heterologous strong promoter (a cassette containing the Ig μ intron enhancer and heavy chain V promoter) results in abundant unspliced transcripts but does not direct switch recombination to IgE (14). Subsequently, it was shown that if the $I_{\gamma 1}$ exon is replaced by a metallothionein promoter switching to IgG1 does not occur. However, if the 114 bp segment containing the $I_{\gamma 1}$ splice donor is inserted just downstream of the metallothionein promoter, switching to IgG1 occurs at normal levels and IL-4 treatment is not required (15). Altogether, these data suggest that splicing of the GL transcript is required to target CSR.

Finally, it was found that none of the I exon sequence itself is required for switching, since an heterologous gene, encoding human hypoxanthine phosphoribosyl transferase (HPRT) driven by the strong, constitutively active, phosphoglycerate kinase (PGK) promoter, can be substituted for the I_{α} exon without loss of ability to switch (16). B cells from these mice switch to IgA with lipopolysaccharide (LPS) stimulation alone and addition of TGF- $\beta 1$, which induces GL α transcripts, is not required. Figure 1(B) presents a diagram of the structure of the targeted allele. Although B cells containing the targeted gene were shown to contain abundant human HPRT mRNA, the structure of the primary transcript synthesized from the targeted allele was not evaluated. Thus, it was unknown if transcription proceeds through the switch region or instead terminates after the HPRT gene. Although the HPRT minigene consists of two exons, it was not clear how splicing of an intron located upstream of the switch region would contribute to CSR. To attempt to learn why HPRT substitution for the I_{α} exon does not disrupt CSR, we have examined the structure

of the RNA transcribed from the targeted C_{α} genes in the I_{α} -deficient HPRT-replacement mice. We also examine the effect of the HPRT substitution at the I_{α} locus on switching to IgG in these mice.

Methods

Mice

Generation of mice with the HPRT minigene replacement of the I_{α} exon has been described previously (16). The HPRT minigene consists of a fusion of exons 1 and 2, the intron between exons 2 and 3, then a fusion of exons 3–9 and a polyadenylation signal from SV40 (17). The promoter is from the PGK gene. Mice were kept in the animal facility of the University of Massachusetts Medical School and were used at 8–16 weeks of age. The same number and ratio of male and female animals for knockout and wild-type mice (at least four in each group) was used in each experiment.

Isolation and culture of splenic B cells

T-depleted splenic cells were obtained as previously described (18) with some modifications. A single-cell suspension of spleen cells was prepared and red blood cells were removed by hypotonic lysis. T cells were depleted by incubation with a cocktail of rat mAb against Thy-1.2 (HO-13.4), Thy-1 (J1Jo.10), CD4 (GK1.5) and CD8 (3.168.3), followed by addition of a mouse anti-rat κ -chain mAb (MAR 18.5) and rabbit complement. All mAb were used at optimal concentrations determined in preliminary experiments. Resting B cells were obtained by separation on a discontinuous density gradient consisting of 50, 60, 66 and 70% Percoll (Pharmacia, Piscataway, NJ) in HBSS, and harvesting the cells from the 66/70 interface. Cells were washed 3 times in RPMI 1640 medium before use.

B cells were cultured ($0.5\text{--}1 \times 10^6/\text{ml}$) in RPMI 1640 medium supplemented with 10% defined FCS (HyClone, Logan, UT), 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 200 U/ml penicillin, 200 mg/ml streptomycin, 0.1 mg/ml kanamycin sulfate (Gibco, Grand Island, NY) and 50 μM 2-mercaptoethanol (Sigma, St Louis, MO). Both B cell activators and cytokines were added at the initiation of culture and were used at the following concentrations: LPS (50 $\mu\text{g}/\text{ml}$) from *Escherichia coli* 055:B5 (Sigma), dextran-conjugated anti-mouse IgD mAb ($\alpha\delta$ -dex) (3 ng/ml) (19), provided by C. M. Snapper (Uniformed Services University of the Health Sciences, Bethesda, MD), TGF- $\beta 1$ (2 ng/ml) (R & G Systems, Minneapolis, MN), mouse rIL-4 (12,000 U/ml), made in a baculovirus expression system and a gift from W. E. Paul (National Institutes of Health, Bethesda, MD), mouse rIFN- γ (10 U/ml), obtained from J. Stevens (American Cancer Society, Atlanta, GA), and mouse rIL-5 (18.7 ng/ml) and mouse rIL-10 (1 ng/ml) (both from PharMingen, San Diego, CA). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2 either in duplicate in 24-well flat-bottom plates for 4 days for induction of class switching or in 25 cm^2 flasks for 1–3 days for RNA isolation.

Analysis of slg expression

Class switch was measured by flow cytometric analysis of mlg expression after stimulation of spleen B cells *in vitro* for

4 days with B cell activators and cytokines. Cells were pelleted and resuspended in 50 μ l of PBS containing 1.5% FCS and 0.2% NaN_3 . To the cell suspension was added 50 μ l mixture of an affinity-purified goat anti-mouse IgM-FITC (1:50) with either 1:50 phycoerythrin (PE)-conjugated goat anti-mouse IgA, 1:50 PE-conjugated F(ab) $_2$ fractions of goat anti-mouse IgG1, anti-IgG2a, anti-IgG2b or anti-IgG3 (Southern Biotechnology Associates, Birmingham, AL). After 60 min on ice, cells were washed 3 times with PBS/1.5% FCS/0.2% NaN_3 and resuspended in 500 μ l of the same solution. Then 200 μ l of 4% paraformaldehyde in PBS was added and cells were vortexed at room temperature for 5 min. Cells were pelleted and resuspended in 1 ml PBS/1.5% FCS/0.2% NaN_3 and analyzed. Cells were analyzed on a FACScan (Becton Dickinson, Mountain View, CA), and forward and side scatter were set to include live lymphocytes. Staining controls were B cell lines expressing either sIgM or sIgA. The IgM $^+$ cell line stains more brightly than splenic B cells treated with LPS, thus explaining the apparent lack of sIgM on LPS-treated splenic B cells. Data were plotted using WinList 3.0 (Verity Software House, Topsham, ME). The gates were set individually for each isotype to maximize the distinction between cells positive or negative for the different isotypes.

RNA isolation

Total RNA was isolated from cultured cells using the Ultraspec RNA isolation system (Biotecx, Houston, TX) according to the manufacturer's protocol.

