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Deletion of a Recombined Ig Heavy Chain Transgene in B-Lineage Cells of Transgenic Mice

Andy Heinzellmann,* Subbiah Kumar,* Scott Noggle,* Ine Goedegebuur,* K. Morgan Sauer,* Satyajit Rath,† and Jeannine M. Durdik*‡

Fully recombined transgenes are stable in their transmission in the germline of transgenic mice, in common with the endogenous genetic complement of most mammalian somatic tissues, including the genes for lymphoid Ag receptors somatically generated from germline mini-genes. There have, however, been isolated reports of unusual low frequency transgene losses in various transgenic mice. Here we show, using Southern blots and PCR-based assays, that plasmablast hybridomas and B cells from three independently derived founder lines of transgenic mice bearing a recombined heavy chain Ig transgene we have been studying show a significant net loss of transgene copies. This loss is more marked in the B cells expressing endogenous heavy chains than in those expressing transgenic heavy chains. We have also examined cells of the B lineage in the bone marrow, and a small degree of deletion is also evident in CD19⁺CD23⁻IgM⁻ immature B-lineage cells. As greater deletion is observed in mature B cells, it is possible that the deletion process either continues into B cell maturity and/or provides a selective advantage. We have investigated the relationship between transgene expression and deletion, and we find that while thymocytes in these mice express the transgene well, T cell hybridomas derived from transgenic thymus do not show any loss of the transgene. Thus, a recombined Ig heavy chain transgene prominently undergoes somatic deletion in B-lineage cells independent of its insertion site or expression. This transgenic instability is significant to the analysis of genomic stability as well as to the design of gene therapy strategies. The Journal of Immunology, 1998, 161: 666–673.

The DNA in differentiated somatic tissues is complete and fully pluripotent (1–3). The DNA sequences of genes in various tissues and organs are assumed to be identical, except for the site-specific DNA rearrangements observed in lymphocytes (4–8; reviewed in Ref. 9). Copy number changes are, however, noted as developmental strategies for a number of genes. These are amplifications or deletions at specific stages of development. For instance, ribosomal genes are amplified in early oogenesis of amphibians (10) as are the genes for the major chorionic proteins of Drosophila (11). In mammalian cells, amplification of drug resistance genes can be selected (12–14), amplification of oncogenes (c-myc) is observed in neuroblastomas (15, 16), and amplification of a LINE-3-related sequence is observed in rat brain (17). Gene deletions are a developmental strategy in nematodes and tetrahymena (18), but have not been reported in mammalian species.

Microinjected transgenes (Tg)³ are generally integrated in multiple copies as tandem arrays. These properties coupled with the ability to follow their unique DNA makes them ideal reporters for examining genetic instability in vivo. It is generally assumed that if a Tg is stable in the germline, it is stable in all somatic tissues. Some Tgs are designed to recombine in lymphoid tissue (or other recombining tissues) by virtue of having a pair of site-specific recombination signals (19–24), but stability is the expectation for all other transgenic mice. However, there have been reports of nonimmunologic Tgs being deleted in rare cells of the tissues expressing them (25, 26). We (27) and other groups (28, 29) have also reported the loss of Ig or TCR Tg copies in B- or T-lineage cells, respectively, at higher frequencies. We have now further characterized the Tg loss we observed and report here that this IgH Tg loss appears to be independent of the insertion site and occurs in vivo in both immature and, more prominently, in mature B cells.

Materials and Methods

Mice

We have described (23) the construction and characterization of transgenic C57BL/6 (IgH⁺) mice bearing a recombined VDJ gene from an anti-p-azobenzenearsenolate (Ars) mAb R16.7 coupled to a µ constant region (Cµ) gene derived from the BALB/c mouse strain (IgH⁻). Three separate founder lines were used: line 5 (with an estimated 30 copies of the Tg), line 33 (60 copies), and line 39 (5 copies). These founders have the Tg inserted in tandem arrays, each at a single insertion site. The Tg is expressed in a lymphoid tissue-specific fashion (23).

DNA preparation

DNA from mouse tissues was isolated by either mincing between sterile frosted glass slides in sterile PBS or by homogenization in 5 ml of lysis buffer (10 mM Tris (pH 8.3), 2.5 mM MgCl₂, 50 mM KCl, 200 µg/ml gelatin, 0.45% Nonidet P-40, 0.45% Tween-20, and 60 µg/ml proteinase K). Red cells were lysed where appropriate by adding 9 vol of sterile water immediately followed by 1 vol of 10× PBS. Samples were incubated at 56°C for 12 h and extracted twice with phenol/chloroform (1/1) and once with chloroform (1/1). The DNA was precipitated with isomyl alcohol and sodium acetate, washed with 70% ethanol, dried, and dissolved in 5 mM Tris (pH 8.0) and 0.5 mM EDTA (30).

