Germline transcription and recombination of a murine VDJ_{\mu\delta\gamma}^1 transgene

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Abstract

To investigate the regulation of Ig switch recombination, we have constructed mice with a 56 kb VDJ_{\mu\delta\gamma}^1 transgene. This transgene included an anti-nitrophenyl VDJ segment, S_{\mu}, C_{\mu}, C_{\delta}, I_{\gamma}^1, S_{\gamma}^1, C_{\gamma}^1 and the C_{\gamma}^1 membrane exons from the murine Igh a haplotype. Two founder lines were produced, with very similar characteristics. Transgenic B cells expressed normal amounts of C_{\mu} (which is \(\geq\) 95% transgenic), C_{\delta} and other cell surface markers, and normal amounts of VDJ and C_{\mu} RNA. \(\gamma^1\) germline transcription of the transgenes is properly regulated since stable transcripts were not expressed in B cells treated with lipopolysaccharide (LPS) alone, nor in thymus or non-lymphoid tissues, but were expressed after treatment of B cells with LPS + IL-4 or CD40L + IL-4. B cells from both lines of transgenic mice expressed transgenic \(\gamma^1\) after in vitro culture with CD40L + IL-4, but not after culture with CD40L alone. However, the CD40L + IL-4 induced IgG1 precursor frequency is much lower for VDJ_{\mu\delta\gamma}^1 transgenic B cells (1:240–760) than for non-transgenic B cells (1:9). Analysis of DNA from transgenic hybridomas indicated that switch recombination can take place in switch (S) regions, but can also take place outside S regions. These results indicate that targeting of switch recombinase to S regions must include regulation beyond the S regions themselves and correct germline transcription. This additional regulation might include cis-acting elements or appropriate spacing or arrangement of the recombining elements.

Introduction

The heavy chain switch is mediated by a deletion event that moves an assembled VDJ region from juxtaposition to C_{\mu} to a new position next to C_{\gamma}, C_{\epsilon} or C_{\alpha}. The deletion event begins and ends in switch (S) regions, 2–10 kb segments of simple sequences repeated in tandem that lie upstream of each heavy chain gene except C_{\delta} (1–2). Switch recombination does not seem to require any specific primary sequence (3), rather it has been termed ‘regionally specific recombination’ (4). One important question is how the unknown switch recombinase is restricted to activity on heavy chain genes—why aberrant switch recombination to DNA like the c-myc gene is apparently infrequent (5–7). Although chromatin accessibility is usually considered in the context of isotypic specificity of the heavy chain switch, part of ‘regional’ specificity for S regions might also be controlled this way. Chromatin accessibility is induced by cytokine signals at the surface of a B lymphocyte; the signals are transduced to the nucleus and direct recombination to the S regions associated with one or two of the six heavy chain genes available (1,2). Cytokines also induce germline transcription of heavy chain C genes (8,9). For example, germline transcription and subsequent switch recombination of the murine \(\gamma^1\) gene is induced by IL-4, and is most pronounced if the B cells are also activated by lipopolysaccharide (LPS) or by CD40 ligation (10–13).

Attempts to study switch recombination on extrachromosomal substrates have emphasized the importance of regionally specific recombination (14–17). The frequency of recombination on transiently transfected episomes is dramatically increased by the presence of S region sequences, and, similar to recombination of heavy chain genes, is influenced by transcription and by enhancer elements (14–

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16. On the other hand, recombination of extrachromosomal substrates occurs often outside the S regions. In the absence of selection for drug resistance genes on the episomes, the vast majority of recombination events may be outside S regions.

Hoping to achieve recombination more like that of endogenous genes, several groups have used chromosomally integrated switch substrates or stable episomes. These have been introduced into cells by retroviruses (18,19), stable transfection (20) and transgenesis (21–25). Taylor and colleagues produced a human minilocus transgene, which includes multiple V, D and J segments, as well as Cμ, Cδ and Cγ1, and apparently undergoes switch recombination. After immunization of transgenic mice, human Cγ1 was produced (22,23). Two hybridomas that expressed human Cγ1 also had rearranged human Sγ1 segments (22). However, the efficiency of switch recombination on the human minilocus, compared to endogenous genes, is unknown. It would be difficult to study regulation of germline transcription or recombination using the human minilocus transgene since the agents that induce human y1 germline transcription and recombination are poorly defined (26–28). It is also unclear if murine regulatory factors and switch recombinase would interact properly with the human gene sequences.

