

Effect of Downregulation of Germline Transcripts on Immunoglobulin A Isotype Differentiation

By Yoshio Wakatsuki and Warren Strober

From the Mucosal Immunity Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary

In this study we determined the role of immunoglobulin (Ig) germline transcripts in the isotype switch differentiation of the cloned lymphoma B cell line CH12.LX. In initial studies, we showed that addition of transforming growth factor β (TGF- β) and interleukin 4 (IL-4), either alone or in combination, augment switching from membrane (m)IgM⁺ to mIgA⁺ cells, and that increased switching is preceded and paralleled by an increase in the steady-state level of α germline transcripts (α GLT). Interestingly, TGF- β and IL-4 affect switching in different ways, as shown by the fact that IL-4 increases and TGF- β decreases the number of dual-positive (mIgM⁺/mIgA⁺) cells; in addition, TGF- β and IL-4 have different effects on the time course of induction of α GLT. In subsequent studies, we established that we could downregulate α GLT levels in CH12.LX B cells by transfecting an expression vector that can be induced to produce transcripts antisense to the I α exon. Using this approach we downregulated α GLT in CH12.LX B cells undergoing switching in the presence of TGF- β and IL-4 and showed that such downregulation led to decreased switching, as evidenced by decreased appearance of dual-positive B cells as well as decreased IgA synthesis relative to IgM synthesis. This result was corroborated by the fact that incubation of CH12.LX cells with phosphorothio-oligo antisense DNA to I α sequence also led to a decrease in the number of dual-positive cells and in the IgA/IgM secretion ratio. In summary, IgA isotype differentiation in CH12.LX B cell, particularly the steps necessary for the elaboration of mIgM⁺/mIgA⁺ switch intermediate cells, is inhibited by downregulation of α GLT; it is therefore apparent that α GLT plays a key role in the initial stage of isotype switch differentiation.

A cell of the B lineage can undergo two types of Ig gene rearrangement during its differentiation. The first involves V region genes, which determine the specificity of the B cell antigen receptor. The second involves C region genes, which determine the isotype and the effector function of the B cell (1–3). It is now well established that lymphokines/cytokines play an important role in the second type of gene rearrangement, i.e., isotype differentiation (4, 5). In this regard, it has been shown that certain cytokines, solely or in combination with other cytokines or activation stimuli, influence Ig isotype differentiation in either primary B cell culture (6–9) or B cell lines adapted to culture (10–12). These studies favor the theory that B cell isotype differentiation is not a stochastic process followed by selection; rather, it is a directed process by which factors outside the B cell act to bring about certain types of differentiation.

Recent studies have provided support for an accessibility theory of isotype switching, which holds that isotype switching is initiated when a 5' region of a C_H gene locus becomes accessible to "switch recombination machinery" and becomes transcriptionally active. Thus, it has been established, using

either normal murine B cells or transformed B cell lines, that germline transcripts (GLTs)¹ relevant to a particular C_H gene are produced before rearrangement of that gene (13–15). In addition, it has been shown that LPS + IL-4 or TGF- β induce DNase-hypersensitive sites at C_H gene loci to which the cell will ultimately switch (16). The accessibility theory obviates the need for isotype-specific recombinases since it implies that a "generic" recombinase could bring about any type of isotype switch after it gains access to a 5' switch region. However, the theory is neutral concerning the role of GLTs in the switch process. On the one hand, it is compatible with the concept that GLTs can simply be byproducts of increased transcriptional activity and have no functional role; on the other hand, it is also compatible with the idea that GLTs have definite function in isotype switch, either directly or through a translated product (17, 18). In the present study we address

¹ Abbreviations used in this paper: GLT, germline transcript; m, membrane.

the question of the role of GLTs in isotype differentiation by studying the effect of downregulation of $I\alpha$ GLT levels on IgA isotype switching in CH12.LX B cells. We show that such downregulation reduces cytokine-driven IgA switching, as evidenced by the occurrence of reduced numbers of dual-positive ($mIgM^+ / mIgA^+$) B cells and reduced secretion of IgA relative to IgM.

Materials and Methods

Cell Lines and Culture Conditions. The CH12.LX B cell lymphoma cell line was a generous gift of Dr. Geoffrey Houghton (University of North Carolina, Chapel Hill, NC) (19). CH12.LX.A2, a subclone of CH12.LX cells expressing and secreting exclusively IgA, was established in our laboratory as previously described (20). CH1 ($mIgM$ -expressing lymphoma) and MOPC-315 (IgA-secreting myeloma) were obtained from American Type Culture Collection (Rockville, MD). Cells were maintained in RPMI containing 10% FCS, 5% NCTC 109 media (both from Whittaker M. A. Bio-products, Walkersville, MD), 20 mM Hepes (Biofluids, Rockville, MD), 2 mM L-glutamine (Gibco BRL, Gaithersburg, MD), and 50 μ M 2-ME (Sigma Chemical Co., St. Louis, MO).

Cytokines. rIL-4 was a kind gift of Dr. William E. Paul (Laboratory of Immunology, NIAID, NIH). It consisted of the supernatant of cells infected with an IL-4-expressing recombinant baculovirus. In some of the studies, rIL-4 expressed in yeast (Genzyme, Cambridge, MA), specified as MIL-4 in text and legends, was also used. Porcine TGF- β 1 was purchased from R&D Systems (Minneapolis, MN).

Expression Vector Constructs and Gene Transfection. A genomic BamHI-HindIII fragment of 5'S α containing $I\alpha$, kindly provided by Dr. Janet Stavnezer (University of Massachusetts, Worcester, MA), was filled in at both restriction sites and ligated to the SmaI site, just downstream to mouse mammary tumor virus (MMTV)-LTR of the expression vector plasmid pMSG (Pharmacia Fine Chemicals, Piscataway, NJ) in both sense (pSE-10) and antisense (pAS-11) orientation. A vector with no insert was used as a nonsense control (pM), which only produces transcripts from a selection marker gene. Transfection was performed by electroporation in phosphate-buffered sucrose media (272 mM sucrose, 7 mM sodium phosphate, 1 mM MgCl₂, pH 7.4) using a Gene Pulsar (Bio-Rad Laboratories, Richmond, CA) at 320 V and 25 μ F capacitance.

Coincubation with Oligo DNA. Antisense (AS-M) and sense (SE-M) phosphorothio-oligo DNA (Synthecell, Houston, TX) to nucleotides 231–245 of the RNA sequence from the $I\alpha$ exon (17) were prepared, as were antisense (AS-2) and sense (SE-1) oligos to nucleotides 188–203 or 126–140 of the same exon sequence, respectively. In coincubation studies, CH12.LX B cells were washed twice in HBSS, then cultured in Opti-MEM (GIBCO BRL) containing 5% NCTC 109, and the cytokines were specified at 10⁵ cells per well in 24-well plates, (Costar Corp., Cambridge, MA) for 8 h. The cells were then coincubated with 20–30 μ M of phosphorothio-oligo DNA for 24 h. Finally, after a 24-h incubation, the cells were transferred again to complete media with the cytokines specified and cultured for an additional 2 d, after which they were processed as indicated in Results.

Flow Cytometric Analysis. Membrane (m)Ig was detected by immunofluorescence using an EPICS 753 (Coulter Electronics Inc., Hialeah, FL) or a FACStar Plus[®] (Becton Dickinson & Co., Mountain View, CA). Cells were washed twice with HBSS (Biofluids, Inc., Rockville, MD) followed by resuspension in HBSS containing 0.2% BSA without phenol red (NIH Media Unit) and stained with

FITC anti-IgA and PE anti-IgM, as previously described (21). In most cases, before addition of fluorochrome-labeled antibodies, 30–50 μ g/ml of anti-Fc γ R (2.4G2; Pharmingen, San Diego, CA) was added to each tube to prevent cytophilic binding of fluorochrome-labeled antibody (22). The FITC goat anti-IgA, FITC goat anti-IgG control, and PE goat anti-IgM were obtained from Southern Biotechnology Associates (Birmingham, AL). Propidium iodide was added to final cell suspension and gates were set so as to exclude dead cells.