Southern blots

EcoRI-digested DNA samples were Southern blotted as previously described (23), using hybridization with a ³²P-labeled pUC DNA probe to

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3 Abbreviations used in this paper: Tg, transgene; Ars, p-azobenzenearsenolate; MBP, myelin basic protein; AC, adenosine triphosphate channel.

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0022-1767/98/802.00
detect the Tg or a pEX-1 probe that hybridizes with the Tg pUC sequences at about 12 kb and the endogenous myelin basic protein (MBP) gene at about 8 kb.

**Polymerase chain reactions**

Multiple dilutions of each DNA sample were added to reaction mixtures containing a primer set(s) (1.5 mM each), dNTPs (200 mM), Tris-HCl (17 mM, pH 8.3), KCl (50 mM), MgCl2 (2.5 mM), Tween-20 (0.09%), Nonidet P-40 (0.09%), gelatin (0.001%), and AmpliTaq DNA polymerase (1 U/μl) diluted 1:40 with 25 μl (30). The DNA samples were amplified using a thermal cycler with denaturation at 93°C for 50 s, annealing at 58°C for 1.25 min, and polymerization at 72°C for 1.25 min for 33 cycles, with extension at 72°C for 10 min. Amplified products were stored at 4°C. PCR products were electrophoresed on a 2.0% agarose gel in TBE buffer containing ethidium bromide (0.5 μg/ml) for 1 h at 300 V. A HueIII digest of pH174 RF DNA was used as a size marker. The gels were photographed under UV illumination, photographs were scanned using Adobe Photoshop 4.0 (Adobe, Mountain View, CA), and were analyzed densitometrically using National Institutes of Health Image (version 1.59) freeware. All figures shown are reversed images.

**Amplification primers**

The pair of 5′ Vh (5′-CTTGGACCTGAGCACACTGTCGTG-3′) and 3′ Vh (5′-GACTCCAAGCTTGTCCCTAGTCCTTCATGACC-3′), giving a band of 782 bp, or the pair of 5′ Vh (5′-CTATGATCGTCTCGTCCACAC-3′) and 3′ Vh (5′-CTTCTTGATGCTGAAAATCCAGAT-3′), giving a band of 647 bp (30, 31), was used for Tg detection. The ATP channel gene was detected using the 5′ primer 5′-GCTCTAGATTGTGATTTAGTGTGCTAAAC-3′ and the 3′ primer 5′-CGAAGGGCTGACTTCCTCCTATGGT-3′. The G stimulatory protein gene (Gsα) was detected as a normalization control in some experiments in place of the ATP channel gene. The 5′ Gsα primer used was 5′-TAACTTCCGATGTTGAGT-3′, and the 3′ primer was 5′-AGGAGGAAACACGCAGACTACCG-3′. The normalization controls were designed to be run in the same reaction tube as the Tg primers. They also were designed to have melting points matching those above and to not form interfering structures with themselves or the Tg primers, using Cprimer (version 1.08) and Amplify (version 1.2) freeware.

**RNase protection assays**

RNase protections were performed on 5 μg of total RNA as previously described (32, 33). Three probes were hybridized with each sample: 1) pGARP, the VDJ of the Arsα Tg (a PsiI doublet) cloned into pGEM (32); 2) pμC4, the mouse μ heavy chain fourth constant region exon also cloned into pGEM (allowing for distinction between IgHμ made in the membrane and secreted forms); and 3) pβ2EII, the second exon of the β2 mRNA (33).

**Hybridomas**

The plasmablast hybridomas used were generated from line 5 mice using the fusion partner Sp2/0 and have been reported previously (27). T cell hybridomas were generated from line 5 and line 33 mice using the fusion partner BW1100 (34) as previously described (35). Hybridoma cells were digested in 200 μl of lysis buffer and proteinase K (30 overnight at 56°C for DNA extraction.

**B cell isolation**

B cells were isolated by staining them with anti-B220-biotin (PharMingen, San Diego, CA), followed by incubation with streptavidin-coated magnetic beads (Dynabeads, Dynal, Chantilly, VA) on a rocker platform at room temperature for 30 min. The samples were then placed in a magnetic particle concentrator (MPC, Dynal) for 2 min, the adherent cell-bead complexes were washed twice in PBS and used for further staining or were suspended in lysis buffer with proteinase K (10° cells/ml) and incubated at 56°C overnight for DNA preparation.