To avoid some of these problems, we designed a minilocus transgene that includes murine VDJ, Cμ, Cδ and Cγ1. Regulation of the murine y1 gene is well-defined. y1 transgenes are subject to the same regulation of germline transcription as is the endogenous y1 gene, even though they are removed from other regulatory elements in the heavy chain locus (24–25). We were able to induce switch recombination of the VDJμδ1 transgene in vitro, using CD40L+ IL-4 activation of transgenic B cells. If the VDJμδ1 transgene included all of the critical substrate and regulatory elements, one would expect switch recombination only after the correct stimuli, in the correct location and at a rate similar to that of the endogenous locus. We studied the regulation of germline transcription, the regulation of switch recombination, the DNA location of recombination events and the frequency of y1 expression from the transgene, relative to the endogenous locus.

Methods

Preparation of transgenic mice

py1/HE17mem included a 10 kb HindIII–EcoRI fragment (l1) and Sγ1, a 6.8 kb EcoRI fragment (Cγ1) and a 5.4 kb EcoRI fragment (y1 membrane exons), cloned into the HindIII and EcoRI sites of pBR325 in the order and orientation found in the mouse genome. The 22 kb HindIII fragment, which included all but the 3′-most 2 kb of py1/HE17mem was moved into the HindIII site of pROR. pROR included pBR322 from nucleotide 1441 to 4363 and a polylinker with several rare-cutting restriction enzyme sites. The resulting plasmid was digested with SfiI at two sites that flank the 22 kb HindIII fragment and the resulting 22 kb SfiI fragment was ligated into the SfiI site 3′ of C9 in the plasmid HC186. HC186 included 4.0 (VDJβ1.8 and Eμ), 12.3 (Sα and Cμ), 10 (Cδ) and 2.0 kb EcoRI fragments, and a 2.4 kb EcoRI–SfiI fragment in the order and orientation found in the genome (29). A final plasmid, VDJμδ1, included VDJμδ and ISγ1 in the same transcriptional orientation. All of the C genes in VDJμδ1 were derived from BALB/cJ (a allotype) mice. The 56 kb insert fragment in VDJμδ1 was separated from the vector by NotI digestion, purified on an agarose gel and then by density centrifugation in cesium chloride, and injected into (SJL×C57BL/6)F2 eggs for the production of transgenic mice. Founders were mated to (SJL×C57BL/6)F2 mice to maintain the transgene with an Igμγ1 endogenous gene.

Southern hybridization

Southern hybridization was carried out in 6×SSC, 2% SDS, 10% dextran sulfate at 61°C for 16–40 h, using nick-translated purified restriction fragments. After hybridization, filters were washed twice in 6×SSC, 0.5% SDS, 61°C for 30 min. In some cases, an additional 1×SSC wash at 61°C for 15 min was used. Probes used included a 4.0 kb EcoRI fragment (VDJ from the B1-8 hybridoma and 5′ flanking DNA), a 1.6 kb BanHI–EcoRI fragment (Jμ and Eμ), a 1.6 kb SstI fragment (Sμ), a 2.1 kb EcoRI–SstI fragment (just 3′ of Sγ1) and a 3.5 kb BanHI–EcoRI fragment (Cγ1).

Flow cytometry and ELISA

Approximately 10⁶ splenocytes were washed twice in RPMI 1640 containing 5% FBS and 0.2% sodium azide. Cells were first stained with biotinylated antibodies to mouse μa (clone DS-1; PharMingen, San Diego, CA), μb (clone AF6-78; PharMingen) or δ (clone SBA-1, Southern Biotechnology Associates, Birmingham, AL) and then counterstained with streptavidin–FITC (Southern Biotechnology Associates) and R-phycocerythrin (PE)–anti-CD45R/B220 (clone RA3-6B2; PharMingen). Cells were stained by standard procedures and analyzed on a FACScan equipped with Lysys II software (Becton Dickinson, San Jose, CA). AutoComp settings were initially established with CaliBRITE beads; additional settings were done by eye on samples of singly stained cells.

ELISA for IgM antibodies used plates coated with 10 μg/ml goat anti-mouse IgM and alkaline phosphatase-conjugated anti-lgM, biotinylated anti-μδ or biotinylated anti-μb. ELISA for IgG1 antibodies used coating with 10 μg/ml anti-lgG1 or 3 μg/ml anti-lgG1* and alkaline phosphatase-conjugated anti-lgG1. Standard curves were generated using purified, monoclonal IgG1 and IgM. Antibodies were purchased from Southern Biotechnology or from PharMingen (allotype reagents).

S1 nuclease protection assay

S1 nuclease protection was performed as described (30). In brief, continuously labeled single-stranded probes were hybridized for 16 h at 43°C to 8–15 μg of total RNA in 20 μl 50% formamide, 0.4 M NaCl, 40 mM PIPEs (pH 6.4) and 1 mM EDTA. The M13 polylinker and primer parts of the probe were isolated by digestion, purified on an agarose gel and then by density centrifugation in cesium chloride, and injected into (SJL×C57BL/6)F2 eggs for the production of transgenic mice. Founders were mated to (SJL×C57BL/6)F2 mice to maintain the transgene with an Igμγ1 endogenous gene.
and endogenous transcripts), MM58 (Iγ1) and pA1.85 (actin) have been described (24).