Proliferation Assays. 8-h [³H]TdR incorporation and viable cell counts using trypan blue to exclude dead cells were used as measures of proliferation as previously described (21). In brief, transfected CH12.LX cells were incubated at a density of 5 × 10⁴ cells/ml in flat-bottomed 96-well plates (Costar Corp.) for 72 h along with various concentrations of IL-4, TGF- β , and dexamethasone. During the last 8 h, 1 μ Ci of [³H]TdR (New England Nuclear, Boston, MA) (sp act, 6.7 Ci/mmol) was added to each well. Radioactivity was measured in a liquid scintillation counter (LS2800; Beckman Instrs., Inc., Fullerton, CA).

ELISA for Ig Secretion. Transfected CH12.LX cells were cultured in 48-well flat-bottomed plates (Costar Corp.) in complete media containing cytokines indicated in Results and/or dexamethasone for 2 or 3 d. Assays were done as previously reported (21). Unlabeled goat anti-IgM, goat anti-IgA and alkaline phosphatase conjugates of goat anti-IgM, and goat anti-IgA were obtained from Southern Biotechnology Associates. Results are presented as the means of assays performed in triplicate. Standard deviations of individual values obtained in these assays were always <10%.

Northern Blot and Southern Blot Analysis. Total RNA was prepared by the acid guanidium thiocyanate-phenol-chloroform extraction method as previously described (23). RNA for reverse transcriptase (RT)-PCR assay was further treated with RQ1 RNase-free DNase (Promega Biotec, Madison, WI) followed by phenol-chloroform extraction. Genomic DNA was prepared by a standard method (24). For Northern analysis, 10 μ g of total RNA from each sample to be analyzed was subjected to electrophoresis in a 1% agarose-formaldehyde gel, transferred onto a Nytran membrane (Schleicher & Schuell, Inc., Keene, NH), and then hybridized to the random-primed ³²P-labeled cDNA probes specified in Results. For Southern analysis, 10 μ g of genomic DNA from various cell lines and liver was digested with the indicated restriction enzymes, subjected to electrophoresis in a 0.8% agarose gel, and transferred onto a Nytran membrane by standard methods. Blotted filters were pretreated with 50% formamide, 5× SSPE, 1× Denhardt's solution, 100 μ g/ml of denatured salmon sperm DNA, then hybridized for 12–16 h at 42°C with probes indicated in Results.

RT-PCR. RT reaction was performed according to the manufacturer's protocol (GIBCO BRL). In brief, heat-denatured RNA (3–5 μ g) was mixed in a final volume of 30 μ l RT reaction buffer containing 1 mM each dNTP (GIBCO BRL), 40 U of RNasin (Promega Biotec), 1.5 μ g of oligo d(pT)₁₆ (Sigma Chemical Co.), 3 μ g of acetyl-BSA, and 300 U of moloney murine leukemia virus (GIBCO BRL) in diethyl procarbonate-treated H₂O. RT was performed at 39°C for 60 min. In a second step, PCR amplification was carried out by adding a small aliquot (5 μ l) of the cDNA obtained in the first step to a PCR reaction mixture that was comprised of 1 mM each of dNTP, 1 μ M each of primers, 1× PCR buffer (10 mM Tris Cl, pH 9.0, 50 mM KCl, 2.5 mM MgCl₂, 0.01% [wt/vol] gelatin), and 0.5 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) in a final reaction volume of 20 μ l. PCR was carried out with a thermal cycler (Perkin Elmer Cetus). The cycle was programmed for initial denaturation by heating to 96°C for 6 min, followed by 25–30 cycles of amplification, each

consisting of a denaturation step at 94°C for 45 s, an annealing step at 60°C for 1 min, and an extension step 72°C for 1.5 min. The final cycle was followed by an extension step at 72°C for 7 min. In the PCR reaction using GAPDH primers, the reaction was terminated at 15 cycles. The number of PCR cycles was adjusted for each primer combination by measuring [³²P]dCTP incorporation so that amplification was carried out during an exponential phase. After agarose separation, PCR products were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc.) and hybridized with specific probes.

Probe DNA. A genomic Styl-PstI fragment of I α that is internal to the I α primer sites was used for detection of I α and I α -C α in RT-PCR, Southern, and Northern blots. A EcoRI-HindIII fragment of p α (J558) for 3'C α , a PstI fragment of p α (J558) for 5'C α , and a PstI fragment of p μ (3741) for 5'C μ were used in Southern blot analysis. Finally, a PstI fragment of GAPDH/pGEM3Z was used as a probe of GAPDH. All probes were labeled by random priming to a specific activity of 5 × 10⁸ to 10⁹ cpm/μg DNA.

Primer Sequences. Primers used in this study were as follows: I α 5' primer, 5'-CAGCCAGACCACAGGCCAGA-3'; I α 3' primer, 5'-GACGACACCGCCTTGTGCCT-3' (nucleotides 1–20 and 481–500 of M29011, respectively); C α 3' primer, 5'-TGTGATGCTGGCATCTGAA-3' (nucleotides 676–695 of V00785); GAPDH 5' primer, 5'-CAGCCAGACCACAGGCCAGA-3'; GAPDH 3' primer, 5'-GCATCGTGGGAAGGGCTCATGAC-3' (nucleotides 330–351 and 554–575 of M32559, respectively).

Data Analysis. Statistical analysis was done by a paired student's *t* test. Blot data were analyzed by densitometry using an Ultrascan XL (Pharmacia LKB, Uppsala, Sweden) and Image Quant™ (Molecular Dynamics, Inc., Sunnyvale, CA).

Results

Effect of IL-4 and TGF- β on IgA Isotype Switching and I α Germline Transcription in CH12.LX B Cells. CH12.LX B cells, in the resting state, are comprised of ~98% mIgM⁺ and 1–2% mIgA⁺ cells. In initial studies, we determined the status of CH12.LX B cells with regard to IgA isotype switching and production of I α GLTs under various culture conditions. In these studies, CH12.LX B cells (5 × 10⁵/ml) were cultured in media containing various concentrations of IL-4 and TGF- β for 3 d and analyzed by flow cytometry on day 4. As shown in Fig. 1, the presence of IL-4 and TGF- β in cultures of CH12.LX B cells additively increased the number of total mIgA⁺ B cells present at the end of the culture. However, while IL-4 led to increased numbers of both mIgM⁺/mIgA⁺ and mIgM⁻/mIgA⁺ cells at every concentration of TGF- β , TGF- β led to decreased numbers of mIgM⁺/mIgA⁺ cells and increased numbers of mIgM⁻/mIgA⁺ cells. Since the effects of IL-4 and TGF- β on IgA isotype switching plateaued at 4–8 × 10³ U/ml and 1 ng/ml, respectively, we have used these concentrations of cytokines in all subsequent studies, unless otherwise specified.