RT-PCR

Genomic DNA was removed from total cell RNA by treating RNA (800 μ g/ml) with RNase-free DNase I (320 U/ml) (Boehringer Mannheim, Indianapolis, IN) in a buffer containing RNase inhibitor (2000 U/ml) (Promega, Madison, MI), 100 mM NaCl, 10 mM MgCl_2 and 1 mM DTT at 37°C for 30 min followed by phenol extraction and ethanol precipitation. For reverse transcription, 20 μ g of RNA was mixed with 800 μ g random hexanucleotide primer (Boehringer Mannheim), heated at 65°C for 5 min and chilled on ice. Then 800 U MuLV reverse transcriptase was added in a final volume of 200 μ l containing 1 \times buffer, 800 U RNase inhibitor and 1 mM each of dNTPs, and the mixture was incubated at 37°C for 60 min. Random primers were removed by differential precipitation with 1 volume of ethanol in the presence of 2.5 M ammonia acetate at room temperature for 10 min and the cDNA was dissolved in 200 μ l distilled H_2O .

PCR was performed using an Expand High Fidelity PCR system (Boehringer Mannheim). The reaction mixture was heated at 95°C for 10 min before addition of enzyme (hot start); followed by 40 cycles of 94°C for 45 s, 55°C for 60 s and 72°C for 90 s, and a final incubation at 72°C for 10 min. A portion (20 μ l) of PCR product was analyzed on a 2% agarose gel and stained with 1 μ g/ml ethidium bromide for 30 min at room temperature.

For detection of GL α RNA, a 239 bp fragment was amplified using an upstream primer (5'-GACATGATCACAGGCA-CAAGGC-3'), derived from the I exon sequences and a downstream primer (5'-TTCCCCAGGTCACATTCATCGT-3'), derived from $C_{\alpha}1$ exon sequences (16). For hHPRT- C_{α} RNA, a 887 bp fragment was amplified using an upstream primer

(5'-CTTTGCTTTCCTTGGTCAGG-3'), which is derived from the human HPRT mRNA sequence (20) (HUMHPRT, GenBank NID g184349) and contains, within its most 3' of 9 nucleotides, three mismatches with mouse HPRT mRNA (MUSHPRT, GenBank NID g193984), and a downstream primer (5'-GGGAAG-GTGTTCACTGTGACTT-3') which spans the junction of $C_{\alpha}2$ and $C_{\alpha}3$ exon (MUSIALPHA, GenBank NID g286082).

Construction of plasmids

Standard molecular cloning techniques were followed in construction of the following plasmids (maps in Fig. 3A).

phHPRT- C_{α} . RT-PCR products were extracted with phenol and ethanol precipitated. The terminus was polished by treatment with T4 DNA polymerase and DNA polymerase I Klenow fragment. The DNA fragment was purified by agarose gel electrophoresis and cloned by blunt-end ligation into the *HincII* site of pBluescript KS(-) (Stratagene, La Jolla, CA). The resultant clones were mapped by restriction enzyme digestion and the selected ones were sequenced. A 547 bp *HindIII*-*PstI* fragment was isolated from one of the type I clones, and subcloned between *HindIII* and *PstI* sites in pBluescript KS(-). The resulting plasmid was linearized with *XhoI* and transcribed *in vitro* using T7 RNA polymerase.

pl α - C_{α} . RT-PCR products of the GL α RNA was treated as above and digested with *PstI*. A 199 bp fragment was gel purified and cloned into gel-purified, *HindIII* (blunt-ended) and *PstI*-cut pBS(+)/BH1.4 plasmid (5). The insert of the resulting plasmid comprises the I_{α} exon joined to $C_{\alpha}1$ exon and was verified by sequencing. This plasmid was then cut with *KpnI*, gel purified to remove the 5' part of the insert and re-circularized. The final plasmid was linearized with *EcoRI* and transcribed *in vitro* using T3 RNA polymerase.

Preparation of RNA probe

The reaction mixture (in 10 μ l) for *in vitro* transcription consisted of 1 μ l RNA polymerase (Stratagene), 1 μ g linearized plasmid, 100 μ Ci [^{32}P]UTP (800 Ci/mM; NEN, Boston, MA), 10 mM DTT, 100 μ g/ml BSA, 1 mM each of ATP, GTP and CTP, 40 U RNase inhibitor, and 1 \times buffer from the manufacturer of the enzyme. The mixture was incubated at 37°C for 2 h followed by addition of 10 U RNase-free DNase I and incubation at 37°C for another 10 min. The reaction was stopped by addition of 30 μ g Proteinase K plus 1% SDS and incubation at 37°C for 2 h. After phenol extraction, the unincorporated [^{32}P]UTP was removed by gel filtration on a spin column.

RNase protection assay

The method of Zinn *et al.* (21) was followed with some modifications. 20 μ g RNA consisting of a mixture of 10 μ g total spleen RNA and 10 μ g yeast RNA, or 20 μ g yeast RNA alone was mixed with 1 $\times 10^6$ c.p.m. of *in vitro* transcribed RNA probe and dried down. The RNA pellet was dissolved in 50 μ l hybridization buffer, heated at 85°C for 5 min and incubated at 45°C overnight. Then 450 μ l of digestion buffer containing nuclease T1 (200 U/ml) and nuclease P1 (20 μ g/ml) (Boehringer Mannheim) was added, and incubation continued at 30°C for 60 min. The reaction was terminated

by addition of 70 μ g Proteinase K plus SDS to 0.5% final concentration and incubation at 50°C for 30 min. Nucleic acids were extracted once with phenol and ethanol precipitated on ice for 30 min with 30 μ g yeast RNA added as carrier. The pellet was dissolved in 15 μ l loading buffer, heated at 85°C for 5 min and electrophoresed on a 4% polyacrylamide/8 M urea gel at 200 V for 4 h. Gels were dried, exposed to X-ray film and signals were quantitated using a Molecular Dynamics Personal Densitometry SI and ImageQuant version 1.1 software.

Results

Detection and sequencing of spliced HPRT-C α GL transcripts

To begin to examine the structure of the RNA transcribed from the HPRT-C α locus, we first determined whether transcription occurs across the entire HPRT-S α -C α segment by performing a series of RT-PCR across the locus. Several pairs of primers spanning the human HPRT gene, S α segment and C α gene were used to amplify fragments from template cDNA transcribed from LPS-activated splenic B cells. LPS induces switching to IgA in the I α ^{-/-} B cells (16). Products were obtained from sets of overlapping primers spanning the entire locus, indicating that transcription occurs across the entire HPRT-S α -C α segment, although no PCR products corresponding to full length transcripts were detected (data not shown).