Cell culture

Splenic leukocytes were prepared by lysis of red blood cells with 0.14 N ammonium chloride, 0.017 M Tris (pH 7.3) or T-depleted splenic leukocytes were prepared by treatment with anti-Thy-1 antibody and complement and purification of surviving cells on Lympholyte M (32). Cells were cultured at 4 x 10⁶/ml for 3 days (for RNA) or at 2 x 10⁵/ml for 7 days (for antibody secretion) in RPMI 1640 supplemented with 10% FBS, glutamine, mercaptoethanol, penicillin and streptomycin. LPS was added to 20 µg/ml (Sigma, St Louis, MO). Recombinant murine IL-4 was added as 10% supernatant of the X63.IL4 cell line (33) or at 35 ng/ml purified protein (Biosource, Camillo, CA). SF9 cells expressing CD40L (13) were added at 1:10 splenic leukocytes in culture and were the kind gift of Wendy Warren and Michael Berton (University of Texas Southwestern Medical Center, San Antonio). In some experiments 10% supernatant of the D10.G4.1 (D10) T cell hybridoma (34), activated for 4 days with conalbumin and irradiated splenocytes (35), was added. Hybridomas were prepared from splenic B cells cultured for 5 days in the presence of CD40L + IL-4, using X63.6.5.3 as a fusion partner.

T-depleted splenic lymphocytes were cultured in limiting numbers in 96-well plates in the tissue culture media described above. Each well included 5–25 (non-transgenic) or 200–1400 (transgenic) T-depleted splenocytes, 1000 CD40L expressing SF9 cells and 20,000 3T3 fibroblasts. After 7 days of culture, 50 µl of culture supernatant was tested for IgG1 production by a sandwich ELISA. Wells treated with CD40L + IL-4 (35 ng/ml) were considered positive for IgG1 production if the absorbance in the ELISA assay was greater than the mean of the CD40L wells + 3 SD (e.g. 0.06 + 3 x 0.02 = 0.12).

Results

Preparation of VDJµδγ1 transgenic mice

Two founders were identified with the VDJµδγ1 transgene. We utilized Southern hybridization to determine the copy number and composition of the transgenic array in the DNA of these founders. As determined by Southern hybridization, DNA from founder 3321 had three or four copies of the transgene inserted in tandem with minimal loss at either the 5' or 3' end. Head to tail arrangements of the transgenes should result in a 7 kb BamHI fragment. About four copies of a 7 kb BamHI fragment hybridized to a Cγ1 probe from the 3' end of the transgene, consistent with a transgene copy number of 4 to 5 (Fig. 1B, lane 7). An additional 8.5 kb SstI fragment (Fig. 1B, lane 1) was probably derived from the 5' most copy of the transgenic array and included sequences from the insertion site. The SΨ part of these transgenes was derived from BALB/c mice, whose SΨ region is 1.6 kb smaller than that of C57BL/6 (the endogenous locus) (36). Since three or four copies of the SstI fragment hybridizing to the SΨ probe was the appropriate size, the transgenic array includes intact SΨ regions, except that one copy is ~1 kb smaller (Fig. 1B, lane 4).

DNA from the 3323 founder included a single copy of the SΨ hybridizing fragment and the 10 kb head to tail VDJ-hybridizing SstI fragment (Fig. 1B, lanes 2 and 6). On the other hand, 3323 DNA had two copies of the 3' Cγ1-hybridizing fragment, one in the head to tail arrangement (7 kb) and an 8 kb fragment that probably represents the 3' insertion site (Fig. 1B, lane 8). 3323 DNA also includes two copies of the intact S1 region (see below). These results indicate that the 3323 founder had one intact copy and an additional partial copy, lacking at least the VDJ and SΨ part of the transgene.

Transgene expression

Flow cytometric analysis was performed to determine the extent of transgenic µ (a allotype) and endogenous µ (b allotype) expression on splenocytes. For both VDJµδγ1 lines allelic exclusion is nearly complete; nearly all of the B220+ cells express µa and only a few percent express µb (Fig. 2). The IgD, µa and µb patterns are indistinguishable from BALB/c splenocytes (Iγh4). Flow cytometric analysis for other B cell markers (CD23, HAS and B7-2) and T cell markers (Ly1 and Ly2) revealed that transgenic splenocytes were indistinguishable from non-transgenic splenocytes (not shown).