**Cell staining and flow cytometry**

For intracellular staining, cells were permeabilized using ice-cold methanol by adding 5 ml of 100% methanol to dispersed cell pellets for 3 min on ice. Cells were pelleted and resuspended in staining buffer on ice for 30 min before staining. Staining was performed by incubation with primary reagents for 45 min in staining buffer (PBS containing 0.05% sodium azide and 1% BSA) on ice. Cells were washed three times with ice-cold PBS and stained in a second step, if required, in the same fashion. They were fixed in 0.05% paraformaldehyde in PBS at the end of staining and stored until analysis. Samples were analyzed and sorted where shown using either an EPICS 752 (Coulter, Hialeah, FL) or a FACSort (Becton Dickinson, Mountain View, CA) flow cytometer. Data analysis was performed using Iso-Contour (Verity Sunnyvale, CA), EASY2 (Coulter), or CellQuest (Becton Dickinson) software packages. Abs and stains were titrated to optimize the working concentrations. The reagents used were anti-B220-biotin, anti-CD19-biotin, anti-CD23-fluorescein, anti-IgM-fluorescein, IgM-biotin, streptavidin-phycerothyrin (PharMingen), streptavidin-Red670 (Life Technologies, Grand Island, NY), anti-Thy-1-fluorescein (Becton Dickinson), mouse anti-rat Ig-fluorescein (Accurate, Westbury, NY), and anti-μ-fluorescein (Southern Biotechnologies, Birmingham, AL).

**Mutation detection by base excision sequence scanning T-scan**

B cell hybridomas containing a single copy of the Tg, liver, and B cell hybridomas from anti-ArS-immunized mice that had previously defined mutations (31) were scanned for mutations by the BESS T-scan mutation detection and localization kit (Epizentech Technologies, Madison, WI). Briefly, 200 ng of DNA was used in each PCR with primers specific for the recombined VDJ region of the Tg (5′Tg flank, 5′-ATTCCAAAGC TACGTATAAACGT-3′; 3′Tg flank, 5′-ATCGTATCCGTTTTCG CAG-3′) in PCR buffer (17 mM Tris (pH 8.3), 2.5 mM MgCl2, 50 mM KCl, 10 μg/ml gelatin, 0.001% nonidet P-40, 0.05% Tween 20). Reactions were begun with a hot start, followed by cycles of denaturation at 94°C for 45 s, annealing at 58°C for 90 s, and extension at 72°C for 95 s, with a final extension time at 72°C for 10 min. Reactions were electrophoresed in 1.25% low melting agarose gels, and the appropriate bands were extracted. This DNA was the template for a second PCR that included limited amounts of dUTP and a nested set of primers internal to the ones described above (5′Tg flank, 5′-AGCAAACTTCCACGAGCATGTTG-3′; 3′Tg nest, 5′-AGTAAGGAAACACGCAGCATGTTG-3′), one of which was labeled with IRD800 (LICOR, Lincoln, NE). The PCR product was then digested with excision and cleavage enzyme (Epizentech) and loaded on a 41-cm 6% Long Ranger gel on an automated sequencer (LICOR).

**Results**

**Detection of Tg loss by PCR**

We have previously reported, using Southern blot analysis, that three independently derived founder lines of Arsα-transgenic mice, lines 33, 5, and 39, have decreasing Tg copy numbers, respectively (23), and that plasma cell hybridomas derived from founder line 5 show significant losses of Tg copy number (27). For analyzing Tg copy numbers in limited numbers of normal, non-transformed cells in vivo, we designed a PCR assay in which the results of the PCR can be seen to match those of the Southern blot analysis closely for genomic DNA from the three founder strains (Fig. 1). Southern blots of EcoRI-digested tail DNAs from two heterozygous individuals each from founder lines 5, 33, and 39 hybridized with pUC DNA are shown in Figure 1A, where the unique flanking bands that characterize the integration site and differ between the founder lines are also shown. Figure 1B shows PCR of kidney DNA from the three founder lines using one set of Tg primers yielding a 782-bp band from the Tg. All three lines show Tg signals, but the intensity of the signal is quite distinct among the lines.