In related studies we determined the effect of TGF- β and IL-4 on I α GLT expression in CH12.LX cells. As shown in Fig. 2, CH12.LX B cells constitutively expressed I α GLTs, and increased such expression upon culture with TGF- β or D10 supernatant, i.e., a Th2 cell line culture supernatant known to contain IL-4 or rIL-4. While maximum induction of I α GLT expression was seen at 12 h in the presence of

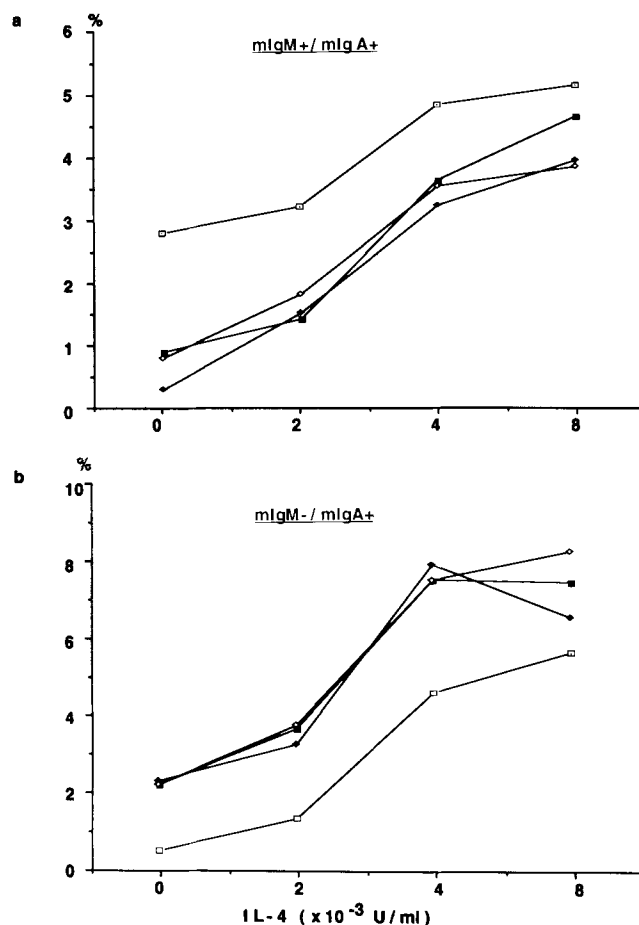


Figure 1. Flow cytometric analysis of CH12.LX B cells cultured in various concentrations of IL-4 and TGF- β for 4 d and stained with PE-labeled anti-IgM and FITC-labeled anti-IgA. (a) mIgM⁺ and mIgA⁺ dual-positive cells, and (b) mIgM⁻ and mIgA⁺ cells. Concentrations of TGF- β are 0 ng/ml (□), 1 ng/ml (◆), 5 ng/ml (■), and 25 ng/ml (◇).

TGF- β , it was seen at 48 h in the presence of either D10 supernatant or rIL-4. Taken together, these data show that TGF- β and IL-4 induce IgA isotype switching in CH12.LX B cells. In addition, they show that such switching is accompanied by increased production of I α germline transcripts as previously reported in other systems (9, 13–15, 25, 26). Finally, these data show that the effects of TGF- β and IL-4 on IgA switching in CH12.LX B cells are distinguishable by their different effects on the number of dual-positive mIgM⁺/mIgA⁺ B cells produced and on the time course of I α GLT expression.

Kinetics of I α GLT Production and Surface Ig Class Switching in Relatively Long-Term Cultures of CH12.LX B Cells. The relationship of I α GLT expression and mIgA switching was further explored in relatively long-term cultures. In this case CH12.LX B cells were cultured in media with or without 1 ng/ml of TGF- β plus 4 × 10³ U/ml IL-4 at the cell density of 5 × 10⁴/ml for ~20 d. At various time points, small aliquots of cells were analyzed for mIgA expression and I α GLT synthesis. Initial cell density was maintained in these cultures by splitting and feeding with fresh media containing

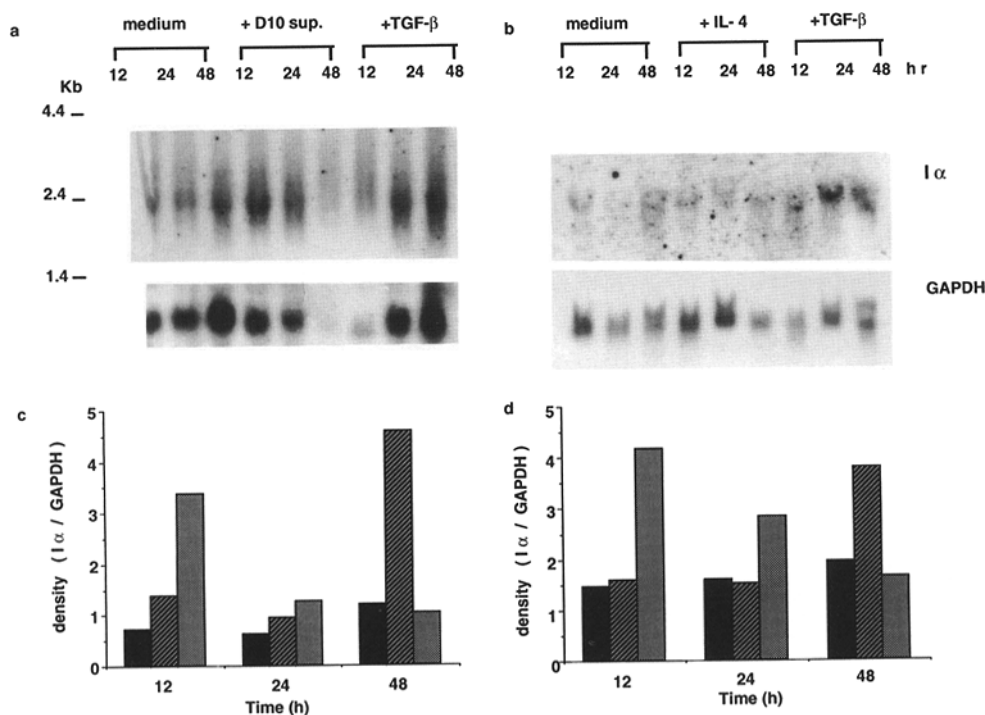


Figure 2. α GLTs of CH12.LX cells induced by D10 cell culture supernatant, TGF- β , and rIL-4. (a and b) Northern blot analysis of α GLTs in different culture conditions and time points: D10 cell supernatant (10%), TGF- β (1 ng/ml), and mouse rIL-4 (MIL-4 100 U/ml). The same filters were rehybridized with a GAPDH probe. (c and d) Histograms of densitometry of α GLT expression normalized with GAPDH in blots a and b, respectively. Media only (filled bar), media + D10 sup. or MIL-4 (hatched bar), and media + TGF- β (stippled bar).

TGF- β /IL-4 every day until day 6 and 2 d thereafter. As shown in Fig. 3, the number of mIgA⁺ cells increased by day 2 and reached a peak at day 8; at this point \sim 20% of the cultured cells were mIgA⁺. Concomitant α GLT expression in cultures containing TGF- β /IL-4 was biphasic with a small initial peak occurring at 24 h of culture and a larger peak occurring after 4–6 d and paralleling in IgA expression; the small peak was not seen in cultures not containing TGF- β (exp. 3). These studies indicate that IgA isotype switching in CH12.LX B cells is quantitatively paralleled by α GLT expression.

Expression Vectors Producing Antisense and Sense α Transcripts. Having demonstrated that α GLT expression correlates with mIgA expression in CH12.LX B cells, we sought to determine the effects of altering the α GLT production on IgA isotype switching. Our approach here was to transfect CH12.LX B cells with an expression vector that, upon induction, would produce antisense transcripts spanning transcription initiation sites and splice donor sites of the α exon to destabilize complementary mRNA produced by host cells (27, 28).