Since these data suggested that the entire locus is transcribed, we hypothesized that a transcript containing HPRT exons and C α exons might be spliced to produce an RNA with a structure analogous to that of GL transcripts (see Fig. 1B). To examine this possibility, we used RT-PCR to attempt to detect a spliced RNA containing HPRT and C α sequences. A 5' primer for the human HPRT minigene, which differs from mouse HPRT, and a 3' primer complementary to sequences spanning the junction of the C α 2 and C α 3 exons were used to amplify cDNA from LPS + TGF- β 1-treated splenic B cells. Figure 2(A) shows that the RT-PCR primed by the HPRT + C α primers yielded two fragments, 887 and 780 bp long, from the I α knockout B cells and none from wild-type B cells. An RT-PCR performed in parallel using wild-type activated splenic B cell RNA and primers derived from the I α and C α 1 exons produced a 239 bp fragment, but no products from the I α ^{-/-} B cell RNA. Control reactions performed without reverse transcriptase (-) yielded no products. These results suggest that splicing occurs between the HPRT and C α sequences in the I α knockout B cells. The finding of two different RT-PCR products suggests that splicing occurs between the HPRT and C α segments at two different sites.

To determine the nature of the two products amplified by RT-PCR from the I α ^{-/-} mice, the two fragments were cloned and analyzed by nucleotide sequencing. The sequences indicate that two different splice donors within the HPRT gene are used to splice to the normal splice acceptor of exon 1 of the C α gene (Fig. 2B). The HPRT sequence in the type II clone ends at nucleotide 649 at which point it is spliced to the C α 1 exon. The type I sequence continues to nucleotide 755 of the HPRT sequence. Consensus splice donor sequences (GT) are found immediately 3' to both of the splice

donor sites used in HPRT. In Fig. 2(B), upper case letters indicate nucleotides in the PCR product and lower case letters indicate nucleotides which are spliced out. These results demonstrate that the I α ^{-/-} mice synthesize a spliced RNA that has a structure very similar to GL α transcripts.

Quantitation of the spliced HPRT-C α RNA by RNase protection experiments

If the spliced HPRT-C α transcripts in the I α knockout mice are present in adequate quantities, this might explain the ability of B cells from these mice to undergo switching to IgA. To quantitate the amount of the spliced RNA in cells, we performed RNase protection assays on total cell RNA from splenic B cells induced to undergo switching for 2 days by LPS in the presence or absence of cytokines, using the cloned I α -C α and HPRT-C α segments as probes (Fig. 3A).

As shown in Fig. 3(B), the I α -C α probe (left half of gel) protects two specific RNA fragments, one corresponding to the 127 nucleotide long C α segment which arises from mature α mRNA. As expected, it protects no other fragment in the I α ^{-/-} B cells, but protects a 384 nucleotide fragment arising from GL α RNA (I α -C α) in wild-type cells treated with TGF- β 1 plus LPS. The larger of the two HPRT-C α PCR products (type I) was used as a probe in the right half of the gel. This probe protects a 187 nucleotide fragment arising from the abundant HPRT mRNA in the I α ^{-/-} cells (16). The HPRT-C α probe also protects a 360 nucleotide fragment (C α) from mature α mRNA in both wild-type and mutant cells. In addition, the HPRT-C α probe protects a 547 nucleotide fragment only in the I α ^{-/-} B cells, whose size matches the predicted size of the spliced HPRT-C α RNA species. Interestingly, densitometry showed that the amount of this fragment appears to be ~3-fold more than the amount of the I α -C α fragment, indicating that the quantity of the HPRT-C α RNA is equal to, if not more than, that of GL α RNA in cells induced to undergo switching to IgA. This estimation was made taking into account the difference in specific activities of the probes, 20% greater for the I α -C α probe, and the difference in sizes of the protected fragments, 42% greater for the HPRT-C α fragment. By comparing the signals from the HPRT-C α fragment and the 187 nucleotide HPRT fragment, correcting for the different lengths of the protected fragments, we estimate that ~1.2% of the transcripts from the I α ^{-/-} allele continue through the S α and C α regions, and are spliced to form the HPRT-C α RNA. These data suggest that B cells from these I α knockout mice switch well because they produce a RNA that corresponds in its structure and its quantity to that of normal GL α RNA.

Switching to other isotypes in the I α knock-out mice

It has been hypothesized that the promoters of individual GL transcripts compete with each other for access to a locus control region (LCR) which is required for their transcription and perhaps also for switching to that isotype (22). Preliminary evidence for competition between C μ genes for induction of their GL transcripts has been obtained from mice with a targeted deletion of their I γ 1 exon (23). B cells from these mice when stimulated with LPS + IL-4 switch ~2 times more frequently to IgE than their wild-type littermates, whereas switching to IgG2b is not increased. These data suggest that

