Templated insertions in the rearranged chicken Ig_L V gene segment arise by intrachromosomal gene conversion

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Chickens create a repertoire for their immunoglobulin light-chain gene by a novel process of sequence substitution within a unique rearranged V gene segment (V_{L1}) during B-cell development in the bursa of Fabricius. Sequence analysis has shown that these nucleotide substitutions are not random. Potential donors for observed sequence substitutions are present within the 25 ψV_L segments located 5' of the V_{L1} gene. In this report, we demonstrate that V_{L1} sequence substitutions: (1) are derived from the ψV_L donor segment templates in *cis*, (2) do not result in reciprocal transfer of V_{L1} gene sequences to the ψV_L segments, and (3) lead to the rapid disappearance of cells with nondiversified rearranged V_{L1} genes during B-cell development in the bursa of Fabricius. Together, these data provide evidence that V_{L1} sequence diversity arises as a result of intrachromosomal gene conversion.

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Gene conversion is the nonreciprocal transfer of genetic information from one genetic element to a homologous but nonidentical genetic element. Nonreciprocal exchange of homologous genetic information has been firmly established only in lower order fungi (for review, see Hastings 1988), in which it is possible to recover and analyze all products of individual meiotic events. Under these circumstances, gene conversion is formally demonstrated by observing non-Mendelian ratios in the segregation of heterozygous markers. In contrast, although numerous events that are consistent with the unidirectional transfer of sequence information during meiosis have been characterized in higher eukaryotic organisms, it has not been possible to exclude the occurrence of alternative recombination events that could result in the apparent gene conversion phenotype of a daughter cell (for review, see Kourilsky 1986). Thus, the term gene conversion has been used loosely to define the observed product of an incompletely defined genetic exchange.

Experimental models to characterize genetic exchange between homologous sequences have also been generated by use of transfected genes determining selectable phenotypes (Liskay and Stachelek 1983; Kucherlapati et al. 1984; Brenner et al. 1985; Subramani and Rubnitz 1985). For example, Liskay and Stachelek (1983, 1986) stably transfected mouse LTK⁻ cells with two contiguous TK genes carrying specific defects. By selection in HAT medium they were able to rescue cells with a functional TK gene. In some instances, the generation of a

functional TK gene occurred by a transfer of sequence information consistent with gene conversion. However, because of the positive selection used to identify these rare events, it could not be determined whether these events occurred by true gene conversion or by double homologous recombination between sister chromatids. Because it has been necessary to use positive selection to identify potential gene conversion events, it has not been possible in current model systems to demonstrate the nonreciprocity of the genetic exchange required to prove that a gene conversion has taken place.

Recently, it has been shown that the immunologic diversity of the chicken immunoglobulin gene repertoire is created by segmental sequence substitutions within the rearranged variable gene segments of both the heavy- and light-chain genes. The Ig_H and Ig_L loci each contain single variable region gene segments capable of participating in the assembly of a functional immunoglobulin gene (Reynaud et al. 1985, 1989). In the chicken, immunoglobulin gene rearrangements occur in peripheral hematopoietic tissues and serve primarily to activate the expression of the immunoglobulin gene loci (McCormack et al. 1989a). Evidence has been presented that in the majority of avian B cells only one Ig_L gene and one Ig_H gene undergo rearrangement, and cells that have undergone a functional rearrangement of both the heavy- and light-chain genes are selected to proliferate within the microenvironment of the bursa of Fabricius (McCormack et al. 1989b; Reynaud et al. 1989). Diversity