Three expression vectors were constructed for this purpose: pAS-11 (antisense), pSE-10 (sense), and pM (nonsense). In preliminary studies, since germline transcripts can be produced by genes on either the productive or nonproductive allele, we transfected the pAS-11 and the pSE-10 into CH12.LX.A2 cells, i.e., an mIgA⁺ subclone of CH12.LX cells that has deleted α genes from both alleles (see Fig. 4), to demonstrate the transfectability and inducibility of the vectors used. As shown in Fig. 5, CH12.LX A2 cells trans-

fected with pSE-10 produced exogenous α mRNA, and such production was increased 6–15-fold in culture containing 10^{-6} M dexamethasone. Maximum induction was seen at 12–24 h and was augmented by IL-4. Similar results were obtained with the pAS-11 vector. Thus, cells of CH12.LX series can be transfected with the vectors used and the latter can be induced to produce α transcripts in a controlled fashion.

Expression of α and α -C α GLT in CH12.LX Cells Transfected with α Antisense, Sense, and Nonsense Vectors. In the next series of studies, we transfected the expression vectors into CH12.LX B cells. After transfection, we cultured the cells in conventional media for 24 h and then added dexamethasone at a final concentration of 1 μ M and/or cytokines (IL-4/TGF- β) at concentrations used in previous studies for an additional 48 h. Finally, aliquots of cells were analyzed for α GLT expression by RT-PCR.

In these experiments, we used primers at the 5' and 3' ends of the α exon to amplify α mRNA transcribed either from the host cell gene (endogenous α mRNA) or from the antisense or sense vectors (exogenous α mRNA). Simultaneously, PCR was performed using primers at the same 5' end of the α exon and at the second exon of C α to amplify α -C α mRNA transcribed only from host cell gene (see Fig. 6). As shown in Fig. 7, a and b, in the presence of cytokines plus dexamethasone, α signals increased in cells transfected with all three vectors. In contrast, while α -C α signals reflecting endogenous α GLT levels were increased in cells transfected with pSE-10 (sense vector) and pM (nonsense vector), the α -C α signal was markedly decreased in cells transfected with pAS-11 (antisense vector). Thus, transfect-

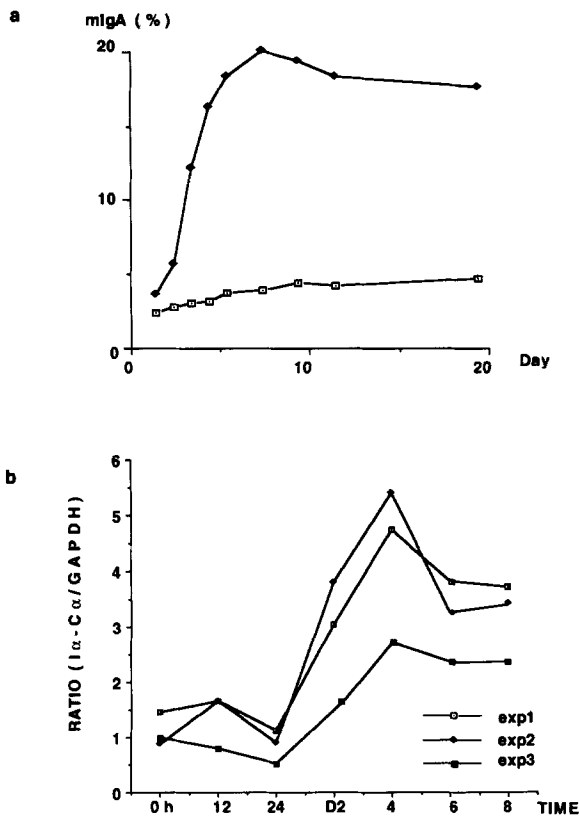


Figure 3. α GLT levels compared with surface IgA expression in CH12.LX B cells in long-term culture with IL-4 and TGF- β . (a) mIgA expression of CH12.LX B cells cultured in media containing 4×10^3 U/ml IL-4 and 1 ng/ml TGF- β (\blacklozenge) and media only (\square). (b) α GLT and GAPDH transcripts measured by RT-PCR at different time points and plotted as the ratio of α GLT/GAPDH signal densities. Media contain IL-4 and TGF- β in exs. 1 and 2, and IL-4 only in exp. 3 (100 U/ml of MIL-4).

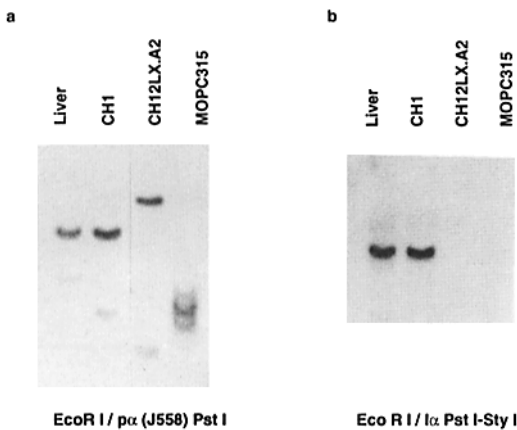


Figure 4. Southern blot analysis of high molecular weight DNA from BALB/c liver, CH1, CH12.LX.A2, and MOPC 315 digested with EcoRI and probed with: (a) PstI fragment of $p\alpha$ (J558) and (b) PstI-StyI fragment of $I\alpha$ genomic DNA.

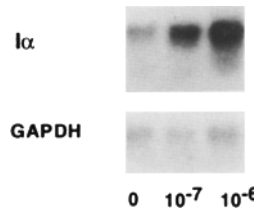


Figure 5. Induction of $I\alpha$ transcripts of pSE-10-transfected CH12.LX.A2 cells, after culture in media containing various concentrations of dexamethasone depicted below the autoradiogram. Shown is one of the representative experiments.

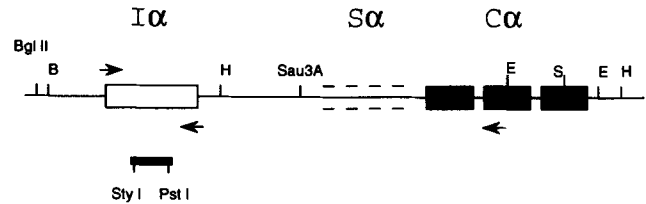


Figure 6. Genomic configuration of IgM-positive CH12.LX cells. Primer sites and the DNA probe used for detection of α GLTs are shown by arrows and a thick bar, respectively. B, BamHI; H, HindIII; E, EcoRI; S, SacI.

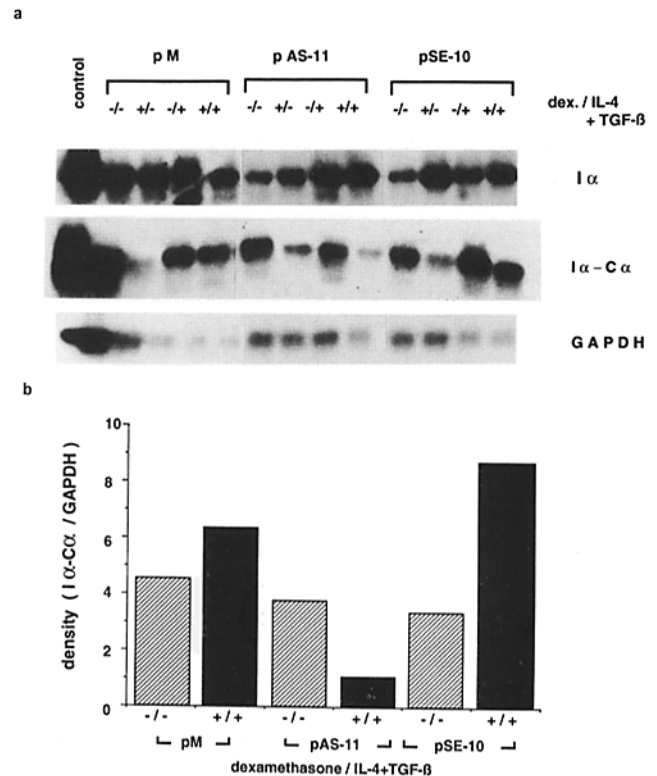


Figure 7. GLTs of CH12.LX B cells transfected with various expression vectors. CH12.LX B cells were transfected with vector constructs and then cultured in the presence or absence of TGF- β (1 ng/ml) + IL-4 (4×10^3 U/ml), and dexamethasone (10^{-6} M) as specified; $I\alpha$, $I\alpha$ -C α , and GAPDH transcripts were then measured by RT-PCR followed by blotting and hybridization with specific probes. As PCR controls, cloned DNA were used for templates. (a) RT-PCR signals; (b) density plots of $I\alpha$ (hatched bar) and $I\alpha$ -C α (filled bar) signals normalized with GAPDH signals.