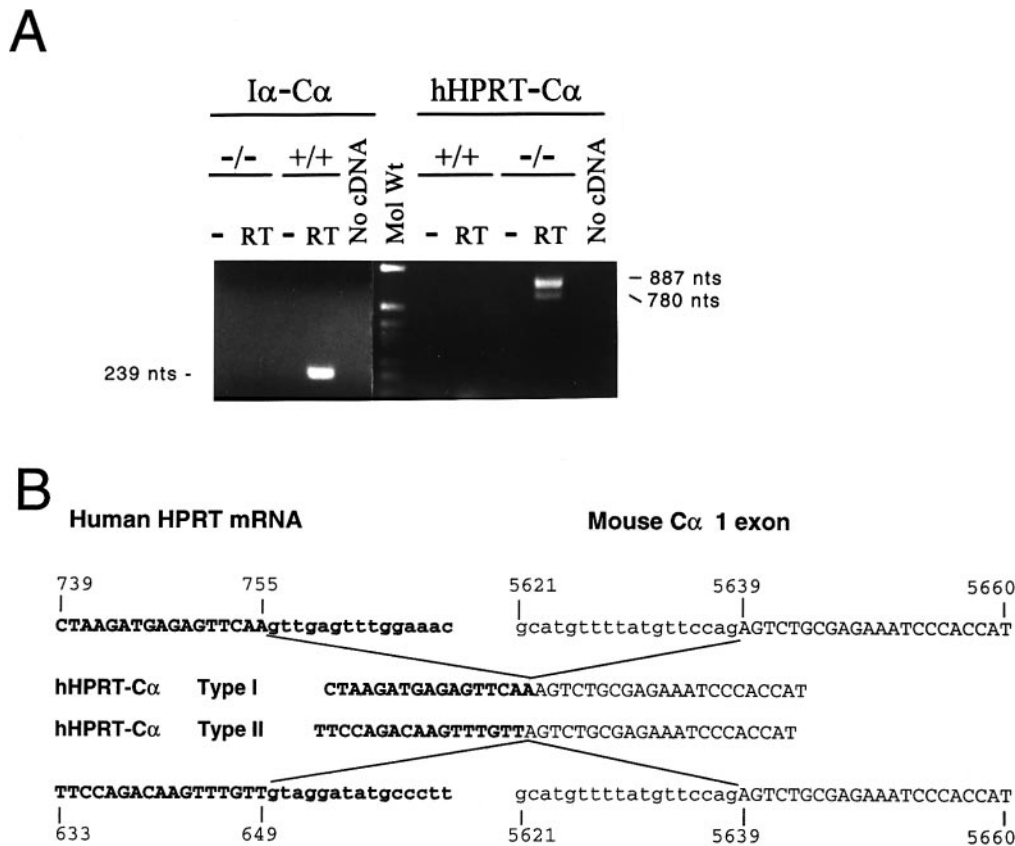


Fig. 2. Detection and sequencing of Ig α locus GL transcripts in I α exon-deficient homozygous mice by RT-PCR. (A) RT-PCR. Resting splenic B cells were isolated from four each of wild-type (+/+) and I α exon-deficient homozygous mice (-/-), and stimulated with LPS (50 μ g/ml) and TGF- β 1 (2 ng/ml) for 3 days. Total RNA was isolated from the stimulated cells, transcribed into cDNA with MuLV reverse transcriptase (RT) or mock transcribed in the absence of the reverse transcriptase (-), and PCR-amplified using primer pairs specific for the mouse I α and C α 1 exons (I α -C α), or specific for chimeric RNA containing human HPRT mRNA spliced to mouse C α RNA (hHPRT-C α). The amplified DNA fragments were electrophoresed on a 2% agarose gel and stained with ethidium bromide. PCR reactions with no *in vitro* transcribed cDNA added were included as additional controls. The mol. wt. marker is DNA marker VI (Boehringer-Mannheim). (B) Sequence of hHPRT-C α clones at the splice junction. The relevant human HPRT mRNA (bold) and mouse C α 1 exon sequences are shown at both the top and the bottom for comparison. The numbering is according to the GenBank loci HUMHPRT for human HPRT mRNA and MUSIALPHA for the mouse C α 1 exon. The nucleotides present in the HPRT-C α cDNA are shown in capital letters and those spliced out are shown in lowercase letters. Two sequences (type I and type II) with different splice donor sites are shown in the middle. Each was represented in three sequenced clones. The control I α -C α segment cloned from the wild-type mice used the identical C α 1 splice acceptor (data not shown). This is also the normal splice acceptor for mature α mRNA.

switching to IgG1 may compete with switching to IgE in B cells treated with LPS + IL-4.

If competition exists among the promoters of GL transcripts, then replacement of the I α gene with the HPRT minigene, which is driven by the strong constitutively active PGK promoter, might inhibit switching to other isotypes. The presence of abundant HPRT mRNA in B cells from these mice, relative to the GL α RNA in wild-type B cells, suggests that the HPRT promoter is much stronger than the GL α promoter (Fig. 3) (16).

We examined the ability of B cells from the I α ^{-/-} mice to switch to IgG isotypes in comparison with B cells from wild-type mice. Splenic B cells from wild-type and I α ^{-/-} mice were induced to switch by LPS treatment with the addition of various cytokines to induce switching to IgA or to IgG. On day 4, cells were stained and analyzed for sIg expression by flow cytometry. As previously demonstrated, switching to IgA by the targeted B cells can be induced by LPS in the absence

of TGF- β 1. Although the percentages of sIgA⁺ cells are quite low at day 4, they are greater than in wild-type cultures treated with LPS + TGF- β 1 (Fig. 4) (16). If one adds the cytokine IL-4, switching to IgA is increased in both wild-type and mutant B cells (Figs 4 and 5). The mechanism of induction of increased IgA switching by IL-4 is unknown and is not due to an effect on GL α transcripts (24–26).

When surface IgG expression was measured in this same experiment, switching to all four IgG subclasses was found to be reduced in the I α ^{-/-} mice in comparison to the wild-type mice by 45–93% (Figs 4 and 5). Figure 4 illustrates some of the flow cytometry data from an experiment in which LPS \pm cytokines were used to induce switching to IgA and to IgG. Figure 5 (left panels) summarizes the data. We considered the possibility that the inhibitory effect may depend on the activator used for inducing class switching. However, when we tested the effect of inducing switching with anti- δ dextran

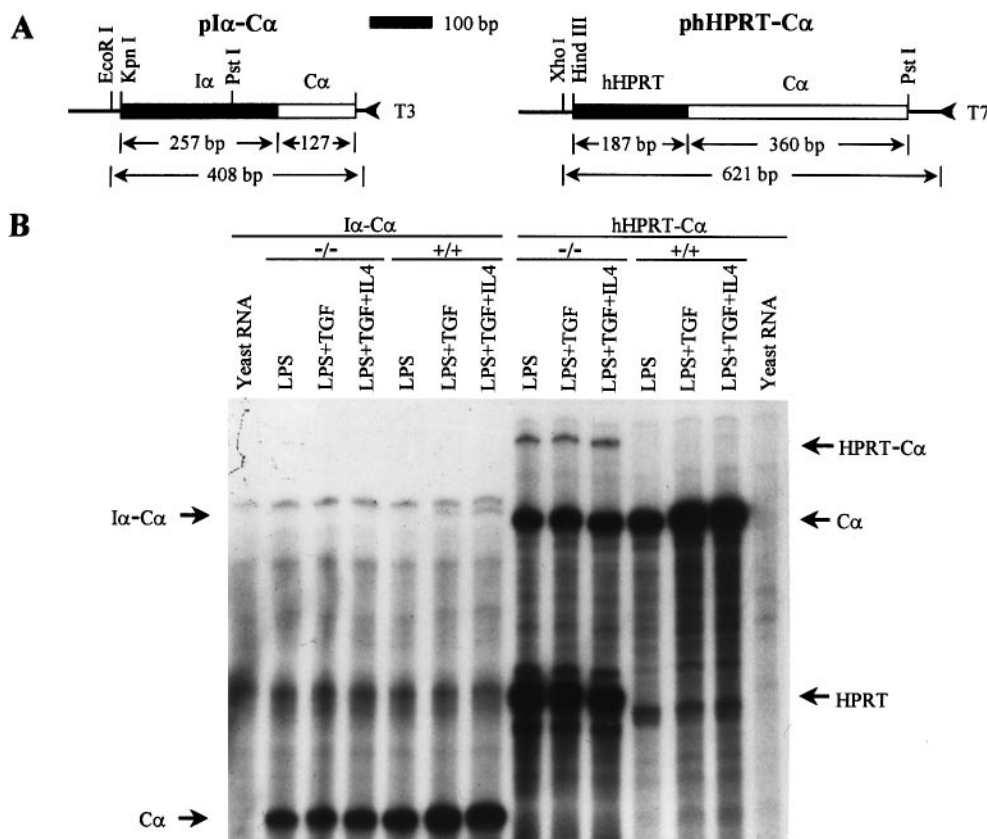
42 Spliced HPRT- C_α RNA in I_α exon-deficient mice

