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*J Immunol* 2005; 175:8133-8137;
doi: 10.4049/jimmunol.175.12.8133

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Sequence Transfers between Variable Regions in a Mouse Antibody Transgene Can Occur by Gene Conversion

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Different vertebrate species show widely differing usage of somatic hyperconversion (SHC) as a mechanism for diversifying expressed Ab V genes. The basis for the differing levels of SHC in different species is not known. Although no clear evidence for SHC has been found in normal mouse B cells, transgenic mice carrying high-copy numbers of a gene construct designed to optimize detection of SHC have previously been shown to exhibit sequence transfers that resemble gene conversion events. However, these transgene sequence transfers could reflect multistep or reciprocal DNA recombination events rather than gene conversions. We now find in low-copy number transgenic mice that transgene sequence transfers can exhibit the unidirectional sequence information movement that is a hallmark of gene conversion. This indicates that gene conversion between V region sequences can occur in mouse B cells; we propose that the lack of efficient SHC contributions to Ab diversification in normal mice may be due, at least in part, to the particular pattern of V gene recombinational accessibility that occurs in differentiating mouse B cells. The Journal of Immunology, 2005, 175: 8133–8137.

D uring B cell development, V(D)J recombination of Ab gene segments is a highly conserved mechanism for the generation of Ab repertoire diversity in all jawed vertebrate species (1, 2). In warm-blooded vertebrates, other processes are also involved in diversifying Ab repertoires. Somatic hypermutation is one mechanism for diversification that reflects the introduction of single point mutations into Ab V(D)J segments (reviewed in Ref. 3). Gene conversion is a second diversifying mechanism that is characterized by transfers of homologous sequences from donor Ab V gene segments to an acceptor V gene segment (4–7). If donor and acceptor segments have numerous sequence differences, then gene conversion can introduce a set of sequence changes into a V region by a single event.

All warm-blooded vertebrate species appear to use somatic hypermutation to diversify their Ab repertoires. However, only some species exhibit frequent gene conversion events during Ab diversification. Hypermutation and hyperconversion have been most extensively analyzed in mice and chickens. In chickens, both processes are observed (4, 8–10) although hyperconversion appears to dominate during early B cell development. In contrast, although somatic hypermutation is common in mice during immune responses, definitive gene conversion events have not been detected in developing or stimulated mouse B cells (11–16). The reasons why mice (or other species such as sheep or humans) do not use gene hyperconversion for Ab diversification are not known.

Hypermutation in mice and hyperconversion in chickens both require the activation-induced cytidine deaminase (AID)3 enzyme that is also important for Ab gene class switching (17, 18). AID deamination of cytidine residues in ssDNAs appears to lead to DNA damage that can result in mutation, gene conversion, or switch recombination events during DNA repair (18–23). It has been suggested that differences in available DNA repair pathways might determine whether AID-induced DNA damage leads to hypermutation or hyperconversion. The strongest evidence supporting this notion comes from the DT40 chicken cell line that undergoes Ab gene hyperconversion during passage in culture; DT40 mutants lacking the XRCC2/3 DNA repair proteins have greatly reduced hyperconversion activity but retain somatic hypermutation activity (24).

The lack of Ab gene conversion events in some species could indicate that hyperconversion pathways have only evolved in some species or, instead, that hyperconversion pathways in some species have become vestigial. Transgenic mice carrying a gene construct (VVCμ), designed to optimize detection of sequence transfers, provide the strongest evidence supporting a possible V-region conversion mechanism in mice (25). The VVCμ transgene (depicted in Fig. 1) contains two tandem VDJ segments; one functional VDJ gene segment (R16.7 VDJ) that encodes a μ H chain capable, with the correct L chain, of binding the hapten, p-azophenylarsonate (Ars); and a second upstream promoterless VDJ segment (2B4 VDJ) that is identical to the R16.7 VDJ except for 17 single base pair differences. Sequence transfers between the 2B4 and R16.7 VDJ segments are easily found in Ars-keyhole limpet hemocyanin (KLH) immunized multicopy VVCμ transgenic mice (25); however, these transfer events could represent either multiple (or reciprocal) recombinations mediated by a mechanism that is not related to the chicken V gene hyperconversion process, or they could represent gene conversions (26). Differences in the importance of the Rad54 repair protein in chicken hyperconversion (27) and in mouse VVCμ sequence transfers (28) might suggest that these two processes are not closely related.