We investigated the ability of transgenic B cells to respond to various stimuli and express transgenic RNA. Spleenic B cells from both lines treated with LPS expressed abundant transgenic VDJ transcripts, as determined by S1 nuclease
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Fig. 2. Surface expression of transgenic and endogenous \(\mu\) and \(\delta\). Total splenocytes from the indicated mice (3–6 months old) were stained and viable lymphocytes (selected after gating on forward and side scatter) were analyzed for surface expression of \(\mu^a\), \(\mu^b\) or total \(\delta\) by flow cytometry.

analysis (Fig. 3, lanes 2 and 4). The amount of these stable transcripts was up-regulated (using actin levels as a control) relative to the level in unstimulated lymph node cells, suggesting that the transgenes are LPS responsive (cf. Fig. 3, lanes 1 to 2 and 3 to 4). Transgenic VDJ transcripts were not detected in thymic RNA. Transgenic lymphocytes expressed significant levels of C_{\mu} transcripts in lymph node and increased levels in LPS-stimulated splenic B cells (Fig. 3, middle panel, lanes 1–4). The levels of C_{\mu} transcripts were comparable to those from non-transgenic lymphocytes. Since almost all of the IgM antibody expressed on the surface of these B220\(^+\) cells was from the transgene (Fig. 2), it is likely that the majority of the C_{\mu} transcripts were also from the transgene. Therefore, expression of the VDJ and C_{\mu} portion of the transgene is roughly the same as expression of the endogenous locus in non-transgenic B cells.

We next investigated the production of \(\gamma1\) germline transcripts in transgenic B cells. Relative to the endogenous \(\gamma1\) gene, the intronic \(\mu\) enhancer is moved much closer to the promoter region for \(\gamma1\) germline transcripts and would flank each \(\gamma1\) promoter region in the transgenic tandem array. It is possible that this powerful transcriptional regulatory element would alter expression of the \(\gamma1\) germline transcripts. We found that \(\gamma1\) germline transcripts were expressed with regulation and quantity like that of the endogenous \(\gamma1\) gene. Using an S1 nuclease analysis that distinguished transgenic (252 bp protection) and endogenous (166 bp protection) transcripts, we observed that both transgenic and endogenous germline transcripts were present in the RNA from B cells cultured with LPS + IL-4 or with CD40L + IL-4, but not in B cells cultured with LPS alone, not in thymus and not in non-lymphoid tissue (Fig. 4). The amount of stable germline transcripts in both lines was approximately the same as the amount of endogenous transcripts.

**Transgenic switch recombination**

We tested serum from unmanipulated transgenic animals for expression of \(\gamma1\alpha\) (transgenic) Ig, using an allotype-specific ELISA. Non-transgenic littermate and C57BL/6 (Igh\(^{h}\)) controls behaved as expected, yielding absorbance readings like that of no serum controls (Fig. 5). However, virtually all of the serum samples from line 3321 mice revealed the presence
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Fig. 3. Transgenic VDJ and Cµ mRNA expression. Total RNA from the indicated cell sources was analyzed by S1 nuclease protection. Probes used were VDJB1-8 (includes 200 bp of intron from the 3′ side of Jµ2, top panel), JC253 (Cµ, middle panel) or pA1-85 (actin, bottom panel). ‘tRNA’ indicates probes hybridized to Escherichia coli tRNA and digested with S1 nuclease. The locations of uncut probes and protected probes are indicated on the left side of the figure.

of significant quantities of γ1α. The majority of serum samples from 3323 mice were likewise positive for γ1α (Fig. 5). The expression of γ1α observed in transgenic sera was always lower than that of BALB/c serum.

We investigated the induction of IgG1 secretion by transgenic B cells cultured in vitro with CD40L-expressing Sf9 cells + IL-4 + supernatant from the activated helper T cell D10 (to supply additional T cell cytokines). We observed secretion of transgenic γ1α Ig by splenocytes activated by this cocktail of reagents (Table 1). Transgenic γ1 secretion is regulated like that of the endogenous locus as it was not observed in supernatants of transgenic B cells cultured with CD40L alone. γ1 expression depended on added recombinant IL-4, as culture of B cells with CD40L + D10 supernatant did not result in elevated levels of γ1 expression (Table 1, Experiment 2). In some experiments, a large portion of the γ1 expressed was of the transgenic (‘a’) allotype. The overall level of γ1 expression was low compared to non-transgenic or BALB/c B cells cultured in a similar way.

