IgA Class Switch in Iα Exon–deficient Mice

Role of Germline Transcription in Class Switch Recombination

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Abstract

Studies have implicated defective Ig class switch in the pathogenesis of IgA deficiency. To understand better the molecular events that regulate IgA class switch, a 1.4-kb region of the IgA locus containing the Iα exon was replaced with a human hypoxanthine phosphoribosyltransferase minigene by gene targeting in murine embryonic stem cells. The Iα exon–deficient mice derived from these embryonic stem cells had normal IgA levels in serum and secretions and normal numbers of IgA B cells in Peyer's patches and spleen. Further, Iα exon–deficient B cells efficiently underwent IgA class switch in vitro, despite the absence of Iα exon–containing germline transcripts. Notably, Iα exon–deficient B cells did not require TGF-β for IgA class switch since stimulation with LPS alone led to IgA expression. Nonetheless, whereas Iα exon–deficient B cells constitutively expressed human hypoxanthine phosphoribosyltransferase transcripts, they did not produce IgA in the absence of LPS stimulation. These results demonstrate that the Iα exon or transcripts containing the Iα exon are not required for IgA class switch. Further, the effects of TGF-β on Iα locus transcription can be supplanted by expression of a heterologous minigene at that locus, but a second signal is required for the induction of IgA class switch. (*J. Clin. Invest.* 1996. 97:477–485.) Key words: gene targeting • genetic transcription • IgA• immunoglobulin class switching • TGF-β

Introduction

Selective IgA deficiency is the most common primary humoral immunodeficiency, occurring with an incidence of 1:500–1:700 (1–3). Whereas the precise etiology and pathogenesis of IgA deficiency remain unknown, previous studies have suggested that B cells from patients with IgA deficiency are defective in their ability to undergo DNA rearrangement leading to IgA class switch (4). Cytokines and cell surface molecules have been shown to play key roles in IgA class switch. For example, TGF-β has been shown to regulate class switch to IgA both in mice (5–8) and in humans (9, 10). This effect of TGF-β is associated with its ability to increase production of germline α transcripts (6, 11) through TGF-β response elements located in the Iα exon promoter (12). Additional signals, besides TGF-β, are required for induction of IgA class switch. For example, stimulation of B cells via surface Ig (sIg)[1] and CD40 is required, in addition to TGF-β, to bring about IgA class switch in human B cells (9). Patients with hyper-IgM syndrome are impaired in their ability to express Ig isotypes other than IgM. Recent studies have demonstrated that these patients have abnormal CD40 ligand molecules expressed on activated T cells, which results in impaired signaling of B cells through CD40 (13–17).

At the molecular level, Ig class switch results in rearrangement of DNA at the Ig heavy chain (Igh) locus, leading to juxtaposition of the VDJ region to a new downstream constant region with deletion of intervening DNA. A consequence of this rearrangement event is the generation of antibodies that retain the same antigen specificity yet perform different effector functions. DNA rearrangement leading to class switch is preceded by specific alterations at the downstream Igh constant region to which the B cell subsequently switches. These alterations, which signal a commitment to class switch at that locus, include changes in chromatin structure with demethylation, generation of new DNase hypersensitivity sites, and de novo transcription. Transcription initiates from I exons located immediately upstream of the switch region producing germline transcripts (18–22).

The precise function of these germline transcripts in control of class switch remains incompletely understood. Nonetheless, recent studies have suggested that I regions containing the I exons play an important role in regulating this process (23–25), and several models have been proposed to explain their function. These models include induction of recombinase accessibility (26, 27) by promoter/enhancer elements (23) or transcription (28); RNA-stabilized DNA conformers (29); transplicing with production of *trans*-mRNA (30); and translation of germline transcripts (31). Recently, studies have suggested that spliced germline transcripts, or the process of splicing, participates in class switch recombination (32). The current studies were undertaken to define further the function of the Iα exon and germline α transcripts in IgA class switch.

Methods

Targeting of the Iα exon in embryonic stem (ES) cells. For construction of the targeting vector, DNA fragments of the murine IgA region were subcloned from the E-6 phage (33) into pBluescript KS$^-$ (Stratagene, La Jolla, CA). The targeting vector consisted of pKS$^-$

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Received for publication 5 April 1995 and accepted in revised form 18 October 1995.

J. Clin. Invest.
© The American Society for Clinical Investigation, Inc.
0021-9738/96/01/477/09 $2.00
Volume 97, Number 2, January 1996, 477–485

1. *Abbreviations used in this paper:* ES, embryonic stem; FIAU, 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5 iodouracil; HAT, hypoxanthine, aminopterin, thymidine; hHPRT, human hypoxanthine phosphoribosyltransferase; hMT, human metallothionein; Igh, Ig heavy chain; PGK, phosphoglycerate kinase; RPA, ribonuclease protection assay; RT, reverse transcriptase; sIg, surface Ig.

containing a 3.7-kb upstream homologous DNA region (XbaI/AvrII fragment immediately 5′ of the Iα exon transcription start sites) and a 3.2-kb downstream homologous DNA region (XbaI/XbaI fragment starting 0.7 kb downstream of the Iα exon and containing the IgA switch region). For positive selection, a human hypoxanthine phosphoribosyltransferase (hHPRT) minigene cassette containing a phosphoglycerate kinase (PGK) promoter and SV40 polyadenylation signal (34) was inserted between these two homologous regions in the same transcriptional orientation as the endogenous Iα exon, whereas for negative selection a *Herpes simplex* thymidine kinase gene (35) was inserted 5′ of the upstream homologous region. The $HPRT^-$ AB2.1 ES cell line (36) was grown on monolayers of mitotically inactivated STO fibroblasts transfected with a leukemia inhibitory factor expression construct (SNL 76/7) as previously described (35). The targeting vector was linearized 5′ of the *Herpes simplex* thymidine kinase gene with KpnI, electroporated into AB2.1 ES cells, and selected in hypoxanthine, aminopterin, thymidine (HAT) and 1-(2-deoxy, 2-fluoro-β-D-arabinofuranosyl)-5 iodouracil (FIAU) as described (34, 35). Selection in HAT alone gave 1,608 colonies per electroporation, whereas selection in HAT and FIAU gave 658 colonies.