Plasmablast hybridomas from a fusion of line 5 spleen cells with the myeloma fusion partner Sp2/0 described previously (27) were then tested for Tg copy loss. Figure 2 shows PCR analyses of titrated amounts of genomic DNAs amplified either with Tg-specific sequences or with primers for an ATP channel gene as an internal control. Line 5 transgenic founder liver and nine cloned plasmablast hybridomas are compared. Of the nine hybridomas tested, only one, 1D5, has Tg signal intensities close to those seen in the liver. This could be due either to loss of Tg copies relative to the liver DNA or to sufficient mutation in the Tg to reduce the ability to prime the PCR response. Since this result is obtained whether the comparison is made by cell number (data not shown) or by the nanogram amounts of DNA added, this result is independent of the potential polyploidy of the hybridomas.
We have used V\(_H\) region primers for PCR amplification, since only one amino acid residue is different between the transgenic and endogenous C\(_{\mu}\) sequences (36). Given this use of V\(_H\) primers, another potential artifact with regard to copy number might occur if extensive mutation of the Tg copies had taken place. This is unlikely, since we find no mutation by sequencing in parallel with one of the most mutated hybridomas known, and Storb et al. (37) have found even when mutation is induced in Ig Tgs that the mutation process proves to be restricted to one Tg copy. In any event, such mutation would not be expected to extend into the pUC-containing sequences and therefore should not affect the Southern hybridization data (Fig. 3). Further, these hybridomas are IgM producers from unimmunized mice and therefore are unlikely to have been subjected to much somatic mutation. As a further technical point, we also found that the PCR primers we employed have always proved capable of producing a PCR band even from heavily mutated hybridomas such as Tg95-2 (31). Similarly, we have sequenced (as described in Materials and Methods) the DNA from several of the hybridomas showing the most extreme cases of apparent Tg loss, DNA from liver (negative control), and DNA from Tg95-2 (heavily mutated positive control), and we found no mutations in these hybridomas. This makes it unlikely that Tg signal was diminished due to inefficient primer binding to a mutated Tg and probable that the cause is loss of Tg copies. All the other eight hybridomas showed significant losses of Tg copies when signal intensities were compared with the copy number in liver DNA (Fig. 2). It is typical in the majority of B cell hybridomas to observe that the end point for the Tg in titrations is earlier than or identical with that seen for the ATP channel gene; this is in contrast to liver cell DNA, which shows a Tg end point two or more steps after the ATP channel end point. The PCR data have the advantage of allowing the use of small numbers of cells for analysis. However, an appreciation of the extent and variability between clones in the extent of deletion is more directly obtained from Southern blot analysis. From densitometric analysis of the signal from kidney DNA compared with hybridoma DNA, the plasmablast hybridomas that only produce \(\mu_b\) show 94 to 98% deletion of Tg copies compared with control desitometric scans (Fig. 3 and Table I), while those that produce both \(\mu_b\) and \(\mu_a\) show a wide range from 19 to 92% deletion of Tg copies. The maximum loss is about 30-fold. Such a great loss cannot be explained by differences in ploidy between the hybridomas and the kidney.

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Comparative copy number detection by PCR and Southern blot. A, Southern blot of EcoRI-digested kidney DNAs from two individuals each from founder lines 5, 33, and 39 hybridized with a \(^{32}\)P-labeled pUC DNA probe, showing differing signal intensities. B, PCRs of kidney DNA from the three transgenic founder lines, using 800, 600, 60, and 6 ng (left to right) of DNA for amplification with either Tg primers or AC gene primers. The signal intensity for the Tg relative to the ATP channel gene is highest for line 33, intermediate for line 5, and lowest for line 39. C, The Tg construct.

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Reduction of Tg copy number in plasmablast hybridomas detected by PCR. Genomic DNAs from liver or plasmablast hybridomas from line 5 were amplified with Tg or AC primers. The DNA was titrated at various concentrations (800, 600, 60, and 6 ng, from left to right) for all samples except 2C8 and 3F1, for which 10-fold higher concentrations of DNA were required to detect a Tg signal (8000, 6000, 600, and 60 ng).