To determine whether mouse VVCμ transgene sequence transfers are due to gene conversions or due to multiple/reciprocal recombinations, we have produced low-copy transgenic mice and analyzed the relationships between donor and acceptor sequences involved in the sequence transfers that occur in these low-copy animals. We find that at least some VVCμ sequence transfers are

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Received for publication May 12, 2005. Accepted for publication October 6, 2005.

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1 This work was supported by National Institutes of Health Grants AI24465 (to E.S.) and CA65441 (to N.D.).

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3 Abbreviations used in this paper: AID, activation-induced cytidine deaminase; Ars, p-azophenylarsonate; KLH, keyhole limpet hemocyanin; ES, embryonic stem.
unidirectional, indicating that gene conversion pathways are available in mouse B cells for the diversification of V regions.

Materials and Methods

Animals

Transgenic mice were produced by cotransferring the VVC\(\mu\) construct (25) and a neoexpression construct into embryonic stem (ES) cells. ES cell clones resistant to G418 were analyzed by Southern blotting to determine approximate copy numbers of integrated VVC\(\mu\) transgenes. ES cell clones having low VVC\(\mu\) transgene copy numbers were injected into blastocysts to produce chimeric mice and these chimeras were bred to establish transgenic lines. All animal studies have been reviewed and approved by the Tufts School of Medicine Institutional Animal Care Use Committee.

Immunization and hybridoma production

Transgenic lines were immunized with Ars-KLH to assess transgene sequence transfers as described previously (25). Mice between 2 and 3 months of age were immunized i.p. three times with 0.1 ml containing 100 \(\mu\)g of Ars-KLH with IFA and then a fourth time with 100 \(\mu\)g of Ars-KLH in saline. Three weeks separated each immunization. Splenocytes or blood lymphocytes for RT-PCR analyses were collected after the third immunization. Four days after the fourth immunization, splenocytes were obtained for hybridoma production. Sequence transfers in splenocytes and blood lymphocytes from immunized transgenic mice were detected using RT-PCR/Southern blot assays that have been described previously (28, 29).

Immunized transgenic mice exhibiting sequence transfers as indicated by the RT-PCR/Southern blot assay were then used to produce hybridomas. Hybridomas were initially screened for production of anti-Ars IgG using an ELISA that has previously been described (30). Anti-Ars IgG-producing hybridomas were then screened for IgG reactive with the ADS anti-idiotypie antigen again using a previously described ELISA (30). Previous studies have shown that many hybridomas from immunized VVC\(\mu\) transgenic mice that exhibit transgene sequence transfers lack ADS reactivity (25). All hybridomas producing anti-Ars IgG that lacked ADS reactivity were selected to be analyzed by sequencing for transgene sequence transfers.

Analyses of transgene copy number and sequences

Transgene-derived \(\gamma\)-chain mRNAs from the selected hybridomas were analyzed for sequence transfers by RT-PCR amplification followed by DNA sequencing as described previously (25). Transgene copy numbers and sequences in genomic DNAs from germline cells and from hybridomas were analyzed by Southern blotting and by sequencing of PCR clones derived using primers specific for the VDJ regions in the VVC\(\mu\) transgene. The transgene-specific primer pair consists of a leader sequence (5'-CCGAATTCACACACTGACTCAAACCATG-3') and a J\(\mu\)2 sequence (5'-CCAGAAATCCTACTGGAAGACTGTGGTAGAG-3') that are found in both the R16.7 and 2B4 VDJ segments of the VVC\(\mu\) transgene.