Southern analysis of ES cell clones. To identify targeted ES cell clones, $HAT^r/FIAU^r$ colonies were picked into individual wells of 96-well feeder plates and analyzed by mini-Southern technique (37), after BamHI digestion, with a probe from the IgA constant region (pm3′Cα) located downstream of the recombination site. Putatively targeted clones were expanded and analyzed further by Southern hybridization (38). Probes used for Southern analysis included a 3.7-kb XbaI/AvrII fragment 5′ of the Iα exon cloned from E-6 phage into pKS (pm5′Iα.3); a 0.7-kb AvrII/XbaI fragment spanning the entire Iα exon, cloned from E-6 phage into pKS (pmIα.5); a 3.6-kb ClaI/BamHI fragment from pPGKhprt mini-5 (34); and a 0.6-kb fragment from the 3′ end of the IgA constant region subcloned from pα(J558) (39) into pGEM2 (pm3′Cα) (provided by Michael Sneller, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD).

Generation of mice with targeted deletion of the Iα exon. Correctly targeted ES cells were injected into C57BL/6cBay blastocysts followed by implantation into foster mothers as previously described (40). Chimeric males with the greatest degree of chimerism, assessed by the amount of agouti coat color, were bred to C57BL/6 female mice. Black agouti offspring from these matings were analyzed for transmission of the targeted allele by Southern hybridization of tail DNA. Male and female heterozygous mice were bred to obtain mice homozygous for deletion of the Iα exon.

Culture of splenic B cells. Spleen non–T cells were obtained as previously described (41). Briefly, 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 anti–Thy 1.2 (New England Nuclear, Boston, MA; final concentration 1:1,000), anti–Lyt 2.2, and anti-L3T4 mAb (Accurate Chemical Co., Westbury, NY; both final concentration 1:1,000) in cytotoxicity media (RPMI 1640 with 0.2% BSA) followed by complement lysis (Low Tox-M rabbit complement; Accurate Chemical Co.). Resting B cells (density 1.081–1.087) were obtained by separation on a discontinuous density gradient consisting of 50, 60, 65, and 70% Percoll (Pharmacia, Uppsala, Sweden) in HBSS with pooling of cells from the 65/70 interface and pellet. By flow cytometry, resting B cells contained $> 90\%$ IgM^+, $< 0.2\%$ IgA^+, and $< 2\%$ $CD3^+$ cells.

B cells ($0.5–2 \times 10^6$/ml) were cultured in RPMI 1640 (M. A. Whittaker Bioproducts, Walkersville, MD) supplemented with 10% FCS (M. A. Whittaker Bioproducts), 15 mM Hepes (GIBCO BRL, Gaithersburg, MD), 5% NCTC 109 medium (M. A. Whittaker Bioproducts), 5×10^{-5} M 2-ME (Sigma Chemical Co., St. Louis, MO), 2 mM glutamine (GIBCO BRL), and 100 U/ml penicillin/100 μg/ml streptomycin (GIBCO BRL) and incubated at 37°C and 6% CO_2 in a humidified atmosphere in 96-well flat-bottom plates, 25-cm^2, or 75-cm^2 flasks (Costar Corp., Cambridge, MA). LPS was added at the initiation of culture, whereas cytokines were added on day 1 of culture. *Escherichia coli* LPS (Sigma Chemical Co., 0127:B8) was used at a final concentration of 10 μg/ml, rIL-2 at 100 U/ml (Pharmingen, San Diego, CA), rIL-5 at 100 U/ml (42), and TGF-β (R&D Systems, Minneapolis, MN) at 1–2 ng/ml.

Measurement of Ig levels. Isotype-specific sandwich ELISAs were performed as previously described (43), except that, for IgA, plates were coated with an mAb (71-14) previously shown to be specific for mouse IgA (44). For measurement of secretory IgA, intestinal secretions were obtained as previously described (45). Serum Ig levels are presented as the mean±SD from analysis of three to six mice per group, whereas secretory IgA levels are derived from two wild-type animals (+/+), two heterozygous animals (+/−), and two homozygous animals (−/−). In vitro Ig production was measured in culture supernatants after 3 and 7 d of incubation. Cultures for in vitro Ig secretion were done in triplicate.

Analysis of sIg expression. Flow cytometric analysis was performed on Peyer's patch and spleen lymphocytes as previously described (43) using a Profile II (Coulter Corp., Hialeah, FL). Cells were stained with phycoerythrin-labeled goat anti–mouse IgM (Southern Biotechnology Associates, Birmingham, AL) and fluorescein-labeled anti–mouse IgA (71-14). Forward scatter and side scatter gates were set to include live lymphocytes, and 10,000 events were collected. Results shown are from one of two experiments that gave similar results.

Measurement of germline α transcripts. After 72 h of culture, total cellular RNA was extracted as described (46) from resting splenic B cells stimulated by LPS in the presence or absence of TGF-β. Germline α transcripts were detected by reverse transcriptase (RT)-PCR (11) using an upstream Iα primer (5′GACATGATCACAGGCACAAGGC3′), which begins 338 bp downstream of the 5′-most Iα exon transcription initiation site and 253 bp upstream of the 3′-most Iα exon splice site, and a downstream Cα primer (5′TTCCCCAGGTCACATTCATCGT3′), which ends 126 bp downstream of the splice acceptor site of the IgA CH1 exon. Reverse transcription and PCR amplification were performed using the GeneAmp® RNA PCR kit (Perkin-Elmer Corp., Norwalk, FL). Specifically, 2.5 U of RT was added to 1 μg of total cellular RNA in a volume of 20 μl, containing 5 mM $MgCl_2$, 1 mM dNTPs, 1× PCR buffer II, 1 U of RNase inhibitor, and 0.75 μM of downstream primer. Reverse transcription was carried out at 42°C for 15 min, followed by 5 min incubation at 99°C, and then 5 min at 5°C. The final volume of reverse-transcribed RNA was adjusted to 100 μl containing 1.5 mM $MgCl_2$, 0.20 mM dNTPs, 1× PCR buffer II, 0.15 μM upstream and downstream primers, and 2.5 U of Taq polymerase. Samples were denatured at 95°C for 1 min, 11 s; then 35 cycles of amplification were performed on a DNA thermal cycler (model 480; Perkin-Elmer Corp.) (each step of the cycle: 1 min, 11 s) with a denaturing temperature of 95°C, an annealing temperature of 55°C, and an extension temperature of 72°C. The primers used in this analysis generate a major PCR product 379 bp in size and a second minor PCR fragment 239 bp in size that is derived from a second Iα exon splice site located 113 bp downstream of the Iα primer. Controls included samples in which no RT or no RNA were added. These controls, plus the fact that primer sites on genomic DNA are separated by ~ 4 kb, indicate that the amplified fragments were not derived from DNA. To confirm the identity of amplified RT-PCR products, Southern blotting of the amplified PCR fragments was performed by standard methods (38) with the Iα exon–specific probe pmIα.5.