tion of CH12.LX B cells with an expression vector producing antisense α GLT downregulates the level of α C α germ-line transcripts in cytokine-treated B cells.

Flow Cytometric Analysis of CH12.LX Cells Transfected with Expression Vectors and Nontransfected Control Cells. To determine the effect of α GLT modulation on the emergence of mIgA-bearing cells, aliquots of transfected CH12.LX B cells were subjected to flow cytometric analysis (Fig. 8, *a* and *b*). In these studies, we found that treatment of untransfected CH12.LX cells with TGF- β /IL-4 in cultures containing dexamethasone leads, after 48 h, to a larger percentage of dual-positive (mIgM⁺/mIgA⁺) B cells ($6.7 \pm 0.9\%$) than obtained in culture not containing dexamethasone ($3.2 \pm 0.5\%$; refer to Fig. 1); in contrast, mIgM⁻/mIgA⁺ B cells were correspondingly decreased in dexamethasone-containing cultures (compare *open bar* in Fig. 8 *b* with *curves* in Fig. 1 *b*). This effect of dexamethasone facilitated our evaluation of the effects of transfection on dual-positive cells.

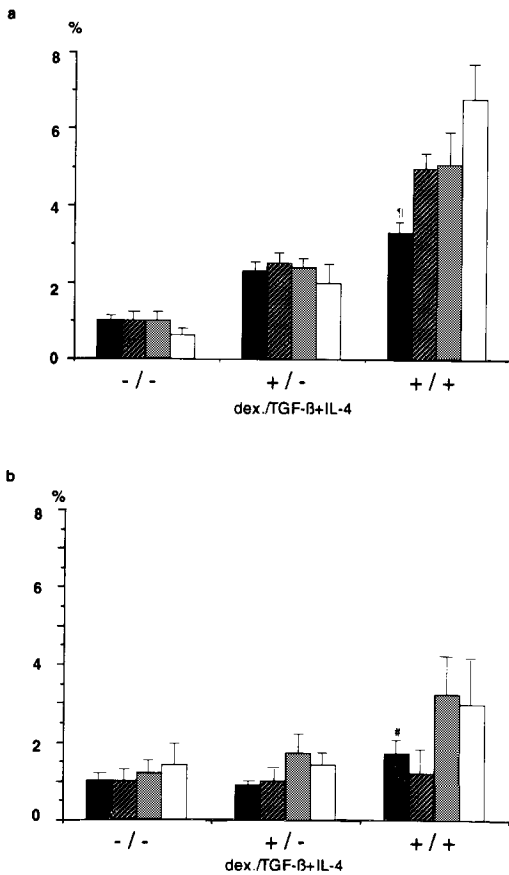


Figure 8. Effects of vector transcripts induction on mIgA expression. Transfected CH12.LX B cells were cultured in media with or without dexamethasone and cytokines (TGF- β /IL-4) as indicated. (Filled bar) pAS-11, (hatched bar) pSE-10, (stippled bar) pM, (open bar) no transfection. (a) Dual-positive mIgM⁺/mIgA⁺ B cells in the presence of dexamethasone and cytokines. Percentage of mIgM⁺/mIgA⁺ double-positive cells in pAS-11 transfected cells (¶) are significantly smaller than the cells transfected with pSE-10 ($p < 0.01$) and pM ($p < 0.05$). (b) mIgM⁻/mIgA⁺ B cells. Percentage of mIgA single-positive cells in pAS-11-transfected cells (#) was not significant ($p > 0.05$) compared with cells transfected with control plasmids.

As shown in Fig. 8 *a*, addition of TGF- β /IL-4 (plus dexamethasone) to cultures of B cells transfected with pSE-11 or pM vectors led to a statistically significant increase in dual-positive mIgM⁺/mIgA⁺ B cells (pSE-10, $p < 0.01$; pM, $p < 0.05$); results shown are the mean \pm SE of at least four separate studies. In contrast, we did not see the significant increase of dual-positive cells by TGF- β /IL-4 in cultures of B cells transfected with pAS-10. In addition, the percentage of B cells that were dual positive after induction with TGF- β /IL-4 was statistically less than that in cultures of pSE-11- or pM-transfected B cells ($p < 0.05$). The effect of transfection followed by culture in TGF- β /IL-4 on the emergence of mIgM⁻/mIgA⁺ B cells was less clear in these studies because, as mentioned, the percentage of such cells generated in the presence of dexamethasone was quite low. In this case, the increase in mIgM⁻/mIgA⁺ cells induced by TGF- β /IL-4 in cultures of both pAS-10- and pSE-11-transfected cells was smaller than the increase in pM-transfected cells but the difference was not significant ($p > 0.05$). Overall, we conclude that CH12.LX B cells transfected with a vector producing antisense transcripts, and thus reducing α GLT levels does in fact undergo decreased cytokine-driven IgA switching, as manifest in the appearance of reduced numbers of dual-positive mIgM⁺/mIgA⁺ switch-intermediate cells.

Effect of Transfection of Expression Vectors on IgM/IgA Secretion. In parallel studies we also determined the ability of the CH12.LX B cells transfected and cultured as described above to secrete IgM and IgA into the culture supernatant in 4 d. Since IgA switch results in decreased IgM and increased IgA production by CH12.LX B cells (these cells do not produce other Ig isotypes), we used the IgA/IgM secretion ratio as the most sensitive indicator of IgA switching.

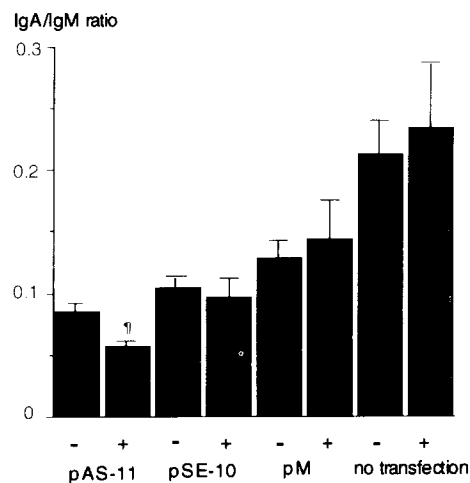


Figure 9. Effects of transfection of CH12.LX B cells on Ig secretion. Results are expressed as ratio of IgA/IgM secreted by transfected CH12.LX B cells. Shown is mean \pm SE of six independent experiments. (-) Media only, (+) media containing dexamethasone (10^{-6} M), TGF- β (1 ng/ml), and IL-4 (4×10^3 U/ml). Significant reduction of IgA/IgM ratio was seen in pAS-11-transfected cells in the culture containing TGF- β , IL-4, and dexamethasone (¶), compared with control (-) and with control plasmids, pSE-10, and pM ($p < 0.05$).