within the rearranged immunoglobulin gene is generated during the expansion of functional B cells in this proliferative phase of B-cell development within the bursa of Fabricius, and is revealed by sequence substitutions that are restricted to the rearranged variable gene segments of both the heavy- and light-chain gene loci (Reynaud et al. 1987, 1989; Thompson and Neiman 1987). Sequence analysis has demonstrated that this diversification does not occur randomly, but results from repetitive substitutions of sequences present in families of V-homologous pseudogenes (ψV) present 5' of both the functional V_{H1} and V_{L1} gene segments (Reynaud et al. 1987, 1989). In the case of the Ig_L locus, all 25 ψV_L segments have been cloned and sequenced, and found to lack both transcriptional and recombination signal sequences (Reynaud et al. 1987). The process of sequence substitution within the variable region genes leads to the generation of cells that no longer proliferate within the bursal environment, and migrate to peripheral lymphoid tissue to comprise the humoral immune system of a mature bird (Reynaud et al. 1987; Thompson and Neiman 1987). Although the sequence information obtained thus far suggests a process of sequence transfer from the ψV segments to the functionally rearranged V gene segments of both the heavy- and light-chain loci, the precise mechanism by which the sequence transfer occurs has not yet been defined.

In this report, we analyze the genetic properties of the sequence transfer between ψV_L donor segments and rearranged V_{L1} genes during B-cell development in the bursa of Fabricius. Using an F_1 cross of two genetically inbred chicken strains, we have been able to demonstrate that gene conversion events occur primarily if not exclusively in *cis*. We developed nondiversifying clonal B-cell lines from these F_1 animals by direct transformation with a *v-rel*-containing retrovirus. In these cell lines, which have undergone multiple sequence substitution within the rearranged V_{L1} gene, there is no reciprocal transfer of sequence information into the intrachromosomal ψV_L segments of either Ig_L allele. Additionally, the frequency with which the undiversified rearranged V_{L1} genes can be obtained during the late embryonic period of bursal development decreases rapidly after the onset of diversification during embryonic development in the bursa of Fabricius. This suggests that individual diversification events within the proliferating bursal lymphocyte population precede DNA replication during the cell cycle. Together, our data demonstrate that the diversification of the chicken immunoglobulin light-chain gene occurs as a result of intrachromosomal gene conversion.

Results

Templated sequence substitutions create diversity in the rearranged V_{L1} gene

The genomic organization of the Ig_L locus of the SC chicken is shown in Figure 1A. A functional Ig_L gene is created by rearrangement of the unique V_{L1} and J_L gene segments. When joined in-frame, this rearrangement leads to transcription of a functional Ig_L gene. Because

the SC bird is an F_1 cross between two inbred chicken strains, designated G4 and S3, it is possible to distinguish between the two parental Ig_L alleles by polymorphic *Bam*HI and *Eco*RI restriction endonuclease sites that flank the V_{L1} - J_L rearranging unit. In all B-cell lines examined to date, these *Bam*HI and *Eco*RI restriction endonuclease sites remain linked, demonstrating that neither rearrangement nor diversification of the chicken Ig_L locus leads to a significant frequency of interallelic recombination.

The nucleotide sequence of a rearranged V_{L1} gene segment from the Ig^+ B-cell line 41LI, derived from transformation of bursal lymphocytes with a *v-rel*-containing retrovirus at the day of hatching, is shown in Figure 1B. This rearranged V_{L1} gene contains 43 nucleotide substitutions with respect to the germ line V_{L1} gene from which it was derived. From the previously published sequence of the ψV_{L5} segment (Reynaud et al. 1987), it can be seen that all 15 of the 5' nucleotide substitutions present in the clone are also present in ψV_{L5} pseudogene, including a 6-bp insertion in the first complementarity-determining region (CDR1). At the 3' end of the clone, the last six nucleotide substitutions are also present in the ψV_{L5} segment. In contrast, the internal 21 nucleotide substitutions present in clone 41LI are all present in the ψV_{L23} gene segment. On the basis of this analysis, it appears that the sequence heterogeneity present in clone 41LI can be entirely accounted for from sequences derived from ψV_{L5} and ψV_{L23} . In the simplest case, this would arise first from substitution of sequences derived from ψV_{L5} beginning somewhere 5' of codon 1 and continuing through codon 85. This diversified segment would then have undergone a subsequent event with sequences derived from ψV_{L23} beginning somewhere between codon 30 and codon 45 and ending between codons 67 and 75. Analysis of this and additional clones confirms the observations of Reynaud et al. (1987) that heterogeneity within the rearranged chicken Ig_L V gene segments arises from sequence information derived from the ψV_L segments (McCormack and Thompson 1990). The purpose of the present experiments was to extend these observations by attempting to define the mechanism by which this sequence substitution occurs.