Fig. 3. Expression of Ig α locus GL transcripts in wild-type and I_α exon-deficient mice. (A) Diagram of DNA templates for production of *in vitro*-transcribed RNA probe. Only restriction sites relevant for this study are shown. The amount of labeled nucleotide incorporated into the I_α - C_α probe was 20% greater than the amount incorporated into the HPRT- C_α probe in this experiment. (C) RNase protection assay using the I_α - C_α RNA probe on the left half of the gel and the HPRT- C_α type I RNA probe on the right half. Equal amounts of c.p.m. (1×10^6) were used in each lane. Resting splenic B cells were isolated from each of wild-type ($+/+$) and $I_\alpha^{-/-}$ mice, and were stimulated as indicated for 2 days. The I_α - C_α probe protects a C_α fragment (127 nucleotides) from mature α mRNA and an I_α - C_α RNA fragment (384 nucleotides) in wild-type cells stimulated with LPS + TGF- β 1. The hHPRT- C_α probe protects fragments from the HPRT mRNA (187 nucleotides), the mature α mRNA (C_α) (360 nucleotides) and from the spliced HPRT- C_α RNA (547 nucleotides).

\pm IL-5 \pm LPS \pm other cytokines (26–29), we again found that the $I_\alpha^{-/-}$ B cells showed reduced switching to the two IgG subclasses tested, IgG1 and IgG3, in comparison to wild-type B cells (Fig. 5, right panels). We have not tested IgE switching in these mice. In conclusion, insertion of an HPRT gene driven by the PGK promoter (PGK-HPRT) at the I_α locus on both chromosomes inhibits switching to all IgG subclasses.

Discussion

Role of GL transcripts in CSR

In this manuscript we demonstrate that a spliced RNA that has the structure of a normal GL transcript is synthesized in B cells in which a PGK-HPRT minigene replaces the I_α exon. The fact that B cells having this chimeric HPRT- C_α transcript switch well to IgA indicates that the sequence of the I exon in the GL transcripts is probably not important for CSR. This is consistent with the lack of conservation of I_α exon sequences between species (30). Thus, any proteins that might bind the I_α exon or any polypeptide that might be translated from the

small open-reading frame of the GL α transcript (5) are not essential for class switching. These data are consistent with a recent report that the I_γ 2b exon can be replaced with an expressed PGK-*neo^r* gene without disruption of CSR to IgG2b (31).

The available data suggest, however, that the general structure of the GL transcript may be essential for class switching. A targeted mutation of the I_γ 2b exon in which it is replaced with a *neo^r* gene driven by the Polyoma virus enhancer/Herpes virus thymidine kinase promoter in the *anti-sense* orientation completely inhibits CSR to the γ 2b gene (13). This suggests that transcription through the switch region may be necessary. However, transcription through the switch region is not sufficient, because transcription in the absence of splicing of the transcript does not direct CSR to that C_H gene (14,15).

Although the data suggest that a transcript of any sequence which is spliced across the switch region may be sufficient for CSR, the data do not allow one to determine the function of splicing. One possibility is that the splicing machinery