Comparisons of gene sequences

Sequences of hybridoma RT-PCR products were compared with the VVC\(\mu\) transgene. R16.7 and 2B4 VDJ sequences to assess whether sequence transfer events had occurred. As described previously (25), only the R16.7 VDJ segments in the VVC\(\mu\) transgene have promoters and can be expressed as mRNA. Sequence transfers are therefore indicated by mRNAs exhibiting a pattern where the expressed R16.7 sequence contains a block of nucleotide changes that match the 2B4 VDJ sequence rather than the R16.7 sequence (25). Sequences of panels of cloned PCR-amplified genomic VDJ segments from germline and hybridoma cells were compared with the VVC\(\mu\) transgene. R16.7 and 2B4 VDJ sequences, as well as the expressed mRNA sequence in the 591A6 hybridoma, to determine the origin of each PCR clone. These clones were all found to correspond to one of the three sequences (either R16.7, 2B4, or 591A6) except for occasional PCR errors that occurred at frequencies of about 1/700 nucleotides. This error frequency was higher than we expected but was similar to errors that we have found in similar PCR amplifications of the endogenous C\(\mu\) gene. Furthermore, the putative PCR errors were scattered throughout the sequences and, except for a few instances, did not occur in more than one clone or at positions that differed between the R16.7, 2B4, and 591A6 sequences. Thus, these PCR errors were ignored in assigning the origin of each PCR clone sequence. Most importantly, in all the clones that were assigned as 2B4 sequences (the donors for sequence transfers) there were no PCR errors that were found at positions that differed between the 2B4 and R16.7 VDJ sequences.

Results

Production of low-copy VVC\(\mu\) transgenic mice that exhibit transgene sequence transfers

To assess whether sequence transfers in the VVC\(\mu\) transgene occur by gene conversion, we wanted to produce low-copy transgenic mice that would facilitate analyses of donor and recipient sequences involved in the sequence transfer event. The VVC\(\mu\) construct was transfected into ES cells and transgene copy numbers were determined for individual clones by Southern blot analyses (see example in Fig. 1). Twenty-four ES cell clones were analyzed and two clones were identified, each of which appeared to carry one transgene copy. The two clones were separately microinjected into blastocysts to obtain chimeric mice and the chimeras were bred. Only one of these ES cell clones yielded chimeras that transmitted the transgene to offspring. However, extensive efforts to obtain IgG-producing anti-Ars hybridomas after immunization of these offspring were unsuccessful, indicating that the single VVC\(\mu\) transgene copy in the ES cell clone was not capable of interchromosomal switch recombination. Because all sequence transfers in multicopy VVC\(\mu\) transgenic mice have been found in IgG-producing B cells (25, 28, 29), and because there is no information indicating whether such transfers can be found in IgM-producing B cells, these mice were not studied further. A third ES clone carrying about two copies of the VVC\(\mu\) construct (Fig. 1) was used to produce another transgenic line (VV29). VV29 mice immunized with Ars-KLH exhibited serum levels of transgene-derived anti-Ars IgG (data not shown) that were similar to previously analyzed VVC\(\mu\) transgenic mice (25, 30, 31). Thus, the

![FIGURE 1. Diagram of the VVC\(\mu\) transgene and Southern blot analyses of VVC\(\mu\) copy number in the ESVV29 cell line and VV29 transgenic mice. In A, the organization of the VVC\(\mu\) transgene is depicted. Boxes represent various exons as labeled. The triangle indicates the promoter of the R16.7 VDJ exon; the 2B4 VDJ segment (black boxes) has no promoter. In B, DNAs from the parental D3 ES cell line are compared with the ESVV29 clone carrying a VVC\(\mu\) transgene. DNAs were digested with BamH1 and EcoRI and hybridized with a probe containing the C\(\mu\)1 exon. Bands at 8 kb correspond to the two germline C\(\mu\) loci present in the ES cells whereas the band at 5 kb corresponds to the VVC\(\mu\) transgene. Quantitative phosphorimager analyses of the band intensities indicate that two copies of the VVC\(\mu\) transgene are found in the ESVV29 cell line. In C, kidney DNAs from VV29 transgenic mice and BALB/c nontransgenic mice are compared. DNAs were digested with BamH1 and EcoRI and hybridized with the pJH1 probe containing J\(\mu\) and E\(\mu\) enhancer sequences. Bands at 2 kb correspond to the two germline J\(\mu\)-C\(\mu\) loci whereas the band at 6 kb corresponds to the VVC\(\mu\) transgene. Analyses of band intensities using densitometry of the autoradiogram indicated that about two copies of the transgene are found in VV29 mice.]
VV29 line provided low-copy VVCμ transgenic mice that were suitable for further analyses of the sequence transfer mechanism.