Ribonuclease protection assays (RPAs). Measurement of hHPRT, Cα, and GAPDH RNA levels was performed using an RPA (Ambion Inc., Austin, TX). Probes used in the RPAs were generated from a 419-bp HindIII/XmnI fragment of the 3′ region of hHPRT subcloned into pSK^+ (phHPRT.419[−]); a 396-bp PstI/HincII fragment containing the 3′ end of Sα and the 5′ region of the first Cα exon subcloned into pSK^+ (pSKE6Sα5′Cα.1); and a control plasmid containing a region of murine GAPDH (pTRI-GAPDH-Mouse; Ambion Inc.). The region of hHPRT chosen was one with significant sequence divergence from murine HPRT, to distinguish human from murine

HPRT. To generate radiolabeled antisense RNA probes, phHPRT.419(−) (linearized with HindIII), pSKE6Sα5′Cα.1 (linearized with BamHI), and pTRI-GAPDH-Mouse (linearized with DdeI) were transcribed in vitro with T3 or T7 polymerase and [^{32}P]UTP according to protocol (Maxiscript; Ambion Inc.). The phHPRT.419(−) probe protects a 419-bp region of hHPRT RNA and the Cα probe protects 202 bp of Cα RNA from exon 1, whereas the pTRI-GAPDH-Mouse probe protects a 149-bp region of mouse GAPDH RNA.

Total cellular RNA was extracted from freshly isolated resting spleen B cells, resting spleen B cells cultured with LPS±TGF-β for 72 h, CH1 or CH12.LX cells cultured with TGF-β for 72 h, or human fibroblasts. Gel-purified ^{32}P-labeled probes (5×10^4 cpm) were mixed with 5 μg of sample RNA in 1.5 ml microfuge tubes and coprecipitated with ethanol and ammonium acetate by incubation at −20°C for 15 min followed by pelleting at 12,000 *g* for 15 min at room temperature. Ethanol supernatant was removed and pellets were resuspended in hybridization buffer (Ambion Inc.), heated to 90°C for 3 min, vortexed, and incubated overnight at 45°C. Samples were digested with RNase A/RNase T1 (diluted 1:100 in RNase digestion buffer) at 37°C for 30 min and precipitated with RNase inactivation/precipitation mixture (Ambion Inc.) at −20°C for 15 min. Supernatants were removed after centrifugation, and pellets were resuspended in gel-loading buffer (95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.5 mM EDTA, 0.025% SDS) and run on a 6% polyacrylamide/8 M urea gel at 100 V for 2 h. Molecular weight markers consisted of PUC BM21 digested with HpaII, DraI, and HindIII (Marker VIII; Boehringer Mannheim Corp., Indianapolis, IN), which was ^{32}P-end labeled with T4 ligase. Gels were exposed to x-ray film at −70°C with or without an intensifying screen.

Results

Generation of Iα exon–deficient mice. Targeted deletion of the Iα exon in ES cells was accomplished using the targeting vector shown in Fig. 1 *A*. Homologous recombination between the targeting vector and genomic sequences resulted in deletion of the entire Iα exon with replacement by an HPRT minigene (34). The region 5′ of the Iα exon, including 5′ *cis*-acting response elements (12), and the IgA switch region located 3′ of this exon were retained. The HPRT minigene is inserted in the same transcriptional orientation as the endogenous Iα exon it replaces and is transcribed from a PGK promoter (47).

From 192 HAT^r/FIAU^r ES cell clones analyzed, two correctly targeted clones were identified. The fidelity of the 5′ and 3′ recombination events was confirmed by Southern hybridization after digestion with SacI or BamHI, respectively (Fig. 1, *B* and *C*). Thus, hybridization of SacI-digested DNA with a probe for the region 5′ of the Iα exon showed a wild-type 6.7-kb fragment in AB2.1 ES cells and an additional 9.1-kb targeted fragment in the two clones, Iα.1 and Iα.2. Rehybridization of this blot with an Iα exon–specific probe recognized the 6.7-kb wild-type fragment but not the 9.1-kb fragment,