![Figure 3](http://www.jimmunol.org/)

**FIGURE 3.** Reduction of the Tg copy number in plasmablast hybridomas detected by Southern blot. DNA from kidney or B cell plasmablast hybridomas from line 5 were used in Southern blot analysis hybridized with a \(^{32}\)P-labeled pUC DNA probe. A, A control kidney (lane 1), hybrids that only produce \(\mu_b\) (lanes 2–6), and one double \(\mu^a\) and \(\mu^b\) producer (lane 7). B, A control kidney (lane 8) and hybrids that express both \(\mu^a\) and \(\mu^b\) (lanes 9–14). The blot shown in A was developed for 7 days, while that shown in B was developed for 2 days. The relative quantitation of band intensities is shown in Table I.
DNA, Massive polyploidy in chromosomes other than the Tg-containing chromosome (chromosome 5) might make a 2- to 3-fold difference in the signal observed. Interestingly, complete deletion of Tg is not observed. There is signal detectable by both Southern blot and PCR. With one exception, these hybridomas produce IgM (27); 1B4 secretes an IgG2a Ab (38) and also shows significant Tg loss. Thus, plasmablast hybridomas show loss of Tg copies at a high frequency independent of whether they have undergone isotype switching. The spectrum of remaining Tg copy numbers is large and stable over months of maintenance in tissue culture and multisubclonings, suggesting that Tg toxicity is unlikely to be a relevant factor.

**Tg loss in nontransformed B cells ex vivo**

To confirm that the Tg losses seen in plasmablast hybridomas are a reflection of losses incurred in vivo by transgenic B-lineage cells, B cells were analyzed directly after isolation. Figure 4 shows the results of PCR analyses of liver DNA and splenic B cell DNA for the Tg or for the ATP channel gene from line 33 transgenic mice. The relative signal for the Tg is significantly lower in B cells than in liver (Fig. 4A), confirming that Tg losses do occur in vivo. Given the heterogeneity of Tg losses from individual plasmablast hybridomas, it is likely that individual B cells have varying degrees of loss. However, the degree of the average Tg loss is high, since the relative Tg signal is only 40% of the liver signal upon quantitation of the relative band intensities (Fig. 4A), showing that the Tg loss occurs at high frequency in B cells in vivo. This finding is not restricted to line 33 mice, since both the other independent transgenic lines, 5 and 39, show significantly lower Tg PCR signals in splenic B cells compared with nonlymphoid tissue such as liver, with the Tg signal from B cells titrating out faster than the signal from liver in both founders (Fig. 4B). Thus, the high frequency Tg loss seen in vivo in B cells occurs in independently derived founder lines. In corroboration of these data, a Southern blot analysis of DNA from kidney, bone marrow, or splenic B cell DNA for the presence of either the Tg or an endogenous control gene, MBP, shows that the Tg/MBP signal ratio is substantially lower in splenic B cells than in kidney or whole bone marrow (Fig. 4C).

**Different degrees of Tg loss in B cells separated based on Tg expression**

In an attempt to examine the possible heterogeneity in Tg loss in these B cells, we next separated transgenic B cells based on their ability to express Tg, since we have previously observed that plasmablast hybridomas expressing transgenic IgM heavy chain had smaller Tg losses than those expressing endogenous IgM heavy chains. Plasmablast hybridomas and serum IgM from these transgenic mice have been shown to express Tg alone, endogenous IgM heavy chain alone, or a mixture of transgenic and endogenous IgM proteins (27). We have therefore used the fact that the transgenic IgH protein uses a Cμ exon derived from the IgH allotype, while the IgH allotype of the C57BL/6 mice themselves is IghH, allowing allotype-specific mAbs to distinguish between B cells making transgenic and/or endogenous IgM heavy chain protein. We therefore separated purely IgM* or IgM*-expressing B cells from splenic cells of transgenic mice of line 5 by flow cytometry and compared Tg levels in their DNAs with reference to a single copy endogenous gene, Gγ, of a Southern blot analysis of EcoRI-digested DNA extracted from splenic B cells, bone marrow, or kidney of the line 5 mice. The blot was hybridized with pEX-1, which contains MBP gene sequences in a pUC vector. The Tg and MBP bands are marked. The ratio of the two bands, shown below each lane, was obtained by scanning and analysis of the autoradiograph. The ratio serves to normalize for any differences in the amount of DNA loaded in each lane. These ratios have not been corrected for the relative lengths of homology of the probes for the Tg vs MBP.