Potential mechanisms of templated sequence substitution in the rearranged V_{L1} gene

A number of potential mechanisms can be hypothesized for the transfer of sequence information from one homologous gene segment to another (Fig. 2). We have taken advantage of the restriction-site and nucleotide-sequence polymorphisms identified within the V_{L1} and ψV_L gene segments of the parental strains of the F_1 SC chicken strain to identify the alleles of the gene segments involved in diversification events. The parental configuration of a B cell with one rearranged Ig_L allele that has not undergone diversification is shown in the center of Figure 2. A sequence polymorphism in the ψV_L segment (labeled "A" on the rearranged allele and "B" on the germ line allele) distinguishes between the parental ψV_L alleles. In a homologous position in the V_{L1}

gene segment is the sequence labeled "C". The V_{L1} parental alleles are distinguished by restriction-site polymorphisms (asterisks) and sequence polymorphisms in the leader intron (data not shown).

By use of these markers, we designed experiments to test six fundamentally distinct mechanisms of sequence

transfer. As depicted in Figure 2, these mechanisms include: (1) intrachromosomal (*cis*) gene conversion, which predicts a unidirectional exchange that replaces C on the rearranged allele with A and leaves the donor A sequence unchanged; (2) interchromosomal (*trans*) gene conversion, which predicts a unidirectional exchange to

Figure 1. Sequence heterogeneity within the avian IgL gene occurs within a single rearranged V_{L1} gene. (A) A schematic map of the germ line (upper line) and rearranged (lower line) chicken IgL locus is depicted (Reynaud et al. 1985). The locus contains a single functional V_{L1} gene, which is capable of rearrangement, producing an in-frame joint of V_{L1} and J_L gene segments by deletion of a circular episome of the DNA between V_{L1} and J_L in the germ line (McCormack et al. 1989a). 5' of the single functional V_{L1} gene are 25 V-homologous gene segments that all lack recombination signal sequence, leader exons, and transcriptional signals. A map of the *KpnI* (K) and *ScaI* (S) restriction sites that we have been able to confirm by sequence analysis of the ψV_L gene segments of both parental G4 and S3 strains of the SC chicken is given. Also shown are the polymorphic *BamHI* (B) and *EcoRI* (R) sites, both of which are present in the G4 strain and absent in the S3 strain. Arrows indicate the orientation of ψV_L segments with respect to V_{L1} transcription. (B) Sequence comparison of the germ line G4 allele of the V_{L1} gene (G), a rearranged V_{L1} – J_L region isolated from the *v-rel*-transformed cell line 41LI, and two potential ψV_L segments capable of donating all of the nongerm-line base pairs of clone 41LI. Identity to the germ line sequence is indicated by dashes. The position of V–J joining is indicated by an arrow.

Figure 2. Potential mechanisms leading to template-mediated sequence substitution within the rearranged variable gene segment. In the central panel, the parental configuration of a B cell that has undergone $V_{L1}-J_L$ rearrangement on one Ig_L allele is illustrated. In all diversification events considered, flanking polymorphic restriction sites remain linked as indicated by the asterisks (*).

Solid black bars represent ψV_L segments homologous to the functionally rearranged V_{L1} gene, and contain polymorphic sites on the rearranged (A) and on the unrearranged (B) parental allele. In a homologous position in the V_{L1} gene is sequence C. Depicted in the upper set of panels are the products of intrachromosomal (*cis*) gene conversion, interchromosomal (*trans*) gene conversion, *cis* double homologous recombination, and *trans* double homologous recombination. The lower set of panels represents events that may occur uniquely after replication of DNA during S phase to generate sister chromatids. These include gene conversion between sister chromatids and double homologous recombination between sister chromatids. As depicted in the lower half of this panel, each of these two mechanisms leads to the production of two daughter cells with distinct phenotypes.