To more accurately define the frequency of transgenic IgG1 expression, relative to the frequency of IgG1 expression of non-transgenic B cells, we utilized the frequency with limiting numbers of B cells. T-depleted splenocytes were cultured with CD40L-expressing cells, IL-4 and 3T3 feeder cells. As predicted by the lower level of transgenic IgG1 expression in serum (Fig. 5) and in bulk cultures (Table 1), ~50-fold more transgenic B cells were required to achieve a reasonable frequency of IgG1+ cultures. The frequency of IgG1+ cultures in three or four different sets of cultures, each with various numbers of either transgenic or non-transgenic B cells, was determined by ELISA. The precursor frequency of IgG1-secreting cells was determined from plots of log [frequency of negative wells] versus cell number per well (Fig. 6A). The average precursor frequencies, from three experiments, are 1:240 (line 3323 B cells), 1:760 (line 3321 B cells) and 1:9 (non-transgenic B cells).

ELISA absorbance data from one cell concentration in one experiment are presented in Fig. 6(B). Transgenic B cells stimulated with CD40L only rarely yield IgG1+ wells. For example in the experiment shown in Fig. 6(B), cultures with 300 line 3323 B cells exposed to CD40L do not secrete IgG1, whereas 68% of cultures with 300 line 3323 B cells, CD40L and IL-4 secrete IgG1. Thus, expression of IgG1 by transgenic B cells is regulated like that of the endogenous locus; it requires signaling by IL-4.

The poor expression of IgG1 by transgenic B cells (Fig. 5 and Table 1) could be due to poor secretion of IgG1 by cells that have switched to the transgenic γ1 heavy chain gene. Statistically, most of the cultures presented in Fig. 6(B) should represent the IgG1 secretion by one, or perhaps two, B cells.
Thus, we used these limiting dilution cultures to compare the IgG1 secretion by single transgenic and non-transgenic B cells. In Fig. 6(B), the portion of wells yielding a given absorbance in an IgG1-specific ELISA (estimating the amount of IgG1 secreted) is shown for both lines of transgenic mice and for non-transgenic mice. Since the distributions of absorbance are comparable for transgenic and non-transgenic cultures, cultures of one or two transgenic B cells secrete IgG1 at approximately the same rate as cultures of one or two non-transgenic B cells. A more quantitative comparison would require matching transgenic and non-transgenic cultures with identical frequencies of IgG1+ cultures, a technically impractical goal. However, data like those in Fig. 6(B) demonstrate that there is not a large difference in secretion by transgenic and non-transgenic B cells. Studies of transgenic hybridomas (see below) support this conclusion.

Characterization of transgenic recombination events

The γ1a expression in transgenic serum and after culture of transgenic B cells with CD40L + IL-4 + D10 supernatant indicated that the VDJµδγ1 transgene can undergo a recombination event. However, that recombination event could involve either recombination within the transgene or between the γ1 part of the transgene and an endogenous VDJ. It is formally possible that the γ1a secretion was the result of extended transcription within the transgenic locus or transsplicing without any recombination. To explore which of these possibilities is more likely, we prepared hybridomas from transgenic B lineage cells. Our goal was to analyze the transgenic and endogenous JH and S regions for DNA rearrangement.

Eight hybridomas, selected for γ1 secretion, were prepared from a 5 day culture of 3323 B cells exposed to CD40L, IL-4 and D10 supernatant. As determined by allotype-specific ELISA, all eight hybridomas secreted γ1a, the transgenic allotype. All eight expressed transgenic VDJ RNA by RT-PCR. Sequencing of the cloned product confirmed the presence of the VDJ junction characteristic of the transgene and Cγ1a-specific polymorphic nucleotides (data not shown).

Southern analysis of BamHI-digested hybridoma DNA with a JH probe will reveal Sµ rearrangements, since BamHI cuts at the 5' end of the JH probe used and within the Cµ gene. With one possible exception, Sµ regions were not retained in the configuration found in non-lymphoid tissue ('3323' or 'NTg'; Fig. 8B) for either the transgene or the endogenous gene. The JH-hybridizing fragment in 4F1.3 DNA that co-migrates with the transgenic fragment in tail DNA may be

Table 1. Transgenic switch recombination induced by CD40 ligation and IL-4

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>µ secretiona</th>
<th>Total γ1 secretiona</th>
<th>γ1a secretiona</th>
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<td></td>
<td></td>
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<tr>
<td>non-transgenic</td>
<td>CD40L</td>
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<td>&lt;2.4</td>
<td>&lt;0.48</td>
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<td>19.5</td>
<td>&lt;0.48</td>
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<tr>
<td></td>
<td>CD40L</td>
<td>&lt;0.48</td>
<td>&lt;2.4</td>
<td>&lt;0.48</td>
</tr>
<tr>
<td>3323</td>
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<td>2.78</td>
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<tr>
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<td>&lt;0.48</td>
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<td></td>
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<td>6</td>
<td>1.2±</td>
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</table>

aValues are ng/ml Ig secreted, as detected by ELISA and comparison to standard curves for purified mAb. The γ1 and γ1a data in Experiment 1 are representative of four experiments; the µ results were more variable.