Transgene sequence transfers in immunized VV29 blood lymphocytes were assessed using an RT-PCR/Southern blot assay that has been described previously (28, 29). In this assay, PCR products that hybridize with the 2B4 probe are indicative of sequence transfers in the VVCμ transgene. As shown in Fig. 2, sequence transfers were detected in two of three immunized VV29 animals, and the levels of sequence transfers in the low-copy VV29 mice were similar to levels in a representative multicopy VV5 mouse that has been analyzed previously (29). In other experiments, splenocytes from four of seven immunized VV29 mice exhibited sequence transfer events either by the RT-PCR/Southern blot assay or by sequencing VDJ segments in cloned splenocyte RT-PCR products (data not shown). These findings showed that sequence transfer events found in high-copy VVCμ mice can also occur in low-copy mice.

Three transgene VDJ regions in VV29 mice

Southern blot analyses had indicated that the VV29 mouse line contained two copies of the VVCμ transgene. However, these analyses had used a hybridization probe located within the Jμ-HV region of the transgenes; this probe would not assess the number of individual VDJ segments that were present within the VV29 mouse line. To determine the number of VDJ segments in the germline of VV29 mice, transgene VDJ gene segments were amplified from VV29 germline (kidney) DNA and then characterized by sequencing. The PCR amplification of VDJ segments used primers that did not distinguish the R16.7 and 2B4 VDJ gene segments. PCR products were cloned and clones were randomly chosen for sequencing. Analyses of the sequences from 14 of these VDJ PCR clones showed that 9 were from the R16.7 VDJ gene segment and 5 were from the 2B4 VDJ segment. Together with the Southern blot analyses that indicated two Jμ-Cμ transgene segments, this 2:1 ratio of VDJ segments indicated that one of the VVCμ transgene copies in VV29 mice was truncated and lacked an upstream 2B4 VDJ segment (Fig. 3B). These results indicate that three transgene-derived VDJ segments are present in VV29 mice; this conclusion was further supported by analyses of VDJ segments in a VV29 hybridoma as described below.

Hybridoma cell lines from low-copy VV29 mice exhibit sequence transfers

Hybridomas were produced from immunized VV29 mice and these were screened for sequence transfer events using the AD8 anti-idiotypic Ab. The AD8 reagent reacts with H chains that carry the R16.7 VDJ CDR2 sequence but not with H chains having the 2B4 VDJ CDR2 sequence (25, 28). Transgene-expressing B cells from VVCμ transgenic mice generally express the AD8-reactive idiotope whereas those VVCμ B cells that exhibit transgene sequence transfers often do not express the AD8-reactive idiotope (25, 28, 29). Candidate hybridomas expressing anti-Ars IgG Abs that were not reactive with AD8 were further screened by sequence analyses of the expressed H chain VDJ from RT-PCR products. Despite the results showing transgene conversion events in immunized splenocytes, it was difficult to find hybridomas that exhibited sequence transfers. Among more than 600 hybridomas that were screened, nine anti-Ars IgG and AD8-negative hybrids were found but only one hybridoma (591A6) exhibiting sequence transfers was obtained. Fig. 3 shows the 591A6 transgene-derived γ-chain mRNA VDJ sequence compared with the R16.7 and 2B4 VDJ sequences present in the VVCμ transgene. The block of nucleotide changes in the expressed 591A6 VDJ that corresponds to the sequence of the 2B4 VDJ indicates a transgene sequence transfer similar to those described previously (25, 28, 29).

Analyses of hybridoma VDJ regions show that sequence transfers occur by gene conversion

The sequence transfer in the 591A6 hybridoma allowed further analyses to determine the transfer mechanism. Sequence transfers that occur by gene conversion are characterized by a unidirectional movement of sequence information from a donor sequence to a homologous acceptor sequence. The donor sequence in a gene conversion event is not altered. Such unidirectional sequence transfers have been directly demonstrated for the chicken hyperconversion mechanism (32). Sequencing of 591A6 transgene VDJ segments was used to determine the status of donor sequences in the hybridoma.

Transgenic VDJ gene sequences in genomic DNA from the 591A6 hybridoma were determined by sequencing cloned PCR products that were generated using primers that did not distinguish 2B4 and R16.7 VDJ gene segments. A total of 69 VDJ sequences were determined; 24 of these were found to come from transgene R16.7 VDJ regions, 25 were from 2B4 VDJ regions, and 20 were from the expressed 591A6 γ-chain VDJ gene (criteria used to assign the sequences are described in Materials and Methods). Because the expressed 591A6 sequence must be derived from a single H chain gene, the observed ratios of VDJ sequences indicate that the 591A6 hybridoma genome contains three transgenic VDJ gene segments: one R16.7 VDJ segment, one 2B4 VDJ segment, and one R16.7-derived VDJ segment that has undergone a sequence transfer event and that is expressed as γ-chain mRNA. These results are consistent with the finding that germline VV29 cells contain three transgenic VDJ segments. The findings indicate that the 591A6 hybridoma retains all of the VDJ copies present in VV29 germline cells, and that one of the two 591A6 R16.7 VDJ segments has undergone both a switch recombination event and a sequence transfer event as depicted in Fig. 3.