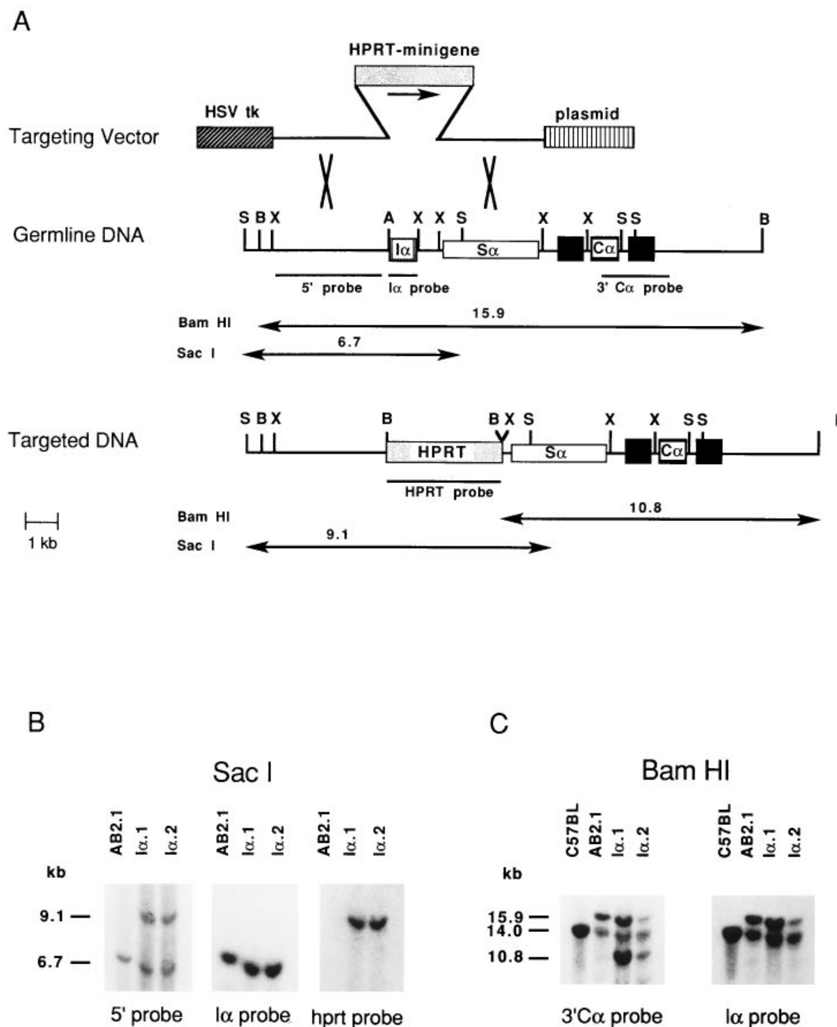

Figure 1. Targeting of the Iα exon in ES cells. (*A*) Strategy for deletion of the Iα exon from the Igh locus in mice. Maps of the targeting vector, germline, and targeted Iα locus DNA are shown. The Iα exon (Iα) and HPRT minigene (▨); the IgA switch region (*Sα*) (□); and the IgA constant region exons (*Cα*) (■). Restriction enzyme sites, predicted restriction fragment lengths, and probes used in Southern analysis are also shown. Homologous recombination between the targeting vector and genomic DNA results in replacement of the Iα exon by the HPRT minigene with retention of the 5′ promoter region and the 3′ switch region. Introduction of the HPRT minigene generates new SacI and BamHI fragments as shown. *A*, AvrII; *B*, BamHI; *S*, SacI; and *X*, XbaI. (*B*) Southern analysis of SacI-digested DNA from targeted ES cell clones. A nylon filter containing SacI-digested DNA was sequentially hybridized with a probe for the region 5′ of the Iα exon (*5′ probe*), a probe specific for the Iα exon (*Iα probe*), and, finally, a probe for the HPRT minigene (*hprt probe*). (*C*) Southern analysis of BamHI-digested DNA from targeted ES cell clones. A nylon filter containing BamHI-digested DNA was sequentially hybridized with the 3′Cα probe, which recognizes a region within the Cα locus lying outside the recombination site, and with the Iα exon probe.

whereas the 9.1-kb fragment was recognized by a probe for the hHPRT minigene.

Hybridization of BamHI-digested DNA with the 3′Cα probe demonstrated a 15.9-kb fragment in AB2.1 ES cell DNA, representing the wild-type (129 SvEv) allele. The additional 14-kb band seen on the blot arises from contaminating feeder cell DNA (STO fibroblasts), which contains a polymorphism in this region similar to that of C57BL/6 (48). DNA from the two Iα exon–targeted ES cell clones demonstrate an additional 10.8-kb BamHI fragment, representing the targeted allele. Rehybridization of this blot with an Iα exon probe detects the wild-type fragment but not the 10.8-kb targeted fragment. Thus, targeting has resulted in deletion of the Iα exon with replacement by the HPRT minigene. Additional Southern analyses confirmed that a concatamer had not integrated at this locus and that the IgA switch region of the targeted locus had not been altered in size (data not shown).

Both targeted ES cell clones were injected into albino C57BL/6cBay blastocysts for generation of chimeras, which were screened on the basis of the level of pigmentation contributed by ES cells to coat color (40). Chimeras derived from the Iα.2 clone had good levels of live births (42%) and high ES cell contribution (50–60%). Germline transmission was achieved with four out of five Iα.2 male chimeras, as evidenced by the presence of black agouti progeny in the F_1 generation of matings with C57BL/6. Southern analysis of tail DNA from these black agouti offspring is shown in Fig. 2 *A*. The 15.9-kb BamHI fragment results from transmission of the wild-type allele from targeted ES cells (129/SvEv), and the 14-kb fragment represents the wild-type C57BL/6cBay allele, whereas the 10.8-kb fragment is derived from the targeted ES cell mutant allele.

Heterozygous targeted animals were bred to generate homozygotes and the offspring were analyzed for wild-type and mutant alleles, as shown in Fig. 2 *B*. Animals homozygous for the Iα exon deletion demonstrate only a single 10.8-kb BamHI fragment detected by the 3′Cα probe, whereas the Iα exon–specific probe fails to detect any genomic sequences confirming that this exon is no longer present. Of 36 offspring analyzed, 8 were found to be homozygous wild type, 22 heterozygous, and 6 homozygous for deletion of the Iα exon.

Figure 3. In vivo production of IgA in Iα exon–deficient mice. (*A*) Total IgA levels in serum and intestinal secretions of wild-type, heterozygous, or homozygous Iα exon–deficient mice. Serum and intestinal secretions were collected from 6–12-wk-old mice and analyzed for IgA levels by ELISA. (*B*) Serum Ig levels in wild-type, heterozygous, or homozygous Iα exon–deficient mice. Serum was collected from 6–12-wk-old mice and analyzed for IgM, IgG, and IgA levels by ELISA.

Iα exon–deficient mice express normal levels of IgA in vivo. To assess the effect of this deletion on IgA expression in vivo, IgA levels in serum and intestinal secretions were determined (Fig. 3 *A*). Homozygous Iα exon–deficient mice had serum and secretory IgA levels equivalent to those of wild-type and heterozygous animals. Thus, serum IgA was 410±180 μg/ml in wild-type mice, 450±160 μg/ml in heterozygous mice, and 370±180 μg/ml in homozygous Iα exon–deficient mice. Similarly, intestinal secretory IgA was 630±70 μg/ml in wild-

Figure 2. Generation of Iα exon–deficient mice. (*A*) Southern analysis of black agouti offspring derived from mating male chimeric mice with C57BL/6 female mice. Tail DNA was digested with BamHI and analyzed with the 3′Cα probe. One allele in the black agouti offspring is a wild-type allele derived from the C57BL/6 mother (14-kb fragment). The other allele is inherited from the chimeric male as either the wild-type (129) allele (15.9-kb fragment) or the targeted allele (10.8-kb fragment). (*B*) Southern analysis of offspring derived from mating heterozygous Iα exon–targeted mice. Tail DNA was digested with BamHI, blotted to nylon, and analyzed by sequential hybridization with the 3′Cα and Iα probes. Mice homozygous for deletion of the Iα exon demonstrate a single 10.8-kb targeted fragment recognized by the 3′Cα probe and no DNA fragments recognized by the Iα probe.

type mice, 620±100 μg/ml in heterozygous mice, and 550±42 μg/ml in homozygous deficient mice. In addition, no significant alterations in serum IgM or IgG were seen in homozygous Iα exon–deficient mice, compared with heterozygous or wild-type mice (Fig. 3 *B*).