bCorresponding absorbance values are significantly different (P < 0.05) compared to γ1a values for transgenic CD40L cultures and all non-transgenic cultures.
not rearranged or rearranged and fortuitously co-migrates. Hence, virtually all JH loci in hybridoma DNA were associated with a 3’ rearrangement. Switch recombination of the endogenous locus begins either 5’ of Sζ or anywhere in Sμ and ends anywhere in Sγ1 (3). Similar recombination in the transgene would result in BamHI fragments from 2.3 to 11 kb in length. A variety of different sized fragments, mostly in the middle of this range, were observed (Fig. 8B).

We examined the hybridoma DNAs for Sγ1 rearrangements, using SstI digestion and a probe from just 3’ of Sγ1. We detected rearranged Sγ1 fragments, many in the middle of the predicted range of 2.8–11 kb and a few that apparently co-migrated with the fragment found in non-lymphoid tissue from either the transgene or the endogenous gene (Fig. 8C). If these co-migrating fragments represented non-rearranged Sγ1 regions, they should be associated with DNA 5’ to Sγ1. Using either PstI or EcoRI–BglII and a probe that lies 5’ to Sγ1, we found that only one hybridoma, 1H5.4, retained a non-rearranged transgenic Sγ1 like that in 3323 tail DNA (data not shown). Thus, virtually all of the Sμ and Sγ1 regions in hybridoma DNA were rearranged (Fig. 8B and C). Since each of the eight hybridomas expressed the transgenic VDJ and Cγ1 (Fig. 6 and data not shown), these combined results suggested that expression of transgenic γ1 heavy chains occurred by recombination in S regions.

In addition to recombination in S regions, another set of deletion events were indicated by three observations. This second set of recombination events removed one of the Sγ1 regions from the transgenic array and extended through the Cγ1 gene, ending 5’ of the transgenic VDJ complex (Fig. 8, recombination 2). First, three or four of the hybridomas lacked the 5’ Cγ1 copy of the transgene (Fig. 8D). Second, those hybridoma DNAs that lacked the 5’ Cγ1 locus also lacked a transgenic VDJ locus in the configuration found in tail DNA.

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**Fig. 6.** Expression of IgG1 by limiting dilution cultures of transgenic VDJμδγ1 splenocytes. (A) Limiting dilution cultures were performed and IgG1+ cultures determined as described in Methods. Plots of percent IgG1+ wells versus cell number are shown for non-transgenic, 3321 and 3323 cultures. Plots of percent IgG1+ wells versus cell number are shown for non-transgenic, 3321 and 3323 cultures. (B) T-depleted splenocytes were cultured at five (non-transgenic), 750 (line 3321) or 300 (line 3323) lymphocytes per well in CD40L (24 wells for each cell concentration) or CD40L + IL-4 (64 wells). After 7 days of culture (see Methods), IgG1 expression was determined by ELISA for each well. The percentage of wells yielding an ELISA absorbance within a given range is shown. A 2-fold difference in ELISA absorbance reflects a 4-fold difference in IgG1 concentration. In order to present more detail for positive cultures, the non-transgenic negative cultures (less than the mean ± 3 SD of the CD40L only cultures) are shown off scale (actual percentage, 74%).

**Fig. 7.** Transgenic VDJ RNA expression in hybridomas. Between 1 and 3 µg of total cellular RNA from the indicated hybridomas or 15 µg of total cellular RNA from non-transgenic lymphocytes cultured for 3 days with LPS + IL-4 was tested for expression of VDJ RNA, using the VDJB1-8 probe by S1 nuclease protection. The migration position of uncut probe, full-length VDJ protection and endogenous V protection are indicated. The amount of full-length VDJ protection in hybridoma RNA is proportional to protection of an actin probe (not shown). Hybridoma 1B5 represents a negative control, as it is a hybridoma that lacked both transgenes and endogenous genes after growth in bulk culture.
almost complete allelic exclusion (Fig. 2). Like C IgM and IgD on the surface of transgenic B cells, and to this high-level expression leads to normal levels of transgenic RNA expression by transgenic B cells is equivalent in level and LPS inducibility to that of non-transgenic B cells (Fig. 3).

Transcriptional expression of the VDJ transgene RNA expression from the endogenous locus, transgenic transcripts are not expressed in thymocytes (Fig. 3). Finally, germline transcripts from the γ1 part of the transgene are expressed with correct regulation, in quantities equivalent to those from the endogenous locus (Fig. 4).