![Figure 4](http://www.jimmunol.org/)

**FIGURE 4.** Transgenic B cells from independent founders show loss of Tg in vivo. A, DNA from splenic B220<sup>+</sup> B cells purified by magnetic sorting and DNA from liver or kidney of line 33 mice were titrated from 800, 600, 60, and 6 ng (left to right) in PCRs with Tg or AC primers. The densitometric scans of the data are shown below each panel. DNA from transgenic line 5 (B, left) or line 39 (B, right) splenic B cells or liver was titrated (800, 600, 60, and 6 ng left) in PCRs with Tg primers. C. Results of a Southern blot analysis of EcoRI-digested DNA extracted from splenic B cells, bone marrow, or kidney of the line 5 mice. The blot was hybridized with pEX-1, which contains MBP gene sequences in a pUC vector. The Tg and MBP bands are marked. The ratio of the two bands, shown below each lane, was obtained by scanning and analysis of the autoradiograph. The ratio serves to normalize for any differences in the amount of DNA loaded in each lane. These ratios have not been corrected for the relative lengths of homology of the probes for the Tg vs MBP.

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**Table I. Loss of Tg copies in hybridomas measured by band intensity in Southern blots**

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TABLE I.

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<td>14</td>
<td>3F7</td>
<td>μα + μβ</td>
<td>7,784</td>
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Occurrence of Tg deletion in B cells in the bone marrow

Since B cells clearly show deletion of the Tg in these mice, the next step was to examine the relative Tg copy number in early B-lineage cells in the bone marrow. In the bone marrow, cells committed to the B lineage express CD19 (39). Mature cells that have left the bone marrow and recirculate into marrow also express CD23. Similarly, cells expressing high levels of B220 (B220bright) generally represent mature B cells that have cycled back into the bone marrow and those with intermediate to low B220 (B220dim), the newly emerged surface IgM-positive B cells and their precursors. We used both these marker sets to follow deletion of Tg copies. In Figure 6A, the titration of the ATP channel signal is quite similar in both populations, whereas the Tg signals titrate out more quickly for the B220bright cells, indicating that there is greater deletion of Tg copies in the B220 bright population than in the B220 dull population. More interestingly, in Figure 6C, CD19+/CD23-/IgM- cells from bone marrow were sorted using flow cytometry (sort gates shown in Fig. 6B), and their DNA were compared with that obtained from B220- cells from the spleen and to liver cell DNA. Figure 6C shows the PCR analysis of these B-lineage cells from the bone marrow and spleen and liver control, and Figure 6D shows the densitometry of those Tg and ATP channel PCR bands. The liver Tg signal is detectable one titration point beyond its ATP channel, whereas the Tg and AC signals end at the same point for both the CD19-CD23-/IgM- bone marrow and the B220- spleen samples. This is the first indication of loss in early B-lineage cells. Densitometry and calculation of the Tg/AC ratios at a sensitive part of the titration curve (a point beyond template DNA saturation and yet before loss of AC signal) reveals that the Tg/AC ratio in the most sensitive part of the curve is 3.7 for liver, 2.0 for the CD19+ pre-B cell population from bone marrow, and 1.3 for the B220+ splenic B cell population. There is thus less overall Tg loss in the bone marrow pre-B pool than in the peripheral B cell pool. However, given the loss in the pre-B cells, environmental Ag-mediated selective influences cannot be solely responsible for the Tg deletion observed in peripheral B cells.
Thymocytes express transgenic protein but do not delete Tg

Given the association between Tg expression and deletion in peripheral B cells, it was possible that transcription of the Tg would be sufficient to drive deletion. It is known that the IgH enhancer can be used for transcription by T cells as well as by B cells (40), and it has been shown in many systems that recombined IgH Tgs are transcribed in the thymus (23, 41, 42). It was therefore confirmed that the Ars\(_m\)-transgenic mice used here also showed such a pattern of expression. RNase protection analysis was performed on thymic RNA using riboprobes from three plasmids: 1) pGARP, containing the VDJ of the Ars\(_m\) Tg; 2) p\(\mu\)Ch4, containing the mouse IgM heavy chain fourth constant region exon, allowing distinction between the membrane and secreted forms of the IgM heavy chain; and 3) p\(\beta\)2EII, containing the second exon of the \(\beta_2\)m gene. As shown in Figure 7A, Tg VDJ expression is seen in R16.7, the hybridoma from which the Tg VDJ region is derived, and in J\(\mu\)4, a Tg transfectant of the J558L myeloma cell line, which also shows the expression of the secreted form of the IgM heavy chain transcript. Transgenic brain and kidney do not show any expression of Tg VDJ or IgM heavy chain transcripts, although they show the presence of \(\beta_2\)m mRNA. In addition to transgenic spleen, transgenic thymus also shows the presence of Tg and IgM heavy chain transcripts, although they show the presence of \(\beta_2\)m mRNA. In addition to transgenic spleen, transgenic thymus also shows the presence of Tg and IgM heavy chain transcripts. Figure 7B shows that, in contrast to fresh live thymocytes, permeabilized thymocytes from transgenic thymus stain well for the presence of IgM heavy chain, demonstrating that the transgenic protein is expressed intracellularly in practically all thymocytes. Thus, if transcription is the determining factor involved in Tg deletion, the thymus should also be susceptible to Tg deletion.