To determine whether IgA expression was normal in lymphoid tissues of Iα exon–deficient mice, flow cytometric analysis for sIgA expression was performed on Peyer's patch and spleen B cells. As shown in Fig. 4, mice that were homozygous for deletion of the Iα exon contained Peyer's patch B cells that express sIgA in numbers comparable to those seen in wild-type or heterozygous mice. Thus, wild-type mice contain 25.3% $sIgA^+$ B cells in Peyer's patches versus 22.8% in heterozygous mice and 29.5% in homozygous Iα exon–deficient mice. Interestingly, in younger Iα exon–deficient mice, larger numbers of double-positive, sIgM/sIgA-expressing B cells were seen in the Peyer's patches. However, these differences were not observed in older animals. Small but similar numbers of $sIgA^+$ B cells (2–4%) were seen in the spleens of wild-type, heterozygous, and homozygous Iα exon–deficient mice.

B cells from Iα exon–deficient mice undergo IgA class switch in vitro. Additional studies were performed to assess the ability of Iα exon–deficient B cells to undergo IgA class switch in vitro. Spleen B cells from wild-type and Iα exon–deficient mice were stimulated in vitro with LPS, either in the presence or absence of TGF-β, IL-2, and IL-5. As shown in Fig. 5, 3-d culture supernatants from B cells of both wild-type and Iα exon–deficient mice contained very low levels of IgA, regardless of the stimulus, indicating that the IgA present in culture supernatants after 7 d of culture was not derived from B cells precommitted to IgA production in vivo. After 7 d of culture, wild-type B cells stimulated with LPS and TGF-β produced large amounts of IgA (12,460±2,310 ng/ml), which was further enhanced by addition of IL-2 and IL-5 (89,100±7,300 ng/ml). Wild-type B cells secreted virtually no IgA when cultured in medium (99±29 ng/ml), and small amounts of IgA when cultured with LPS alone (809±169 ng/ml) or LPS with IL-2 and IL-5 (1,319±264 ng/ml). In contrast, Iα exon–deficient B cells produced large amounts of IgA upon stimulation with LPS alone (7,530±1,200 ng/ml), resulting in levels comparable to those seen in wild-type B cells stimulated with both LPS and TGF-β. Further, the addition of TGF-β to Iα exon–deficient B cells resulted in only a modest increase in secreted IgA (11,330±710 ng/ml). Again, addition of IL-2 and IL-5 resulted in further enhancement of IgA secretion (23,600±1,800 ng/ml), as seen with wild-type B cells. Notably, Iα exon–deficient B cells produced virtually no IgA when cultured in medium alone (68±12 ng/ml), indicating that they did not express IgA in the absence of LPS stimulation.

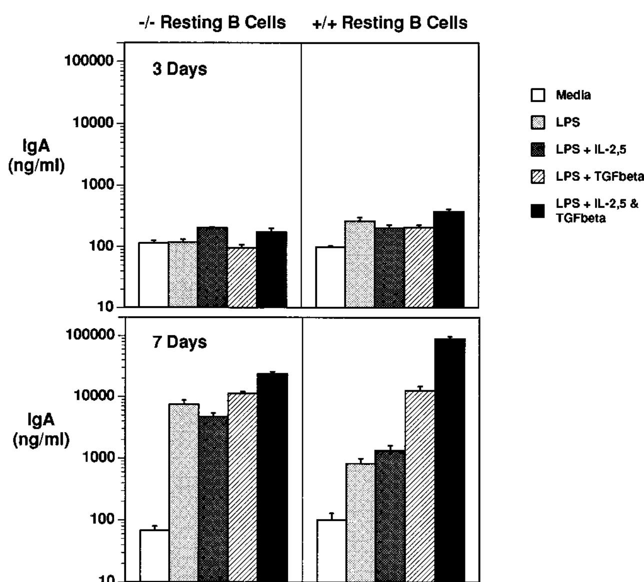

Figure 5. In vitro induction of IgA secretion in Iα exon–deficient B cells. Resting spleen B cells from wild-type and Iα exon–deficient mice were stimulated with LPS with or without TGF-β, IL-2, and IL-5. Supernatants were assayed by ELISA after 3 and 7 d of culture for the presence of IgA. Results shown are representative of three separate experiments.

The ability of Iα exon–deficient B cells to undergo IgA class switch in vitro was further assessed by analysis of sIgA expression. As shown in Fig. 6, wild-type B cells stimulated

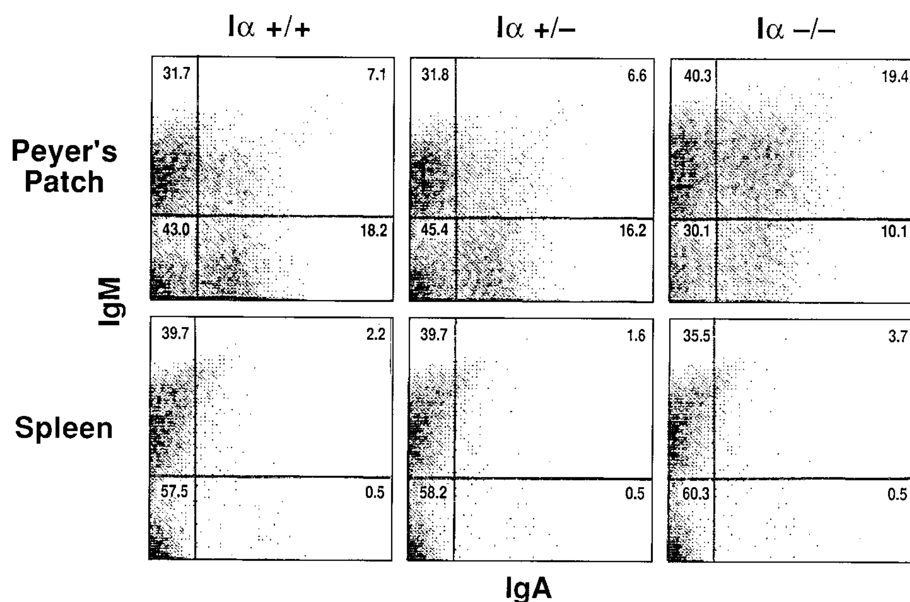

Figure 4. IgA B cells in lymphoid tissues of Iα exon–deficient mice. Lymphocytes were obtained from Peyer's patches and spleens of 6–12-wk-old mice and analyzed by flow cytometry for expression of sIgM and sIgA. Upper panels show Peyer's patch B cells from wild-type ($I\alpha^{+/+}$), heterozygous ($I\alpha^{+/-}$), or homozygous Iα exon–deficient ($I\alpha^{-/-}$) mice, whereas lower panels show spleen B cells from the same animals.