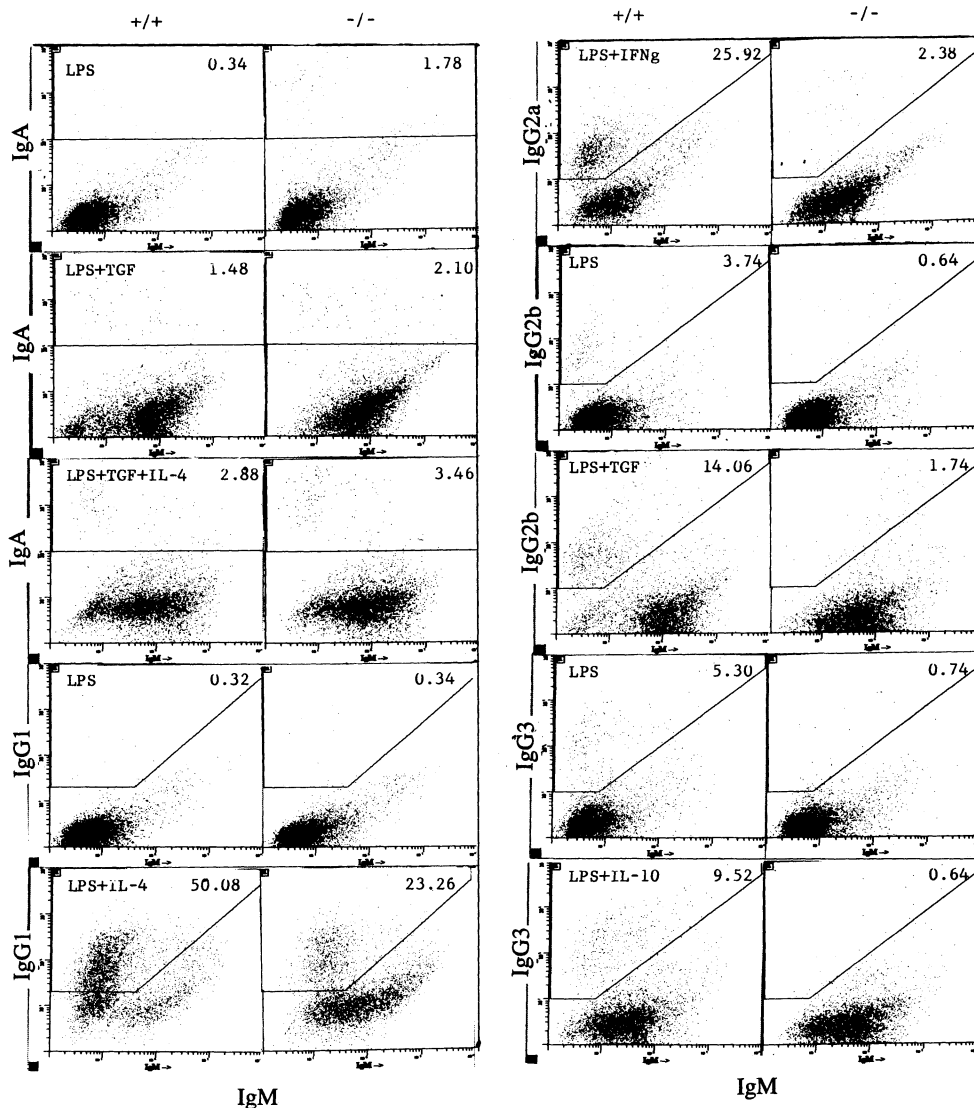


Fig. 4. Normal switching to IgA but reduced switching to IgG isotypes in I α exon knockout mice. Resting splenic B cells from six wild-type (+/+) and six I α exon-deficient homozygous mice (-/-) were pooled and stimulated with LPS and various cytokines for 4 days, and mIg expression was quantitated by flow cytometry analysis as detailed in Methods.

contributes to the process of switch recombination. Alternatively, splicing results in cutting of the primary transcript, perhaps thereby allowing the intron RNA which contains the switch region sequences to remain associated with the genome (32–34). The DNA–RNA complex might somehow serve as a target for enzymes involved in CSR or otherwise facilitate some step required for switch recombination.

Mechanism of inhibition of IgG expression in I α HPRT-replacement mice

Insertion of the PGK-*neo^r* gene by gene targeting into a number of different loci has been shown to inhibit expression of nearby genes that are regulated, or thought to be regulated, by the same LCR. Examples are insertions into the granzyme B gene (35) and into a regulatory gene for myogenesis, *MRF4* (36). It has also been shown that replacement of elements

within the LCR for the β -globin gene cluster with a PGK-*neo^r* gene inhibits expression of several genes within the β -globin gene cluster (37,38). If the same elements of the β -globin LCR are simply deleted from the chromosome, the effects on expression of the genes are minimal. A similar finding has been obtained for the 3' Ig κ enhancer. Replacement of the 3' Ig κ enhancer with the PGK-*neo^r* gene inhibits development of κ^+ B cells to a greater extent than does deletion of the 3' κ enhancer (39).

Altogether these data suggest that insertion of a PGK-*neo^r* gene into one gene within a gene cluster might disrupt interactions between the promoters of other genes within that cluster with enhancer elements of the LCR and that insertion of the PGK-*neo^r* gene into the LCR itself disrupts regulation of many of the genes of the cluster. In agreement with this hypothesis, Cogne *et al.* (22) found that insertion of a PGK-

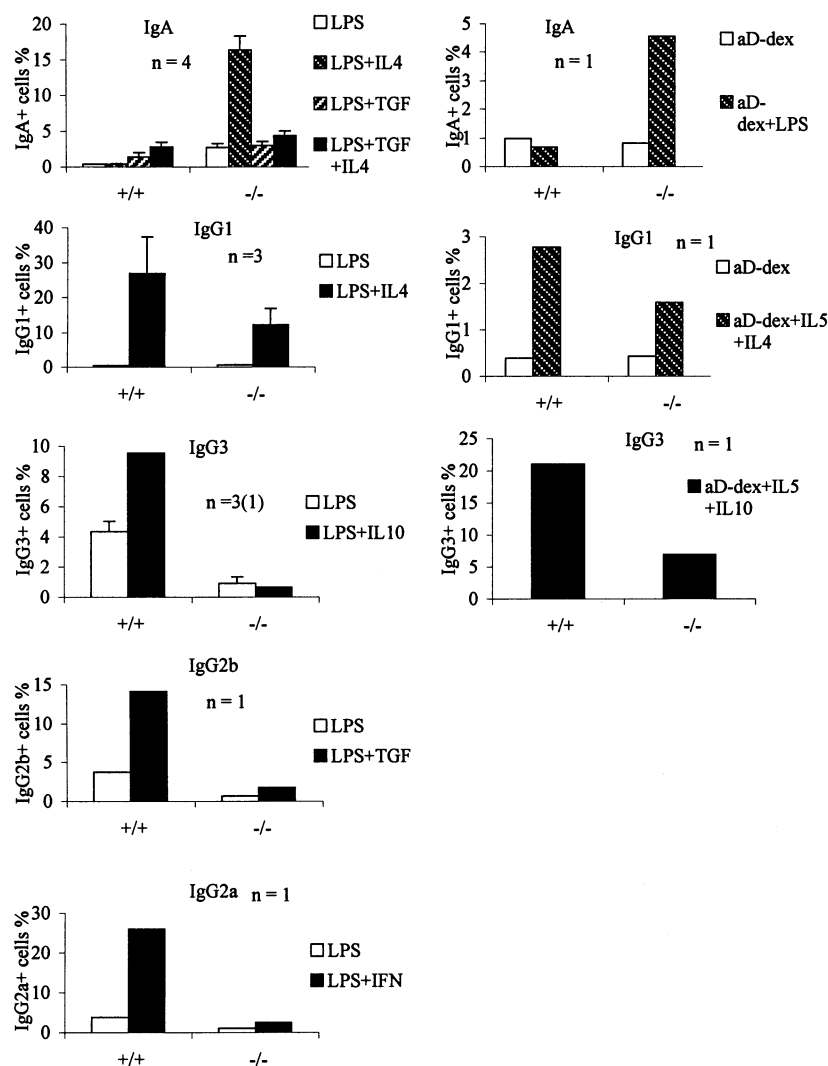
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Fig. 5. Summary of flow cytometry data measuring slg expression in splenic B cells induced to undergo CSR. Resting splenic B cells from wild-type (+/+) and I_{α} exon-deficient mice (-/-) were stimulated with either LPS and/or dextran-conjugated anti-mouse IgD mAb (aD-dex) plus various cytokines for 4 days, and mlg expression was quantitated by flow cytometry analysis. *n* indicates the number of experiments performed. When three experiments were performed, the means + SE values are shown.

neof gene into the IgH 3' enhancer reduces the levels of GL transcripts for several, but not all, isotypes. These data further indicate that the 3' IgH enhancer is involved in activating transcription of several isotypes and thus may function as a LCR (22,40). Thus, we hypothesize that the inhibition of switching to all four IgG subclasses in B cells from the I_{α} -HPRT replacement mice may be because the PGK-HPRT gene may be able to sequester the LCR, thereby disrupting interactions of the promoters for these GL transcripts with the IgH 3' enhancer and/or other regulatory sequences and that this inhibition results in inhibition of CSR.