Recombination by the VDJγ1 transgene

IgG1 is produced from the transgene (Fig. 5) that, like IgG1 from the endogenous locus, is induced by CD40L + IL-4 (Table 1 and Fig. 6). In hybridomas that express transgenic IgG1α with the transgenic VDJ, both S0 and S1 regions are rearranged (Fig. 8). Even though we have not directly demonstrated a S0S1 junction, these data strongly suggest recombination in S regions. Therefore, the VDJγ1 transgene is capable of recombination events that resemble the heavy chain switch in that they are induced by the same agents that induce switch recombination and take place in S regions.

Nevertheless, in remarkable contrast to the endogenous levels of Cγ RNA and γ1 germline RNA, expression of IgG1 from the transgene is not a frequent event. Levels of IgG1α in serum and in tissue culture supernatants after B cell culture with CD40L + IL-4 are much lower than that of non-transgenic mice. The transgenic mice produce virtually no IgG1 antibody to nitrophenyl (NP) after repeated injection with NP-conjugated proteins (not shown—the transgenic VDJ is from an anti-NP antibody). The precursor frequency of IgG1-expressing cells is 25- to 75-fold lower than the precursor frequency of non-transgenic B cells (Fig. 6). γ1 expression by the human minilocus transgene could be similarly inefficient (22–23).

However, the in vitro experiments required to determine this would be more difficult for the human transgenics, as the agents that would specifically stimulate recombination to human γ1 in a mouse cell are not defined.

There are two categories of explanations for the poor expression of IgG1 from the VDJγ1 transgene: (i) explanations that involve faulty switch recombination and (ii) explanations that do not involve the switch recombination process per se. Included in the second category would be a scenario in which the transgenic B cells could not be activated by CD40L + IL-4 sufficiently to induce switch recombination. This scenario does not account for several results. The transgenic B cells appear normal by cell surface markers and are activated by both LPS and CD40L. After immunization with NP-protein conjugates, transgenic mice produce abundant anti-NP IgM (not shown). Most importantly, transgenic B cells respond to CD40L + IL-4 treatment by production of non-transgenic levels of γ1 germline transcripts (Fig. 4).

Another scenario in the second category is that transgenic B cells may switch efficiently, but fail to secrete large amounts of IgG1 antibody. We have found that some transgenic hybridomas secrete IgG1 at a rate equivalent to that of non-transgenic hybridomas. However, a subset of transgenic hybridomas secrete IgG1 at about one-fourth the rate (data not shown). This small reduction in rate of secretion by some transgenic B cells will not, in general, alter our analysis of precursor frequency (Fig. 6). This is largely a ‘plus or minus’ test. We cannot exclude the possibility that we have a few false negative wells whose secretion of IgG1 is so low that it cannot be distinguished from background. However, adding a few more positive wells to the transgenic data would change

Discussion

Transgene RNA expression

Transcriptional expression of the VDJγ1 transgene in B cells from both founder lines is very similar to the endogenous locus. Transgenic VDJ RNA transcripts are abundant, and Cγ RNA expression by transgenic B cells is equivalent in level and LPS inducibility to that of non-transgenic B cells (Fig. 3). This high-level expression leads to normal levels of transgenic IgM and IgD on the surface of transgenic B cells, and to almost complete allelic exclusion (Fig. 2). Like Cγ RNA

(Fig. 8A); they presumably retained a transgenic VDJ complex (albeit with a DNA environment different than that found in non-lymphoid tissues), because they expressed the VDJ RNA. Third, we detected at least eight fewer fragments hybridizing to the 3′ Sγ1 probe than would have been predicted by the JH content of these hybridomas (Fig. 8A and C). Hence, recombination takes place outside Sγ1, deleting the sequences that hybridize to this probe.

Fig. 8. Heavy chain gene content of transgenic hybridomas. DNA from the indicated transgenic line 3323-derived hybridomas, fusion from the indicated transgenic line 3323-derived hybridomas, fusion

Transgenic B cells may switch efficiently, but fail to secrete large amounts of IgG1 antibody. We have found that some transgenic hybridomas secrete IgG1 at a rate equivalent to that of non-transgenic hybridomas. However, a subset of transgenic hybridomas secrete IgG1 at about one-fourth the rate (data not shown). This small reduction in rate of secretion by some transgenic B cells will not, in general, alter our analysis of precursor frequency (Fig. 6). This is largely a ‘plus or minus’ test. We cannot exclude the possibility that we have a few false negative wells whose secretion of IgG1 is so low that it cannot be distinguished from background. However, adding a few more positive wells to the transgenic data would change
the precursor frequency by 2-fold or less. The precursor frequency would remain dramatically lower than that of nontransgenic B cells.