We therefore compared the Tg signals, relative to the ATP channel gene signal, from line 33 thymus and liver. As shown in Figure 8, there is no loss of Tg copies in the transgenic thymus. We further confirmed this by making T cell hybridomas from thymus of line 5 and line 33 origin using fusing thymocytes with BW1100. PCR analysis of nine T cell hybridomas from line 5 (Fig. 9A) and six T cell hybridomas from line 33 (Fig. 9B) as well as 11 subclones of a single T cell hybridoma from line 33 (Fig. 9C) are shown in Figure 9. The T cell hybridomas and subclones show uniformly high signals consistent with no significant loss of Tg copies in any T cell line, while the plasmablast hybridomas show great variation (compare 3G4 and 2C8; 2C8 clearly demonstrates the loss of some Tg copies; Figs. 9A and 2). These data suggest that the process of cell fusion itself is unlikely to be responsible for inducing Tg loss in vitro, and more importantly, that Tg transcription and translation are not sufficient for causing Tg deletion, which is thus probably a B-cell lineage-specific event.

Discussion

We have shown here that a recombined Ig heavy chain Tg undergoes copy losses in the B cell compartment at high frequency, although it is stable in other tissues including the thymus despite being transcribed there.

Using both Southern blots and PCR assays, hybridomas derived from transgenic spleen cells showed significant losses of Tg copies. However, many possible explanations for such loss could be...
plausible. The fusion partner or the fusion process itself or some artifact of selective culture could be responsible for inducing Tg deletion in vitro. The fact that the T cell hybridomas do not show any Tg deletion (Fig. 9) make it unlikely that tissue culture artifacts are involved. Our direct demonstration that splenic B cells purified and tested directly by PCR and Southern blot analysis for Tg copy numbers clearly show fewer copies than other somatic tissues rules out any artifactual explanations in vitro. All three independently derived founder lines of these transgenic mice show Tg deletion in their splenic B cells (Fig. 4). Thus, it is unlikely that any insertion site-specific events are involved in the deletion observed.

We found loss of Tg copies in eight of nine plasmablast hybridomas at extremely high frequency compared with the highest reported frequencies measured for homologous recombination of two adjacent copies, which range between $10^{-5}$ for one gene ($hprt$) and $10^{-3}$ for another ($a$ box gene) (43). Similarly, the estimates for Tg loss in sperm transgenic for a herpes simplex virus thymidine kinase gene (26) and for liver cells transgenic for an albumin-promoter-driven urokinase-type plasminogen activator (25) are $10^{-4}$. In these two cases, the Tg expressed is not native to the tissue deleting the Tg, making explanations based on Tg toxicity plausible. However, in the TCR Tg losses described (28, 29) and in the Ig Tg loss reported above, the Tg product is native to the deleting cell type. In the TCR transgenic mice, expression is completely lost. However, in the case of the Ig Tg described above, the transgenic product is present in cells that have undergone some Tg loss and is also seen in B cells in the bone marrow, making selection (for cells that do not express Tg) based on toxicity or environmental Ags unlikely.

When and where does deletion occur? The plasmablast hybridomas are normally efficiently generated not from mature B cells, but from plasmablasts in the late stages of differentiation to plasma cells (34, 35). Thus, deletions of Tg copies in them represent an event detected in the last stages of the life of a B cell. Since our data readily detect the presence of deletions in splenic B cell populations (which contain very small numbers of plasma cells), it is not likely that deletion occurs only in plasma cells. Thus, mature, circulating peripheral B cells in these transgenic mice show Tg deletion.

Since allelic exclusion of the endogenous IgH loci by the transgenic locus is imperfect (27, 44), we could examine the relative Tg loss based on endogenous usage. Our data show (Fig. 3) (27) that in the plasmablast hybridomas, Tg loss was correlated with the expression of endogenous IgM heavy chain proteins. Correspondingly, the relative Tg losses in these subpopulations of transgenic B cells ex vivo showed that endogenous-only B cells have far fewer Tg copies left compared with the Tg-only B cells (Fig. 5).