The inhibition of switching to other isotypes by replacement of the I_{α} exon with the PGK-HPRT gene may be specific to the PGK promoter. In all examples mentioned above in which insertion of the *neof* gene inhibited expression of several genes, the inserted *neof* gene was driven by the PGK pro-

motor. Three examples have been reported in which a *neof* gene driven by the Polyoma enhancer/Herpes virus thymidine kinase promoter replaced an I exon (13,14,23). B cells from these mice showed no alteration in ability to switch to any of several isotypes, except for the one corresponding to the targeted I exon. However, since in these three cases the *neof* gene was inserted in antisense polarity, it is possible that the lack of inhibition could be due to the polarity of the *neof* gene.

In conclusion, B cells from mice having the I_{α} exon replaced by an expressed HPRT minigene in the sense orientation synthesize a spliced RNA with the HPRT coding sequences replacing the I_{α} exon. This RNA appears to function similarly to the *bona fide* GL α transcript in promoting CSR. Replacement of the I_{α} exon with the HPRT gene, which is driven by the strong PGK promoter, results in decreased CSR to all four IgG subclasses, possibly due to disruption of interactions

between the promoters for GL γ transcripts and the 3' IgH enhancer.

Acknowledgements

We thank Drs W. E. Paul (Office of AIDS Research, NIH, Bethesda MD) for mouse IL-4, C. M. Snapper (Uniformed Services University of Health Sciences, Bethesda MD) for anti- δ dextran and the American Cancer Society (Atlanta, GA) for IFN- γ . We thank Dr Carol Schrader, Mr Ching Hung Shen and Ms Bidisha Dasgupta for helpful comments on the manuscript. The research was supported by a grant from the National Institutes of Health AI23283 to J. S. Its contents are solely the responsibility of the authors and do not necessarily represent the views of the NIH.

Abbreviations

| | |
|------|---|
| CSR | class switch recombination |
| GL | germline |
| HPRT | hypoxanthine phosphoribosyl transferase |
| LCR | locus control region |
| LPS | lipopolysaccharide |
| PE | phycoerythrin |
| PGK | phosphoglycerate kinase |
| TGF | transforming growth factor |