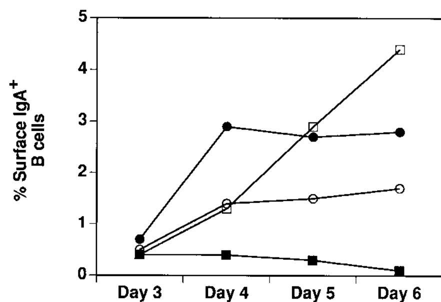

Figure 6. In vitro induction of sIgA in Iα exon–deficient B cells. Resting spleen B cells from wild-type and Iα exon–deficient mice were stimulated with LPS with or without TGF-β. Cells were assayed by flow cytometry for expression of sIgA between days 3 and 6 of culture. $I\alpha^{+/+}$ B cells, LPS (*solid square*); $I\alpha^{+/+}$ B cells, LPS + TGF-β (*solid circle*); $I\alpha^{-/-}$ B cells, LPS (*open square*); $I\alpha^{-/-}$ B cells, LPS + TGF-β (*open circle*).

with LPS alone expressed little sIgA (< 0.5%); however, in response to LPS and TGF-β, they demonstrated a significant increase in sIgA expression by day 4 (2.9%). On the other hand, Iα exon–deficient B cells stimulated with LPS alone manifested increased sIgA by day 4 (1.3%), and this increased further to 4.4% by day 6. The addition of TGF-β to Iα exon–deficient B cells resulted in less sIgA expression (maximum of 1.7% on day 6) than that seen after stimulation with LPS alone.

Production of germline α transcripts does not correlate with IgA expression. IgA class switch occurred in Iα-deficient mice despite the absence of germline α transcripts, as shown in Fig. 7. Thus, whereas B cells from wild-type or heterozygous mice expressed readily detectable germline α transcripts after stimulation with LPS and TGF-β, Iα exon–deficient B cells produced no detectable Iα exon–containing transcripts. Despite the absence of germline α transcripts, Iα exon–deficient B cells produced substantial amounts of IgA after stimulation with LPS alone (2,270±553 ng/ml and 2,970±358 ng/ml), resulting in levels approaching those seen in wild-type (6,740±1,987 ng/ml) or heterozygous (4,937±1,444 ng/ml) B cells stimulated with LPS and TGF-β. TGF-β had little if any enhancing effect on IgA secretion in Iα exon–deficient B cells (4,277±316 ng/ml and 2,410±340 ng/ml). Of note, heterozygous B cells also produced large amounts of IgA after stimulation with LPS alone (9,400±600 ng/ml) in comparison with wild-type B cells (640±47 ng/ml). IgA secretion by heterozygous B cells was not enhanced by TGF-β, despite the fact that a significant increase in germline α transcripts was seen upon addition of TGF-β.

Expression of human HPRT RNA and Cα RNA in $I\alpha^{-/-}$ B cells. Whereas no germline transcripts containing the Iα exon were detected in $I\alpha^{-/-}$ B cells, it was important to determine whether transcription was occurring at the Iα locus in $I\alpha^{-/-}$ B cells. It was reasoned that, since the hHPRT minigene contained a PGK promoter, which induces ubiquitous and constitutive expression of the PGK gene (47, 49), expression of the hHPRT minigene would be likely to occur in resting $I\alpha^{-/-}$ B cells. To assess this, RPAs measuring levels of hHPRT RNA in $I\alpha^{-/-}$ spleen B cells were performed. As shown in Fig. 8 *A*, freshly isolated resting spleen B lymphocytes from $I\alpha^{-/-}$ mice expressed readily detectable levels of hHPRT RNA, as demonstrated by protection of a 419-bp fragment with the hHPRT probe. $I\alpha^{-/-}$ spleen B cells stimulated for 3 d with LPS with or

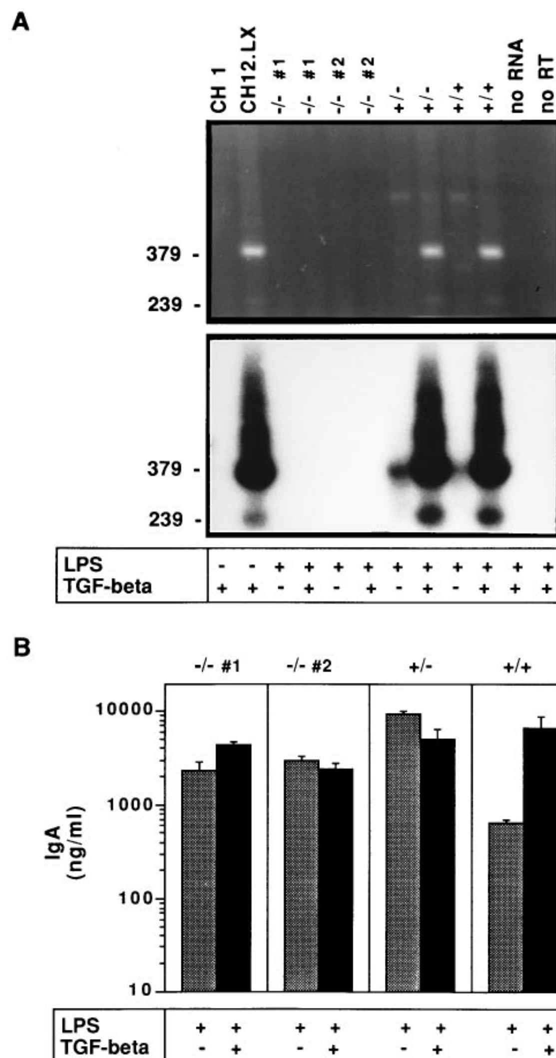

Figure 7. Measurement of germline α transcripts in Iα exon–deficient B cells. Resting spleen B cells from Iα exon–deficient (−/−; 1 and 2), heterozygous (+/−), and wild-type (+/+) mice were stimulated with LPS with or without TGF-β. After 3 d of culture, RNA was extracted and assessed for expression of germline α transcripts by RT-PCR. (*A*) Upper panel shows ethidium bromide–stained gel containing amplified products. Lower panel shows Southern blot hybridized with Iα exon–specific probe. Reactions containing no RNA or no RT were performed as additional controls. *CH1*, B cell line that does not switch to IgA and does not express germline α transcripts; *CH12.LX*, B cell line that switches to IgA and expresses germline α transcripts. (*B*) Supernatants from parallel cultures were assayed by ELISA for IgA after 7 d of culture. Cultures assayed for IgA secretion also contained IL-2 and IL-5. LPS (▒), LPS + TGF-β (■).

without TGF-β expressed levels of hHPRT RNA not substantially different than those seen in freshly isolated resting spleen B cells after normalization to the level of GAPDH RNA expression. This assay was specific for human and not mouse HPRT, since RNA from $I\alpha^{+/+}$ spleen cells produced no 419-bp protected fragments whereas human fibroblast RNA did.