replace C on the rearranged allele with B from the donor ψV_L segment of the germ line allele, whereby the donor B sequence remains unchanged; (3) *cis* double homologous recombination, which predicts a reciprocal exchange between C and A on the rearranged allele; and (4) *trans* double homologous recombination, which predicts a reciprocal exchange between C on the rearranged allele and B on the germ line allele. In addition, two mechanisms that could occur only after DNA replication during cell proliferation were considered: (5) gene conversion between sister chromatids; and (6) double homologous recombination between sister chromatids. These two mechanisms predict unidirectional and reciprocal exchanges, respectively, and each leads to the production of two daughter cells with distinct phenotypes. Because it is not possible in our system to identify all of the products resulting from a single sequence-substitution event, we designed a series of experiments to address each of the potential molecular mechanisms individually in the hope of defining the molecular mechanism of sequence transfer by the process of elimination.

Rearranged V_{L1} gene substitutions occur in cis

As noted in Figure 1B, sequence information present within the ψV_L segments appears to be capable of inserting additional codons in the rearranged V_{L1} gene. Six of the ψV_L segments identified by Reynaud et al. (1987) contain distinct insertions of 2–5 codons within CDR1. The sequences of all six of these pseudogene segments were determined from germ line DNA of both parental strains of the SC bird. Two of the pseudogenes, ψV_{L11} and ψV_{L12} , were found to have polymorphisms that flanked the insertion (Fig. 3A). On the basis of these polymorphisms, it was possible to design unique 20-mer oligonucleotide probes spanning these polymorphisms for the G4 and S3 alleles of ψV_{L11} and ψV_{L12} . These oligonucleotide probes were then used to screen a recombinant library containing rearranged V_{L1} – J_L regions derived from polyclonal bursal lymphocytes at day 18 of embryogenesis. Positive clones were sequenced to identify the parental allele from which they were derived, as well as to identify definitively the pseudogene donor used in the diversification event. Sequence polymorphisms present in a nondiversifying portion of the leader intron of these clones allow assignment of individual V – J recombinants to the parental allele from which they were derived. The sequences of two clones identified in this manner are presented in Figure 3B. In both of these clones, large-scale substitutions of sequences derived from the ψV_{L12} segment were observed. In clone 89-21, which is derived from a rearranged G4 allele, the sequence at each of eight base pairs that are polymorphic between the ψV_{L12} sequences of the parental strains is derived from the G4 ψV_{L12} allele. In contrast, clone 122-8, which is derived from a rearranged S3 allele, encodes sequence at the same eight polymorphic bases derived from the ψV_{L12} S3 allele. As defined by unique V_{L1} – J_L joints and the beginning and end of the templated ψV_{L12} sequence substitutions, 11 ψV_{L12} -templated sequence substitutions were defined (Fig. 3C). Of these,

five occurred on rearranged V_{L1} – J_L segments from the G4 parental allele, and in all five cases, the polymorphic base pairs were derived from the G4 ψV_{L12} allele. In six clones, sequence substitution occurred on a rearranged S3 allele, and in all six clones the polymorphic base pairs defined the donor sequence template as being from S3 ψV_{L12} allele. Similar analyses of four events involving the ψV_{L11} segment identified two sequence-substitution events on a rearranged G4 allele. Both were derived from G4 ψV_{L11} donor segments. Two events on a rearranged S3 allele that involved sequence substitutions from a S3 ψV_{L11} donor segment were also observed (Fig. 3C). This analysis indicates that the sequence information used for the generation of diversity within rearranged V_{L1} genes arises from homologous pseudogene segments primarily in *cis*. Sequence analysis of 52 randomly cloned V_{L1} – J_L regions has identified 24 similarly defined diversification events involving a number of additional pseudogene donors (McCormack and Thompson 1990). All of these events occurred in *cis*. Of 39 events analyzed, we failed to define a single sequence substitution mediated in *trans*. Therefore, on the basis of this analysis, we eliminated *trans* gene conversion and *trans* double homologous recombination from consideration as the primary source of sequence substitution within rearranged V_{L1} genes during B-cell development in the bursa of Fabricius.