Thus, these cells express the endogenous allele only despite still having some copies of the Tg. The expression of an allelic endogenous product instead of the Tg was suggested as a driving force in TCR Tg deletion by others (29). One reason for our observations could be a selective advantage, that the use of endogenous polyclonal origin IgM heavy chains is likely to give a greater range of Ag binding repertoire to the B cells. Therefore, it is possible that the few cells that undergo some Tg deletion as a low frequency event express endogenous alleles and therefore offer greater chances of recognition for environmental Ags. This would lead to the selective expansion of such cells in the peripheral B cell pool and would account for the prominence of the deletional phenomenon we observed even if it were a low frequency event, as envisaged by other workers (28). If this were the only mechanism, pre-B cells and emerging B cells in the bone marrow should not show evidence of deletion. As there is some degree of Tg deletion clearly seen in CD19+CD23−, surface IgM−pre-B cells (Fig. 6), it is unlikely that peripheral antigenic selection is the only mechanism driving or selecting Tg loss. Tg deletion may still be a B cell receptor-selected event. B cell repertoires undergo positive and negative selection events immediately upon expression of a B cell receptor. Some studies suggest that cells expressing an endogenous $\mu$-chain are more readily positively selected than those expressing a transgenic Ig (45). If positive selection plays a role in Tg deletion, the earliest deleting cells would be present in the bone marrow, as our data indicate. The importance of lineage-specific signals is reinforced by our results with thymocytes and T cell hybrids.

Transcription of Ig and TCR genes appears to be correlated with their recombination (9, 46, 47). It is possible that such transcripts simply demonstrate that the locus is open and accessible for the recombining mechanisms. If the mechanisms mediating Tg deletion were analogous, it is possible that tissues showing Tg transcription would also show Tg deletion. To investigate this, we have taken advantage of the fact that thymocytes can use the IgH enhancer for transcription (41, 42). We have confirmed that the transgenic mice used here express both transgenic mRNA and protein in their thymocytes (Fig. 7). Interestingly, all thymocytes appear to express transgenic protein (Fig. 7B). Tg copy number variation was analyzed in T cell hybridomas derived from such thymocytes so as to permit detection of individual variation. However, despite excellent Tg expression, none of the thymocyte-derived T cell lines had any evidence of variation in Tg copy numbers (Fig. 8). Thus, expression of the Tg is not by itself sufficient to mediate deletion of Tg copies, even in a cell lineage that undergoes DNA recombination at other loci.

Several mechanisms for the Tg copy number reduction are possible: homologous recombination initiated by random double stranded breaks or by VDJ site-specific recombinase initiated events or by Ig switch recombination (either intra- or interchromosomal events). The latter is unlikely to be the sole mechanism because deletion is observed in plasmablast hybridomas that have not undergone Ig switching (Fig. 2) as well as in hybridomas that have undergone such switches (hybridoma 1B4, Fig. 2). In vitro investigations of homologous recombination of $\mu$ (48–52) show much lower frequencies than we observed here. None of our analyses with plasmablast hybridomas revealed any cells in which the Tg had been completely deleted; at least one copy appeared to

![FIGURE 9. PCR analysis of transgenic T cell hybridomas from lines 5 and 33. A. Tg PCR signal for nine T cell hybridomas from line 5 thymus compared with plasmablast hybridomas that either do (2C8) or do not (3G4) show evidence of significant Tg loss. B. PCR signals for the Tg or the AC gene in titrated amounts (threefold beginning at 5000 ng) of DNA from six T cell hybridomas from line 33 or from line 33 liver. C. PCR signals from 11 subclones of one of the T cell hybridomas from B, 6D1.](http://www.jimmunol.org/Downloaded.png)
remain despite the use of probes widely separated from each other in the Tg construct (27) (Fig. 3). It thus appears that intrararray recombinations may be responsible for Tg loss. Because the most extreme case of Ig deletion still leaves one copy remaining, there is an apparent requirement for homology for intrararray recombination. This requirement may be for survival and resolution rather than for initiation of deletion.

Thus, it is possible that homologous recombination events mediate gene losses at high frequency within the Tg tandem arrays. If the repetitive nature of the Tg array was responsible for its sensitivity to such deletion, there would be a difference between the deletion frequency seen in high copy number and low copy number transgenic lines, which there does not appear to be (Fig. 4). Thus, regardless of the precise mechanism of loss, some B-cell lineage-specific contributory factor appears to be crucially involved in the deletion. The identity of such a factor(s) and the delineation of Tg properties encouraging such deletion would help address mechanisms contributing to genomic stability and would also aid in the design of therapeutic Tgs.

References