This also allowed us to normalize for different numbers of surviving B cells under the different culture conditions. As shown in Fig. 9, only in cultures of B cells transfected with the vector pAS-11 was there a significant reduction in the IgA/IgM secretion ratio. These results indicate that down-regulation of α GLT transcripts in CH12.LX B cells leads to decreased IgA switching, as shown by decrease of cytokine-induced IgA secretion.

Effects of Expression Vector Transcription on CH12.LX Cell Proliferation. One possible explanation of the effect of the expression vector producing antisense transcripts on IgA isotype differentiation was that this vector selectively altered cell proliferation, thereby modulating the number of cells that undergo isotype differentiation. We therefore measured cell proliferation in cultures containing various doses of dexamethasone along with IL-4 and TGF- β . We found that while dexamethasone reduced cell proliferation in a dose-dependent manner, there were no significant differences in proliferation among the cultures transfected with pAS-10, pSE-11, and pM (Fig. 10). In addition, we did not see a significant difference in the proliferation between CH12.LX and post-switch cell CH12.LX.A2 when these cells were transfected with pAS-11 (data not shown). Thus, it is unlikely that the effects of the antisense expression vector were due to selective suppression of cell proliferation.

Effect of Modulation of α GLT on IgA Switching of CH12.LX B Cells as a Result of Incubation of Cells with Phosphorothioate Oligo DNA. In separate studies we modulated α GLT expression in CH12.LX B cells by incubation of these cells with antisense or sense phosphorothioate oligo DNA (27, 28). This approach allowed us to determine effects of α GLT modulation in the absence of the effects of electroporation or dexamethasone in culture. Accordingly, we cultured CH12.LX B cells with phosphorothioate oligo DNA (25 μ M) in serum-free media in the presence and absence of TGF- β /IL-4 for

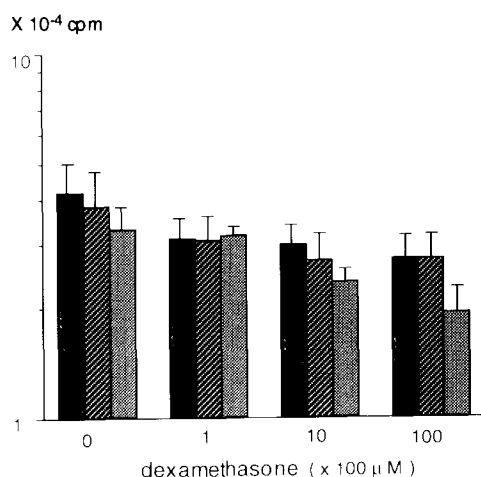


Figure 10. Effects of transcription from expression vectors on proliferation of transfected cells. CH12.LX B cells were transfected with expression vectors and cultured in media containing TGF- β (1 ng/ml), IL-4 (4×10^3 U/ml), and various concentrations of dexamethasone as indicated. pAS-10 (filled bar), pSE-11 (hatched bar), and pM (stippled bar).

24 h and recultured them in complete media containing TGF- β /IL-4 for an additional 72 h. We then measured IgM and IgA in the culture media and analyzed cells for the presence of mIgA⁺ cells. As shown in Table 1, the presence of antisense oligo DNA led to a significantly reduced IgA/IgM ratio ($p < 0.01$) as compared with sense oligo DNA (triplicate studies). Furthermore, as shown in Table 2, antisense

Table 1. Effect of Antisense and Sense Oligo DNA on Ig Secretion by CH12.LX Cells

	IgA	IgM	IgA/IgM ratio
	ng/ml	ng/ml	
Media	375 \pm 75.1	1,880 \pm 117	0.199 \pm 0.025
+ TGF- β /IL-4	640 \pm 133	2,443 \pm 132	0.262 \pm 0.037
+ AS-M	231 \pm 22.8	2,091 \pm 144	0.110 \pm 0.015*
+ SE-M	435 \pm 65.2	1,907 \pm 60.5	0.228 \pm 0.027
+ AS-2	204 \pm 18.9	2,068 \pm 83.9	0.098 \pm 0.005*

Effects of sense (SE-M) and antisense (AS-M, AS-2) phosphorothio-oligo DNA on Ig secretion. The results represent mean \pm SE of four experiments of triplicate assay for each experiment. IgA/IgM ratio was significantly reduced in culture containing antisense oligo compared with culture containing sense oligo.

* $p < 0.01$.

Table 2. Effect of Antisense and Sense Oligo DNA on Cytokine-induced mIg Expression of CH12.LX Cells

	mIgM ⁺ /IgA ⁺		mIgM ⁻ /IgA ⁺	
Media	0.88 \pm 0.16		1.69 \pm 0.35	
Media + IL-4/TGF- β	3.35 \pm 0.63		5.55 \pm 0.76	
Exp.	AS*	SE*	AS [†]	SE [†]
1	1.52	1.88	3.85	4.79
2	1.98	2.08	5.45	5.48
3	1.89	2.39	4.63	5.17
4	2.23	2.47	4.87	5.95
5	1.70	2.45	4.61	3.81
6	2.69	3.11	4.25	4.48
7	1.99	3.53	5.48	5.62
8	2.83	3.85	5.81	6.20

Effects of sense (SE-M, SE-1) and antisense (AS-M, AS-2) phosphorothio-oligo DNA on mIgA and mIgM expression in cytokine-treated CH12.LX B cells. Oligo DNA of AS-M and SE-M in expts. 1–4 and AS-2 and SE-1 in expts. 5–8 were used as antisense and sense oligo, respectively. Difference in AS and SE is significant in mIgM⁺/mIgA⁺ cells and not significant in mIgM⁻/mIgA⁺ cells, by paired student's t test.

* $p < 0.01$.

† $p > 0.1$.

oligo DNA led to a decrease in the number of mIgM⁺/mIgA⁺ B cells occurring at the end of culture as compared with sense oligo DNA ($p < 0.01$). These results are thus concordant with the results obtained by transfection of expression vectors as described above.

Discussion

In previous studies, we have shown that when CH12.LX B cells are incubated with IL-4, the number of mIgA⁺ cells increases, and that this increase is not due to preferential increase of mIgA⁺ cells, but rather is due to switching from mIgM⁺ to mIgA⁺ cells (20). In the present study, we have shown that TGF- β also enhances the differentiation of mIgM⁺ CH12.LX B cells into mIgA⁺ B cells and that TGF- β acts additively with IL-4 in this respect. In addition, we have shown that TGF- β increases the production of α GLTs and thus is similar to IL-4 in respect to the latter's effect on B cells undergoing IgG1 and IgE differentiation (6, 22).

In the course of these studies, we observed a clear-cut difference between the effect of TGF- β and IL-4 on CH12.LX B cell isotype differentiation. In short-term culture, addition of TGF- β to CH12.LX B cell cultures caused a decrease in mIgM⁺/mIgA⁺ B cells and an increase in mIgM⁻/mIgA⁺ B cells, whereas IL-4 caused an increase in both cell types. Moreover, TGF- β induced α GLT production peaking as early as 12 h, whereas IL-4 induced α GLT production peaking at 48 h or later. Similarly, in long-term culture, whereas addition of TGF- β and IL-4 to CH12.LX B cell culture led to an initial α GLT peak at 12 h followed by a second, larger peak at day 4 (which correlated with peak mIgA⁺ B cell expression), addition of IL-4 alone led to the appearance of the second peak, but not the only early peak. Taking the results of Northern studies and both short- and long-term cultures together, it seems likely that the early peak is produced by TGF- β acting early on B cells already committed to α GLT synthesis, whereas the second peak is cell cycle related and due to IL-4 acting later to increase the number of cells producing α GLT.