Clonal B-cell lines do not contain reciprocal sequence substitutions in the ψV_L segments

Previous experiments using polyclonal B cells or avian leukemia virus (ALV)-immortalized B-cell lines failed to demonstrate evidence of gross rearrangements within the ψV_L gene region. However, because these populations are heterogeneous with respect to the diversification events they have undergone (Thompson and Neiman 1987; Thompson et al. 1987), it has not been possible to determine whether individual sequence substitution events lead to the reciprocal exchange of genetic information with the pseudogene donor. To address this issue in detail, it was first necessary to obtain clonal populations of B cells that had undergone defined diversification events within their rearranged V_{L1} gene. Recently, Barth and Humphries (1988a) showed that a *v-rel*-containing retrovirus can directly transform avian B cells. These cells do not undergo sequence diversification of their rearranged V_{L1} gene in culture (Barth and Humphries 1988b). Therefore, these cell lines were analyzed to address the question of whether reciprocal transfer of genetic information into the pseudogene region has occurred. To ensure that these cell lines reflect events that normally occur during B-cell development in the bursa of Fabricius, cell lines were derived from two stages of B-cell development. Twenty-two cell lines established after REV-T (CSV) infection of day-old chicks (Barth and Humphries 1988) and 20 cell lines isolated after in vitro transformation of 6-week-old bursal cells by REV-T (CSV) were examined for the presence of V_{L1} restriction endonuclease sites known to be diversified during B-cell development (Thompson and Neiman

Figure 3. Sequence substitutions into the rearranged V_{L1} genes occur in *cis*. (A) The germ line sequences of both parental alleles of ψV_{L12} and ψV_{L11} are depicted for the CDR1 domain. Polymorphisms between the parental strains relative to the V_{L1} gene are indicated by bold underlined characters. (B) Using oligonucleotide probes specific for the CDR1 sequence of ψV_{L12} , clones were isolated from a library of rearranged V_{L1} - J_L regions isolated from day-18 embryo bursal lymphocyte DNA. Sequences of the G4 allele ψV_{L12} germ line segment, clone 89-21 (a rearranged G4 allele), clone 122-8 (a rearranged S3 allele), and the S3 allele ψV_{L12} germ line segment are compared to the germ line G4 allele V_{L1} sequence. Polymorphic bases between the G4 and S3 ψV_{L12} alleles are underlined. (C) The utilization of G4-derived and S3-derived donor ψV_{L12} and ψV_{L11} sequences in diversified V_{L1} genes of each parental allele.

1987). As shown in Table 1, 20 cell lines derived from the day of hatch have the phenotype of one rearranged and one germ line allele, and two cell lines contain both alleles in the rearranged configuration. Analysis of the *ScaI* restriction endonuclease site at the 3' end of the rearranged V_{L1} gene showed that 14 of the alleles retained the germ line-encoded *ScaI* site, whereas 10 had undergone sequence modification at this site. Analysis of the *KpnI* restriction endonuclease site within CDR1 of the rearranged V_{L1} gene showed that 15 of the clones retained the *KpnI* site, whereas nine had undergone sequence modification at this site. In the 20 cell lines derived from 6-week-old bursal lymphocytes, 18 contained one rearranged and one germ line allele and two contained both alleles in rearranged configuration. In contrast to cell lines derived from the day of hatch, all 22 of the rearranged alleles in cell lines derived from 6 weeks of age showed evidence of sequence modification at the *ScaI* site, and 16 of the alleles demonstrated sequence modification of the *KpnI* site. This evidence of progressive sequence diversification at these restriction sites is consistent with the diversification at these same sites that has been observed in polyclonal B cells derived at equivalent developmental time points (Thompson and Neiman 1987). Therefore, these cell lines appear to reflect the events that occur normally during B-cell development.