To assess expression of Cα-containing RNA, resting spleen B cells from $I\alpha^{+/+}$ or $I\alpha^{-/-}$ mice were stimulated for 3 d with LPS alone or with LPS and TGF-β. RNA from these cells was then analyzed in an RPA using a Cα-specific probe. As ex-

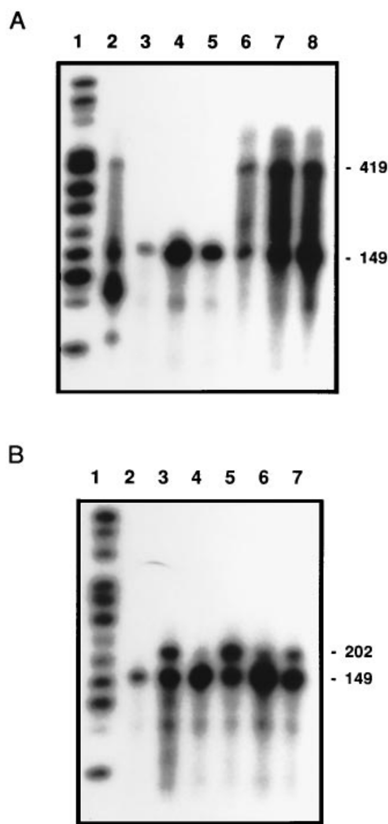

Figure 8. (*A*) Expression of hHPRT RNA by Iα exon–deficient B cells. Total cellular RNA was extracted from $I\alpha^{+/+}$ or $I\alpha^{-/-}$ resting spleen B cells, either immediately after isolation or after 72 h of stimulation with LPS±TGF-β. Human fibroblast RNA was used as a positive control. To assess levels of expression of hHPRT or mGAPDH RNA, an RPA was performed with probes specific for hHPRT or mGAPDH. The hHPRT probe protects a 419-bp fragment of hHPRT RNA. The mGAPDH probe protects a 149-bp fragment of mouse GAPDH RNA. Lane *1*, molecular weight markers; lane *2*, human fibroblasts; lane 3, resting $I\alpha^{+/+}$ B cells; lane *4*, $I\alpha^{+/+}$ B cells stimulated with LPS; lane *5*, $I\alpha^{+/+}$ B cells stimulated with LPS + TGF-β; lane *6*, resting $I\alpha^{-/-}$ B cells; lane *7*, $I\alpha^{-/-}$ B cells stimulated with LPS; and lane *8*, $I\alpha^{-/-}$ B cells stimulated with LPS + TGF-β. (*B*) Expression of Cα RNA by Iα exon–deficient B cells. $I\alpha^{+/+}$ or $I\alpha^{-/-}$ spleen B cells were stimulated with LPS or LPS + TGF-β for 3 d. Total cellular RNA was extracted and an RPA was performed with probes specific for Cα and mGAPDH. The Cα probe protects a 202-bp fragment of Cα RNA, whereas the mGAPDH probe protects a 149-bp fragment of mouse GAPDH RNA. Lane *1*, molecular weight markers; lane *2*, CH1, a murine B cell lymphoma line that does not express IgA; lane *3*, CH12.LX, a murine B cell lymphoma line that switches to and expresses IgA; lane *4*, $I\alpha^{+/+}$ B cells stimulated with LPS; lane *5*, $I\alpha^{+/+}$ B cells stimulated with LPS + TGF-β; lane *6*, $I\alpha^{-/-}$ B cells stimulated with LPS; and lane *7*, $I\alpha^{-/-}$ B cells stimulated with LPS + TGF-β.

pected, $I\alpha^{+/+}$ B cells stimulated with LPS and TGF-β expressed large amounts of Cα-containing RNA but not when stimulated with LPS alone. In contrast, $I\alpha^{-/-}$ B cells expressed detectable levels of Cα RNA after stimulation with LPS alone. The addition of TGF-β to $I\alpha^{-/-}$ B cells had only modest effects on Cα RNA expression over that seen with LPS alone.

Discussion

Recent studies have demonstrated an important role for Ig I regions in control of class switch recombination. For example, it was shown that deletion of the Iγ1 (23) and Iγ2b (24) regions in mice prevented class switch to IgG1 and IgG2b, respectively, whereas deletion of the Iε region significantly impaired class switch to IgE (25). In contrast to these previous studies, our results demonstrate that B cells lacking the entire Iα exon are capable of undergoing class switch to IgA. Thus, Iα exon–deficient mice have normal IgA levels in serum and gastrointestinal secretions, and normal numbers of IgA B cells in Peyer's patches and spleen. In addition, Iα exon–deficient B cells can be induced in vitro to express sIgA and secrete IgA with levels comparable to that seen in wild-type B cells. From these results, we conclude that the Iα exon or products derived from this exon are not essential for IgA class switch.

The reason for the apparent discrepancy between results reported here and previous studies targeting other I regions may be evident from analysis of the different targeted mutations that were generated. For example, in targeting of the Iγ1 and Iγ2b regions, both the I exon promoter and I exon were deleted. Further, the selection gene (*neo^r*) was either inserted in the opposite transcriptional orientation to that of the I exon it replaced or was deleted during a second recombination event. As a consequence, transcription from the endogenous Igh locus would no longer occur, whereas any transcription of the inserted *neo^r* gene would proceed in the direction opposite to that of the switch region. Thus, these studies do not allow a distinction to be made between a requirement for the I exon itself, for the I exon promoter, for transcription per se, for germline transcripts, or for translation products of these transcripts in Ig class switch. In contrast, whereas the entire Iα exon was deleted in the studies reported here, the Iα promoter was retained. Further, the hHPRT selection gene was inserted in the same transcriptional orientation as the Iα exon it replaced. Although no Iα exon–containing transcripts were produced, transcription of the hHPRT minigene was observed in Iα exon–deficient B cells. In addition, transcription of the hHPRT minigene occurred in the direction of the Sα region. These observations and the finding of unimpaired IgA class switch in Iα exon–deficient B cells are consistent with the hypothesis that events associated with transcription participate in IgA class switch.