The CH12.LX B cell differentiation pathway as defined above allowed us to delineate the role of α GLT in IgA switching. In our main approach to this question, we transfected CH12.LX B cells with an inducible expression vector producing antisense, sense, or nonsense transcripts with respect to the I α exon (29–33). In initial studies, we found that transfection of an I α antisense expression vector into cells followed by induction led to a marked decrease in endogenous I α -C α GLT levels; in contrast, in cells transfected with sense or nonsense control vectors, endogenous I α -C α GLT transcripts were increased. Thus, the transfection of CH12.LX B cells with antisense expression vectors followed by appropriate induction did indeed downregulate α GLT transcripts.

In further studies, we found that in CH12.LX B cells transfected with an antisense-producing vector and then treated with TGF- β /IL-4 there was a smaller increase in the occurrence of dual-positive mIgM⁺/mIgA⁺ B cells as compared with cells transfected with the control vectors. The effect of

antisense vector transfection on the emergence of dual-positive cells correlated with the effect in TGF- β /IL-4-induced IgA secretion since such transfection also led to a decrease in secretion of IgA relative to IgM.

In accompanying studies we determined that the transfection and induction procedures used do not, in themselves, explain the effects on CH12.LX B cell switching. First, these effects were not due to the inducing steroid since addition of dexamethasone alone to cultures of CH12.LX B cells led to increased switching to IgA. Second, in six independent experiments, we did not observe a significant difference in the growth of CH12.LX B cells that could be ascribed to differences in the type of vector transfected. This last fact indicates that the observed effects of the antisense expression vector were not due to the preferential inhibition of cell cycling, which is presumed to be necessary for B cells to undergo switch (34, 35).

In a second approach to the question of how α GLT transcription is related to IgA switching, we cultured CH12.LX B cells in the presence of phosphorothio-oligo DNA that were antisense and sense to either transcription initiation sites or putative translation initiation sites of the I α exon. This approach was complementary to the first since it avoided the use of transfection and steroid exposure. The results were consistent with those obtained with transfection in that we again observed decreased appearance of dual-positive mIgM⁺/mIgA⁺ cells and a decreased IgA/IgM secretion ratio when cells were exposed to antisense but not sense oligo DNA.

In both of the experimental approaches, the primary effect of reducing α GLT level appears to be on the number of mIgM⁺/mIgA⁺ dual-positive cells. In previous studies, we have shown that when CH12.LX B cells are incubated with IL-4, the number of dual-positive mIgM⁺/mIgA⁺ cells increases (20). In addition, murine Peyer's patch B cells are composed of mIgA⁺ cells, of which ~50% are mIgM⁺/mIgA⁺ dual-positive cells (Ehrhardt, R. O., and W. Strober, manuscript submitted for publication). Finally, stable dual-positive subclones of CH12.LX (36) and BCL₁ (10, 37) can be derived from cells undergoing switching, and these dual-positive cells can become single-positive cells in culture (20). Thus, dual-positive mIgM⁺/mIgA⁺ B cells appear to represent a switch-intermediate cell producing two class of Ig simultaneously even though the C_H genes are in germline configuration (38–40). It has been recently suggested that such cells arise as a result of a "discontinuous transcription and splicing" mechanism, which allows widely separated stretches of DNA to be transcribed and spliced (37, 41). One possible explanation of our results, therefore, is that germline transcripts containing I region transcripts may facilitate such a mechanism. A second molecular mechanism for production of dual-positive cells, i.e., the "alternative splicing of long-transcripts model" (38), is also compatible with our results since such transcripts would theoretically contain I region sequences, which can be destabilized by antisense transcripts.

When α GLT levels were decreased by either of the methods used here, we observed a reduction in IgA secretion relative to IgM secretion. We can envision two possible explanations for this phenomenon. The first and more likely explanation

is that reduced α GLT levels lead to decreased formation of dual-positive mIgM⁺/mIgA⁺ B cells, which then ultimately give rise to a decreased number of IgA-secreting B cells. A second explanation is based on recent studies by Gaff and Gerondakis (42) showing that I α GLT produced by I.29 μ cells can be spliced to C α transcript containing either a secretory (α_s) or membrane (α_m) domain. They suggested that when the 3' most splice donor site was used, the 3' most potential transcription initiation codon in I α is in phase with the C α open reading frame. On this basis, it is possible that a truncated form of secretory IgA is produced by mIgM-

positive CH12.LX cells, which produce I α GLTs. Therefore, destabilization of such a form of GLTs by antisense methods would theoretically lead directly to decreased IgA secretion.

Overall, the results presented here provide strong evidence that α GLT production is quantitatively associated with IgA switching and, more importantly, that such production is not simply an epiphenomenon of the switch process. Rather, such transcripts appear to play a key role in an early stage of IgA isotype switching, during which cells express C μ and C α simultaneously before deletional rearrangement.

We thank Gregory R. Harriman for his suggestions, and Akira Shimizu, Jeffrey Cohen, and Fred D. Finkelman for reading manuscript. We also acknowledge Susan Barbieri and David Stephany for help with flow cytometric analysis.

Address correspondence to Yoshio Wakatsuki, Mucosal Immunity Section, Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, Building 10, Room 11 N 250, National Institutes of Health, Bethesda, MD 20892.

Received for publication 20 October 1992 and in revised form 1 April 1993.

References

1. Kataoka, T., T. Kawakami, N. Takahashi, and T. Honjo. 1980. Rearrangement of immunoglobulin γ 1-chain gene and mechanism for heavy-chain class switch. *Proc. Natl. Acad. Sci. USA*. 77:919.
2. Alt, F.W., T.K. Blackwell, R.A. DePinho, M. Reth, and G.D. Yancopoulos. 1986. Regulation of genome rearrangement events during lymphocyte differentiation. *Immunol. Rev.* 89:5.
3. Honjo, T., A. Shimizu, and Y. Yaoita. 1989. Constant-region genes of the immunoglobulin heavy chain and the molecular mechanism of heavy chain class switching. In *Immunoglobulin Genes*. T. Honjo, F.W. Alt, and T. Rabbits, editors. Academic Press Limited, London. 123-149.
4. Finkelman, F.D., J. Holmes, I.M. Katona, J.F. Urban, Jr., M.P. Beckman, L.S. Park, K.A. Schooley, R.L. Coffman, T.R. Mossman, and W.E. Paul. 1990. Lymphokine control of in vivo immunoglobulin isotype selection. *Annu. Rev. Immunol.* 8:303.
5. Esser, C., and A. Radbruch. 1990. Immunoglobulin class switching: molecular and cellular analysis. *Annu. Rev. Immunol.* 8:717.
6. Isakson, P.C., E. Pure, E.S. Vitteta, and P.H. Krammer. 1982. T cell derived B cell differentiation factor(s). Effect on the isotype switch of murine B cells. *J. Exp. Med.* 155:734.
7. Leberman, D.A., and R.L. Coffman. 1988. Interleukin 4 causes isotype switching to IgE in T cell-stimulated clonal B cell cultures. *J. Exp. Med.* 168:853.
8. Snapper, C.M., F.D. Finkelman, and W.E. Paul. 1988. Differential regulation of IgG1 and IgE synthesis by interleukin 4. *J. Exp. Med.* 167:183.
9. Berton, M.T., and E.S. Vitteta. 1990. Interleukin 4 induces changes in the chromatin structure of the γ 1 switch region in the resting B cells before switch recombination. *J. Exp. Med.* 172:375.
10. Chen, Y.-W., C.J. Word, S. Jones, J.W. Uhr, P.W. Tucker, and E.S. Vitteta. 1986. Double isotype production by a neoplastic B cell line. *J. Exp. Med.* 164:548.
11. Stavnezer, J., S. Sirlin, and J. Abbot. 1985. Induction of immunoglobulin isotype switching in cultured I.29 lymphoma cells: characterization of the accompanying rearrangements of heavy chain genes. *J. Exp. Med.* 161:577.
12. Alberini, C., R. Biassoni, S. DeAmbrosio, D. Vismara, and R. Sitia. 1987. Differentiation in the murine B cell lymphoma I.29: individual μ + clones may be induced by lipopolysaccharide to both IgM secretion and isotype switching. *Eur. J. Immunol.* 17:555.
13. Stavnezer-Nordgren, J., and S. Sirlin. 1986. Specificity of immunoglobulin heavy chain switch correlates with activity of germline heavy chain genes prior to switching. *EMBO (Eur. Mol. Biol. Organ.) J.* 5:95.
14. Lutzker, S., P. Rothman, R. Pollack, R. Coffman, and F.W. Alt. 1988. Mitogen- and IL-4-regulated expression of germline Ig γ 2b transcripts: evidence for directed heavy chain class switching. *Cell.* 53:177.
15. Stavnezer, J., G. Radcliff, Y.-C. Lin, J. Nietupski, L. Berggren, R. Sita, and E. Severinson. 1988. Immunoglobulin heavy-chain switching may be directed by prior induction of transcripts from constant-region genes. *Proc. Natl. Acad. Sci. USA*. 85:7740.
16. Schmitz, J., and A. Radbruch. 1989. An interleukin 4-induced DNase I hypersensitive site indicates opening of the γ 1 switch region prior to switch recombination. *Int. Immunol.* 1:570.
17. Radcliffe, G., Y.-C. Lin, M. Julius, K.B. Marcu, and J. Stavnezer. 1990. Structure of germ line immunoglobulin α heavy-chain RNA and its location on polysomes. *Mol. Cell. Biol.* 10:382.
18. Reaban, M., and J.A. Griffin. 1990. Induction of RNA-