Thus, we favor explanations in which the poor IgG1 expression from the transgene is due to problems in switch recombination per se. Switch recombination may occur infrequently on the transgenic substrate, so that the transgenes do not switch at all. Alternatively, switch recombination may occur frequently, but inaccurately. Inaccurate switch recombination would result in deletion of the transgenic VDJ or Cγ1, so that IgG1 cannot be produced from the transgenic locus. At a minimum, switch recombination of endogenous S regions requires germline transcription and switch recombinase (1,2). Deletion of the germline promoters and I exons has demonstrated that germline transcription is a prerequisite for recombination, even though the substrates for recombination, the S regions, are intact (37,38). Transcription per se is not sufficient for switch recombination; it has been proposed that germline transcripts must retain some important structural characteristics (39–40).

There are several situations in which switch recombination is dramatically altered in the absence of changes in germline transcription (41). Although some of the changes in switch recombination could be attributed to changes in recombinase activity (42), it is also likely that factors in addition to accessibility and recombinase regulate switch recombination (41). Our analysis of the VDJµδγ1 transgene provides direct evidence for additional regulation. If S regions, germline transcription and recombinase were the only requirements for switch recombination, γ1 expression from the VDJµδγ1 transgene should be efficient. The Sγ region is intact compared to that in BALB/c genomic DNA (Fig. 1). The Sγ region is somewhat smaller than that from BALB/c, but is equivalent in size to that of other haplotypes and 2 kb larger than Sγ1 in the Iγh locus (Fig. 8) (43). Thus, Sγ1 regions much smaller than that in the VDJµδγ1 transgene can support efficient and accurate switch recombination. Abundant germline transcripts, with the same structure as those from the endogenous gene, are expressed from the γ1 part of the transgene. Therefore, we conclude that factors or elements in addition to germline transcription and S regions target switch recombinase.

The VDJµδγ1 transgene may be missing some cis-acting element that either enhances switch recombination or directs the location of switch recombination, e.g. the regulatory elements 3′ of the heavy chain locus (44–48). Alternatively, inefficient switch recombination might be due to the unusual organization of the transgenic array. As one example, the VDJµδγ1 transgenic loci include two to five copies of perfectly homologous S regions spaced only 50 kb apart; a situation not found in the endogenous heavy chain locus. Secondly, the VDJµδγ1 construct places the Eµ intronic enhancer in an unusual position relative to Sγ1, closer to its 5′ end and directly 3′ to it in the transgenic array. Eµ has dramatic effects on recombination both in the endogenous locus and on episomes (14,49). Thirdly, in the line 3323 mouse, the 5′ Sγ1 has no donor S region 5′ of it, unlike the endogenous locus. These or other aspects of the unusual arrangement in the transgenic array might alter targeting of recombinase.

Some results favor the explanation that switch recombination on the transgenes is frequent, but inaccurate. Many of the transgenic loci in hybridoma DNA are missing a copy of the sequences just 3′ of the Sγ1 region (Fig. 8C). In three hybridomas the putative deletion extends beyond the SstI site 3′ of Cγ1, resulting in deletion of Cγ1 and rearrangement of the SstI fragment including the transgenic VDJ (Fig. 8, recombination 2). By selecting for hybridomas that express γ1 (and thus the transgenic γ1t, since allelic exclusion is so strong), we selected for relatively normal switch recombination in the 3′ part of the transgenic locus in line 3323 (Fig. 8, recombination 3). If recombination of the transgenic locus were inefficient, the Sγ1 should not undergo recombination and would usually remain in the germline configuration. This is not the case, in at least seven of eight hybridomas, the upstream Sγ1 region was rearranged. As discussed above, the rearrangement seemed to frequently include the deletion of Cγ1.

If switch recombination on the VDJµδγ1 transgene is both frequent and inaccurate, it would be similar to that on episomal substrates (14–17). Like the endogenous locus, recombination on retroviral substrates is largely restricted to S regions (19). We note that the retroviral switch substrates lack Eγ1 (18), which our transgenic construct includes in each copy of the array. From published data, it is difficult to estimate either the efficiency or the accuracy of switch recombination of the human mini-locus (22,23). To summarize, loss of a cis-acting element, organizational factors or spatial factors could alter recombinase recognition of the VDJµδγ1 transgene, resulting in inefficient or inaccurate switch recombination in spite of intact S regions that are efficiently transcribed.

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Abbreviations

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>C</td>
<td>constant</td>
</tr>
<tr>
<td>D</td>
<td>diversity segment</td>
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<tr>
<td>Eµ</td>
<td>enhancer in the VDJ–Cµ intron</td>
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<td>J</td>
<td>joining segment</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
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<tr>
<td>NP</td>
<td>nitrophenyl</td>
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<td>S</td>
<td>switch</td>
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<td>V</td>
<td>variable segment</td>
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Recombination of a murine VDJγ1 transgene


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