However, previous deletion of the Iε promoter and exon with replacement by the μ enhancer (E_{μ}) and a V_H promoter (25) led to substantial abrogation of IgE class switch, despite the finding that transcription through the Sε region occurred in the Iε-deficient B cells. This result suggests that transcription per se is not sufficient for class switch recombination. In support of this, a recent study found that B cells in which the Iγ1 region was replaced by a human metallothionein (hMT) promoter were incapable of undergoing class switch to IgG1 (32). However, insertion of 114 nucleotides from the 3′ region of the Iγ1 exon (containing a splice donor site) immediately downstream of the hMT promoter led to efficient IgG1 class switch. Despite the dramatic differences in the ability to switch to IgG1, both B cells targeted with the hMT promoter alone and B cells containing the hMT promoter along with 114 nucleotides of the Iγ1 exon produced transcripts containing IgG1 switch region sequences, when stimulated with LPS. The authors interpreted these findings as indicating that spliced switch transcripts or the splicing of germline transcripts, rather than transcription per se, controls DNA rearrangement leading to class switch.

The results of that study, however, do not exclude the possibility that the 114 nucleotides from the 3′ region of the Iγ1 exon inserted downstream of the hMT promoter contain a necessary recombination control element, aside from the splice donor site. For example, previous studies have suggested that germline transcripts might encode a functional polypeptide that could play a role in class switch recombination (31, 50). Further, transplicing between a VDJ-containing IgM

mRNA transcript and a germline Ig transcript giving rise to a *trans*-mRNA has been proposed to play a role in class switch recombination events (51–54), suggesting that transcripts containing the 3′ region of the Iγ1 exon might participate in transplicing with productive Ig transcripts. Our studies clearly demonstrate that no Iα exon sequence–derived peptides are required for IgA class switch. In addition, given the finding that IgA class switch occurs in Iα exon–deficient B cells that lack any Iα exon sequences, it is unlikely that transplicing between productive Ig transcripts and Iα exon–containing germline transcripts plays an obligatory role in class switch. Thus, our studies demonstrate that no recombination control elements required for IgA class switch are contained within the Iα exon.

The studies reported here are consistent with, but do not prove, the hypothesis that spliced switch transcripts or splicing participates in class switch recombination. hHPRT transcripts are expressed in Iα exon–deficient B cells and, of note, the hHPRT minigene contains an intron between exons 2 and 3 that is spliced out of transcripts during RNA processing. Insertion of the HPRT minigene immediately upstream of the IgA switch region and in the same transcriptional orientation as the endogenous Iα exon may allow transcriptional read-through of the IgA switch and constant regions with subsequent splicing of these transcripts. Recent experiments have shown that transcription occurs through the Cα region in Iα exon–deficient B cells (Harriman, G. R., unpublished observations). Whether splicing or spliced products are required for IgA class switch in these cells remains to be determined. Nonetheless, it is interesting to note that previous studies have shown transcription of IgA switch region–containing plasmids produces RNA transcripts from the switch region that can stabilize a unique switch region DNA conformer via RNA–DNA hybrids (29). In normal B cells, primary germline α transcripts initiating from the Iα exon and containing switch region sequences might, either directly or as splice products, participate in stabilization of switch region DNA conformers, thereby targeting the recombinase to that locus. In Iα exon–deficient B cells, processing of hHPRT read-through transcripts may provide the appropriate RNA substrate required for formation of such RNA–DNA hybrids.

Interestingly, Iα exon–deficient B cells were capable of producing IgA upon stimulation with LPS, in the absence of TGF-β. The constitutive expression of the hHPRT minigene at the Iα locus appears to obviate the need for TGF-β. Consequently, the only requirement for TGF-β in IgA class switch appears to be regulation of transcriptional activity at the Iα locus. In fact, addition of TGF-β to LPS-stimulated Iα exon–deficient B cells was inhibitory in the case of sIgA expression. The reason for this inhibition is not readily apparent. Whereas TGF-β induces B cells to undergo IgA class switch, previous studies have shown that it inhibits B cell survival (41) and IgA secretion by B cells already committed to IgA expression (5, 9). Thus, TGF-β might inhibit survival and/or IgA expression in Iα exon–deficient B cells undergoing DNA rearrangement at the IgA locus after LPS stimulation. With regard to delayed expression of sIgA seen in vitro with Iα exon–deficient B cells, the reason is also not readily apparent. It is possible that transcription rates of the HPRT minigene in Iα exon–deficient B cells, although constitutive, are lower than those induced by TGF-β at the Iα locus in normal B cells. Further studies comparing such transcriptional rates will be required to resolve this issue.

Notably, IgA expression was not seen in resting Iα exon–deficient B cells, despite the presence of hHPRT minigene transcription. Rather, IgA class switch was only seen when these B cells were stimulated with LPS. This suggests that LPS provides a signal, independent of transcription at the Iα locus, which is required for IgA class switch. In this regard, B cells from IgA-deficient patients are capable of expressing germline transcripts when stimulated with TGF-β in vitro (4). Nonetheless, B cells from IgA-deficient patients are defective in their ability to undergo IgA class switch in vivo and do not produce IgA in vitro when PBMCs are stimulated with pokeweed mitogen. These findings suggest that, although B cells from patients with IgA deficiency have the capacity to produce germline α transcripts, there is defective or ineffective delivery of a second stimulus that is required for DNA rearrangement leading to IgA class switch.

In conclusion, mice with targeted deletion of the Iα exon were found to express normal levels of IgA in vivo, and B cells from these mice were capable of undergoing class switch to IgA in vitro. These studies demonstrate that the Iα exon or products derived from this exon are not essential for IgA class switch. Further studies are needed to determine whether events related to transcription at the Iα locus or the processing of transcripts from this locus are central to control of class switch. Nonetheless, the current studies in Iα exon–deficient mice further refine our understanding of the Iα region and its function as a recombination control element. These mice promise to be a useful model for elucidating the molecular events mediating IgA class switch and for further defining the pathogenesis of IgA deficiency.

Acknowledgments

The authors thank Milton Pyron for valuable technical assistance and Yoshio Wakatsuki for providing the E-6 phage. We also thank Lynn Huston and Molly Bogue for helpful discussions.

This work was supported by grants from The Methodist Hospital Foundation and National Institutes of Health (AI33469) to G. R. Harriman, and by a postdoctoral fellowship from the Medical Research Council of Canada to A. C. Davis.

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