- stabilized DNA conformers by transcription of immunoglobulin switch region. *Nature (Lond.)*. 348:342.
19. Haughton, G., L.W. Arnold, G.A. Bishop, and T.J. Mercolino. 1986. The CH series of murine B cell lymphomas: neoplastic analogues of Ly-1⁺ normal B cells. *Immunol. Rev.* 93:35.
 20. Kunimoto, D.Y., G.R. Harriman, and W. Strober. 1988. Regulation of IgA differentiation in CH12.LX B cells by lymphokines. *J. Immunol.* 141:713.
 21. Harriman, G.R., D.Y. Kunimoto, J.F. Elliot, V. Paetkau, and W. Strober. 1988. The role of IL-5 in IgA B cell differentiation. *J. Immunol.* 140:3033.
 22. Snapper, C.M., F.D. Finkelman, D. Stefany, D.H. Conrad, and W.E. Paul. 1988. IL-4 induces co-expression of intrinsic membrane IgG1 and IgE by murine B cells stimulated with lipopolysaccharide. *J. Immunol.* 141:489.
 23. Chomczynski, R., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156.
 24. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY. 9.2 pp.
 25. Rothman, P., S. Lutzker, B. Gorham, V. Stewart, R. Coffman, and F.W. Alt. 1990. Structure and expression of germline immunoglobulin γ 3 heavy chain gene transcripts: implications for mitogen and lymphokine directed class-switching. *Int. Immunol.* 2:621.
 26. Berton, M.T., J.W. Uhr, and E.S. Vitetta. 1989. Synthesis of germ-line γ 1 immunoglobulin heavy-chain transcripts in resting B cells: induction by interleukin 4 and inhibition by interferon γ . *Proc. Natl. Acad. Sci. USA.* 86:2829.
 27. Minshull, J., and T. Hunt. 1986. The use of single-stranded DNA and RNase H to promote quantitative 'hybrid arrest of translation' of mRNA/DNA hybrids in reticulocyte lysate cell-free translations. *Nucleic Acids Res.* 14:6433.
 28. Helene, C., and J.-J. Toulme. 1990. Specific regulation of gene expression by antisense, sense and antigenic nucleic acids. *Biochem. Biophys. Acta.* 1049:99.
 29. Sagata, N., M. Oskarsson, T. Copeland, J. Brumbaugh, and G.F. Vande Woude. 1988. Function of *c-mos* proto-oncogene product in meiotic maturation in *Xenopus* oocytes. *Nature (Lond.)*. 335:519.
 30. Holt, J.T., T. Venkat Gopal, A.D. Moulton, and A.W. Nienhuis. 1986. Inducible production of c-fos antisense RNA inhibits 3T3 cell proliferation. *Proc. Natl. Acad. Sci. USA.* 83:4794.
 31. Han, L., J.S. Yun, and T.E. Wagner. 1991. Inhibition of Moloney murine leukemia virus-induced leukemia in transgenic mice expressing antisense RNA complementary to the retroviral packaging sequences. *Proc. Natl. Acad. Sci. USA.* 88:4313.
 32. Hambor, J.E., M.L. Tykocinsky, and D.R. Kaplan. 1988. Functional sequences of antisense RNA-mediated inhibition of CD8 surface expression in a human T cell clone. *J. Exp. Med.* 168:1237.
 33. Stein, C.A., and J.S. Cohen. 1988. Oligodeoxynucleotides as inhibitors of gene expression: a review. *Cancer Res.* 48:2659.
 34. Severinson-Gronowicz, E., C. Doss, and J. Schröder. 1979. Activation to IgG secretion by lipopolysaccharide requires several proliferative cycles. *J. Immunol.* 123:2057.
 35. Dunnick, W., M. Wilson, and J. Stavenezer. 1989. Mutation, duplication, and deletion of recombined switch regions suggest a role for DNA replication in the immunoglobulin heavy-chain switch. *Mol. Cell. Biol.* 9:1850.
 36. Kunimoto, D.Y., M.C. Sneller, L. Claffin, J.F. Mushinski, and W. Strober. 1993. Molecular analysis of double isotype expression in IgA switching. *J. Immunol.* 150:1338.
 37. Nolan-Willard, M., M.T. Berton, and P. Tucker. 1992. Coexpression of μ and γ 1 heavy chains can occur by a discontinuous transcription mechanism from the same unrearranged chromosome. *Proc. Natl. Acad. Sci. USA.* 89:1234.
 38. Yaoita, Y., Y. Kumagai, K. Okumura, and T. Honjo. 1982. Expression of lymphocyte surface IgE does not require switch recombination. *Nature (Lond.)*. 297:697.
 39. Perlmutter, A., and W. Gilbert. 1984. Antibodies of secondary response can be expressed without switch recombination in normal mouse B cells. *Proc. Natl. Acad. Sci. USA.* 81:7189.
 40. Chen, Y.-W., C.J. Word, V. Dev, J.W. Uhr, E.S. Vitetta, and P.W. Tucker. 1986. Double isotype production by a neoplastic B cell line. II. Allelically excluded production of μ and γ 1 chain without C_H gene rearrangement. *J. Exp. Med.* 164:562.
 41. Shimizu, A., M.C. Nussenzweig, H. Han, M. Sanchez, and T. Honjo. 1991. Trans-splicing as a possible molecular mechanism for the multiple isotype expression of the immunoglobulin gene. *J. Exp. Med.* 173:1385.
 42. Gaff, C., and S. Gerondakis. 1990. RNA splicing generates alternative forms of germline immunoglobulin heavy chain transcripts. *Int. Immunol.* 2:1143.