The expressed R16.7 VDJ transgene copy in the 591A6 hybridoma exhibits nucleotide changes that are clearly derived from a donor 2B4 gene. Because there is only one 2B4 gene available in VV29 B cells to serve as a donor for these nucleotide changes, then the single 2B4 VDJ gene that is present in the 591A6 hybridoma must have been the donor for the DNA sequence transfer that has...
altered the expressed γ-chain 591A4 VDJ gene. The 25 PCR clones from the single donor 2B4 VDJ gene in 591A6 show that the sequence of this 2B4 gene is the same as found in the germline and, therefore, has not been altered by the sequence transfer process. These results show that the sequence transfer event in 591A4 cells is unidirectional and has occurred by a gene conversion mechanism.

Discussion

Analyses of the VV29 transgenic mouse line carrying only two copies of the VVCμ transgene show the same types of sequence transfers between Ab V genes in stimulated B cells that have previously been reported for the high-copy VV1 and VV5 transgenic lines (50 and 30 copies, respectively) (25). Thus, high transgene copy numbers are not required for the sequence transfer mechanism. Furthermore, the levels of sequence transfer events in immunized VV29 mice appear to be similar to the levels in VV1 and VV5 mice, indicating that copy number does not have a major effect on sequence transfer efficiency. Characterizations of the donor and acceptor V gene sequences in a hybridoma derived from an immunized VV29 mouse show the unidirectional sequence transfers that are characteristic of a gene conversion mechanism and that have been shown for hyperconversion events in chicken B cells (32). These results also suggest that the numerous sequence transfers found in mice carrying high copy numbers of the VVCμ transgene (25, 28) also occur by gene conversion. The results indicate that mouse B cells can exhibit gene conversions between Ab V genes, similar to the events that have been reported for chicken, rabbit, and cow B cells.

Our results indicating a mechanism for gene conversion between V genes within a transgene construct in mouse B cells raises questions about the apparent lack of such events between the endogenous V genes in nontransgenic mice. The frequency of transgene conversions in VVCμ transgenic mice does appear to be low because preferential Ag selection of cells that have undergone conversion events is required to easily detect the transfers in immunized animals (29). For endogenous mouse Ab genes, only a few studies have found evidence to suggest possible V gene conversion events (33, 34). Given the numerous studies of Ab responses to immunization with various Ags that have been done in nontransgenic mice, it is surprising that no definitive examples of gene conversion events have been detected.

Perhaps there are other features of mouse B cell differentiation that limit gene conversions between germline and recombinant V genes during normal B cell maturation or during B cell responses to Ag. Transgene conversion events appear to occur in Ag-stimulated B cells; all transgene sequence transfers in VVCμ mice have been found together with somatic mutations and in IgG-producing cells. In normal mouse B cells, the germline V genes that would provide donors in V gene conversion events are in “closed” chromosomal chromatin and might not be accessible to a hyperconversion mechanism. Furthermore, in those early normal pre-B cells where germline and recombinant V genes would presumably both be in accessible chromatin, the AID protein does not appear to be expressed (35, 36) and, therefore, hyperconversion is again not likely to occur. Thus, the regulation of V gene accessibility in mouse B cells might inhibit the conversion process. Nevertheless, our results, showing that the VVCμ transgene can exhibit conversion events between V genes, support a hyperconversion mechanism that has been conserved during evolution but that varies in activity in different species due to distinct evolutions of Ab gene organization and regulation in each individual species.

Acknowledgments

We thank Drs. Frederick W. Alt and Jianzhu Chen for help in the production of transgenic ES cell lines, Dr. Hwei-Fang Tsai for help in analyzing the initial single-copy mice, and Dr. Naomi E. Rosenberg for critically reading the manuscript.
Disclosures

The authors have no financial conflict of interest.

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