References

- 1 Stavnezer-Nordgren, J. and Sirlin, S. 1986. Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO J.* 5:95.
- 2 Lutzker, S., Rothman, P., Pollock, R., Coffman, R. and Alt, F. W. 1988. Mitogen- and IL-4-regulated expression of germline Ig γ 2b transcripts: evidence for directed heavy chain class switching. *Cell* 53:177.
- 3 Severinson, E., Fernandez, C. and Stavnezer, J. 1990. Induction of germ-line immunoglobulin heavy chain transcripts by mitogens and interleukins prior to switch recombination. *Eur. J. Immunol.* 20:1079.
- 4 Lutzker, S. and Alt, F. W. 1988. Structure and expression of germline immunoglobulin γ 2b transcripts. *Mol. Cell. Biol.* 8:1849.
- 5 Radcliffe, G., Lin, Y.-C., Julius, M., Marcu, K. and Stavnezer, J. 1990. Structure of germline immunoglobulin α heavy-chain RNA and its location on polysomes. *Mol. Cell. Biol.* 10:382.
- 6 Leberman, D. A., Nomura, D. Y., Coffman, R. L. and Lee, F. D. 1990. Molecular characterization of germ-line immunoglobulin A transcripts produced during transforming growth factor type β -induced isotype switching. *Proc. Natl Acad. Sci. USA* 87:3962.
- 7 Bachl, J., Turck, C. W. and Wabl, M. 1996. Translatable immunoglobulin germ-line transcript. *Eur. J. Immunol.* 26:870.
- 8 Coffman, R. L., Leberman, D. A. and Rothman, P. B. 1993. Mechanism and regulation of immunoglobulin isotype switching. *Adv. Immunol.* 54:229.
- 9 Snapper, C. M. and Finkelman, F. D. 1993. Immunoglobulin class switching. In Paul, W. E., ed., *Fundamental Immunology*, 3rd edn, p. 837. Raven Press, New York.
- 10 Stavnezer, J. 1996. Antibody class switching. In Dixon, F., ed., *Advanced Immunology*, 61:79. Academic Press, San Diego.
- 11 Stavnezer, J. 1996. Immunoglobulin class switching. *Curr. Opin. Immunol.* 8:199.
- 12 Jung, S., Rajewsky, K. and Radbruch, A. 1993. Shutdown of class switch recombination by deletion of a switch region control element. *Science* 259:984.
- 13 Zhang, J., Bottaro, A., Li, S., Stewart, V. and Alt, F. W. 1993. A selective defect in IgG2b switching as a result of targeted mutation of the I γ 2b promoter and exon. *EMBO J.* 12:3529.
- 14 Bottaro, A., Lansford, R., Xu, L., Zhang, J., Rothman, P. and Alt, F. W. 1994. I region transcription (*per se*) promotes basal IgE class switch recombination but additional factors regulate the efficiency of the process. *EMBO J.* 13:665.
- 15 Lorenz, M., Jung, S. and Radbruch, A. 1995. Switch transcripts in immunoglobulin class switching. *Science* 267:1825.
- 16 Harriman, G. R., Bradley, A., Das, S., Rogers-Fani, P. and Davis, A. C. 1996. IgA class switch in I α exon deficient mice. Role of germline transcription in class switch recombination. *J. Clin. Invest.* 97:477.
- 17 Matzuk, M. M., Finegold, M. J., Su, J. G., Hsueh, A. J. and Bradley, A. 1992. Alpha-inhibin is a tumour-suppressor gene with gonadal specificity in mice. *Nature* 360:313.
- 18 Lin, S. C. and Stavnezer, J. 1996. Activation of NF- κ B/Rel by CD40 engagement induces the mouse germline immunoglobulin C γ 1 promoter. *Mol. Cell. Biol.* 16:4591.
- 19 McIntyre, T. M., Klinman, D. R., Rothman, P., Lugo, M., Dasch, J. R., Mond, J. J. and Snapper, C. M. 1993. Transforming growth factor- β 1 selectively stimulates immunoglobulin G2b secretion by lipopolysaccharide-activated murine B cells. *J. Exp. Med.* 177:1031.
- 20 Jolly, D. J., Okayama, H., Berg, P., Esty, A. C., Filpula, D., Bohlen, P., Johnson, G. G., Shively, J. E., Hunkapillar, T. and Friedmann, T. 1983. Isolation and characterization of a full-length expressible cDNA for human hypoxanthine phosphoribosyl transferase. *Proc. Natl Acad. Sci. USA* 80:477.
- 21 Zinn, K., DiMaio, D. and Maniatis, T. 1983. Identification of two distinct regulatory regions adjacent to the human β -interferon gene. *Cell* 34:865.
- 22 Cogne, M., Lansford, R., Bottaro, A., Zhang, J., Gorman, J. and Alt, F. W. 1994. A class switch control region at the 3' end of the immunoglobulin heavy chain locus. *Cell* 77:737.
- 23 Jung, S., Siebenkotten, G. and Radbruch, A. 1994. Frequency of immunoglobulin E class switching is autonomously determined and independent of prior switching to other classes. *J. Exp. Med.* 179:2023.
- 24 Kunimoto, D. Y., Harriman, G. R. and Strober, W. 1988. Regulation of IgA differentiation in CH12LX cells by lymphokines. IL-4 induces membrane IgM-positive CH12LX cells to express membrane IgA and IL-5 induces membrane IgA-positive CH12LX cells to secrete IgA. *J. Immunol.* 141:713.
- 25 Shockett, P. and Stavnezer, J. 1991. Effect of cytokines on switching to IgA and α germline transcripts in the B lymphoma I. 29 μ : transforming growth factor- β activates transcription of the unrearranged C α gene. *J. Immunol.* 147:4374.
- 26 McIntyre, T. M., Kehry, M. R. and Snapper, C. M. 1995. Novel *in vitro* model for high-rate IgA class switching. *J. Immunol.* 154:3156.
- 27 Mandler, R., Chu, C. C., Paul, W., Max, E. E. and Snapper, C. M. 1993. Interleukin 5 induces S μ -Sy1 DNA rearrangement in B cells activated with dextran-anti-IgD antibodies and interleukin 4: a three component model for Ig class switching. *J. Exp. Med.* 178:1577.
- 28 Snapper, C. M., Kehry, M. R., Castle, B. E. and Mond, J. J. 1995. Multivalent, but not divalent, antigen receptor cross-linkers synergize with CD40 ligand for induction of Ig synthesis and class switching in normal murine B cells. *J. Immunol.* 154:1177.
- 29 Shparago, N., Zelazowski, P., Jim, L., McIntyre, T. M., Stuber, E., Pecanha, L. M. T., Kehry, M. R., Mond, J. J., Max, E. E. and Snapper, C. M. 1996. Interleukin-10 selectively regulates murine isotype switching. *Int. Immunol.* 8:781.
- 30 Nilsson, L., Islam, K. B., Olafsson, O., Zalcberg, I., Samakovlis, C., Hammerstrom, L., Smith, C. I. E. and Sideras, P. 1991. Structure of TGF- β 1 induced human immunoglobulin C α 1 and C α 2 germ-line transcripts. *Int. Immunol.* 3:1107.
- 31 Seidl, K. J., Bottaro, A., Vo, A., Zhang, J., Davidson, L. and Alt, F. W. 1998. An expressed *neo^r* cassette provides required functions of the I γ 2b exon for class switching. *Int. Immunol.* 10:1683.
- 32 Reaban, M. E., Lebowitz, J. and Griffin, J. A. 1994. Transcription induces the formation of a stable RNA-DNA hybrid in the immunoglobulin α switch region. *J. Biol. Chem.* 269:21850.
- 33 Reaban, M. E. and Griffin, J. A. 1990. Induction of RNA-stabilized DNA conformers by transcription of an immunoglobulin switch region. *Nature* 348:342.

46 *Spliced HPRT-C_α RNA in I_α exon-deficient mice*

- 34 Daniels, G. A. and Lieber, M. R. 1995. RNA:DNA complex formation upon transcription of immunoglobulin switch regions: implications for the mechanism and regulation of class switch recombination. *Nucleic Acids Res.* 23:5006.
- 35 Pham, C. T. N., MacIvor, D. M., Hug, B. A., Heusel, J. W. and Ley, T. J. 1996. Long-range disruption of gene expression by a selectable marker cassette. *Proc. Natl Acad. Sci. USA* 93:13090.
- 36 Olson, E. N., Arnold, H.-H., Rigby, P. W. J. and Wold, B. J. 1996. Know your neighbors: three phenotypes in null mutants of the myogenic bHLG gene *MRF4*. *Cell* 85:1.
- 37 Fiering, S., Epner, E., Robinson, K., Zhuang, Y., Telling, A., Hu, M., Martin, D. I. K., Enver, T., Ley, T. J. and Groudine, M. 1995. Targeted deletion of 5' HS2 of the murine β -globin LCR reveals that it is not essential for proper regulation of the β -globin locus. *Genes Dev.* 9:2203.
- 38 Hug, B. A., Wesselschmidt, R. L., Fiering, S., Bender, M. A., Epner, E., Groudine, M. and Ley, T. J. 1996. Analysis of mice containing a targeted deletion of the β -globin locus control region 5' hypersensitive site 3. *Mol. Cell. Biol.* 16:2906.
- 39 Gorman, J. R., Stoep, N. V. d., Monroe, R., Cogne, M., Davidson, L. and Alt, F. W. 1996. The Ig κ enhancer influences the ratio of Ig κ versus Ig λ B lymphocytes. *Immunity* 5:241.
- 40 Madisen, L. and Groudine, M. 1994. Identification of a locus control region in the immunoglobulin heavy-chain locus that deregulates *c-myc* expression in plasmacytoma and Burkitt's lymphoma cells. *Genes Dev.* 8:2212.