To identify definitive sequence substitution events within the rearranged V_{L1} genes of these clonal cell lines, the rearranged V_{L1} genes from individual cell lines were rescued by the polymerase chain reaction (PCR) as previously described (McCormack et al. 1989a) and then sequenced both as single clones and as pools of 8–20 clones. For each of 11 rearranged V_{L1} – J_L regions sequenced from the day-of-hatch cell lines, unambiguous unique sequence was obtained by both approaches, demonstrating that there was no significant rate of further sequence substitution during propagation of these clonal cell lines in culture. Three of these 11 cell lines displayed evidence of sequence substitution derived from ψV_{L7} . In each of these three cell lines the ψV_{L7} segment from both alleles was sequenced to determine whether reciprocal sequence substitutions had occurred. Because

the G4 and S3 parental ψV_{L7} genes are polymorphic for the first base pair of codon 17, we could assure ourselves in each clonal cell line that we had successfully sequenced both of the parental ψV_{L7} segments. In all instances, the ψV_{L7} segments from both parental alleles retained their germ line sequence. The nucleotide sequence of one of the cell lines displaying a ψV_{L7} -derived sequence substitution, clone 14S4, is shown in Figure 4. The ψV_{L7} sequence substitution found in 14S4, which is derived from a rearranged G4 allele, has been derived in *cis* from the G4 parental allele, but has not led to the reciprocal transfer of V_{L1} sequence information to the ψV_{L7} segment in either the G4 or S3 allele of that cell line. On the basis of this sequence information, we were able to exclude the possibility that sequence substitution is derived from intrachromosomal double homologous recombination. These data also provide further evidence for excluding *trans* double homologous recombination as a mechanism of sequence diversification of chicken V_{L1} genes.

Sequences derived from the rearranged V_{L1} do not appear within the pseudogene region of bursal lymphocytes

Although the above data rule out intrachromosomal double homologous recombination as a mechanism of sequence diversification, they do not exclude the possibility of double homologous recombination between sister chromatids. Because sister chromatid exchange appears to be a common mechanism of sequence transfer following chromosomal duplication during S phase, we also sought to exclude this as a mechanism of sequence substitution within the rearranged V_{L1} gene. As depicted in Figure 2, sister chromatid exchanges would lead to the generation of two distinct daughter cells. One daughter cell would contain the observed product of a gene conversion event involving sequences derived from the same parental allele. The other daughter cell would have acquired a V_{L1} -derived sequence substitution within a pseudogene but would contain an unmodified rearranged V_{L1} gene segment. Because it has previously been shown that the expression of an unmodified rearranged immunoglobulin gene leads to selection of B cells within the bursa of Fabricius (McCormack et al. 1989b; Reynaud et al. 1989), this second product of double homologous recombination between sister chromatids should be retained within the polyclonal B-cell population of the bursa of Fabricius and be capable of undergoing further sequence diversification events. Because multiple pseudogenes can be used to diversify the functionally rearranged V_{L1} genes (e.g., see Fig. 1), sequence alteration of a single pseudogene is not likely to affect the ability of the bursal lymphocyte to develop, and additional alterations in the ψV_L region may accumulate. After several rounds of diversification by sister chromatid sequence exchange and cell division, some B cells would accumulate sequence substitutions in the ψV_L cluster that are detectable as a gain or loss of restriction endonuclease sites, but are not re-

Table 1. Characteristics of bursal lymphoma cell lines derived from transformation with a v-rel-containing retrovirus at the day of hatching or at 6 weeks post-hatching

Characteristic	Time of transformation	
	day of hatching	6 weeks post-hatching
One rearranged Ig_L allele ^a	20	18
Both Ig_L alleles rearranged	2	2
Loss of V_{L1} restriction endonuclease sites in rearranged alleles		
<i>KpnI</i>	9 (38%)	16 (73%)
<i>ScaI</i>	10 (41%)	22 (100%)

^aAnalyses performed as described previously (Thompson and Neiman 1987).

Figure 4. Partial nucleotide sequence of clone 14S4, a rearranged G4 allele, is compared to the germ line G4 V_{L1} gene. Sequence modifications are also compared to the G4 allele of the ψV_L7 segment. The S3 allele of the ψV_L7 segment contains a polymorphism in codon 17 that is not present in clone 14S4. In both the ψV_L7 segment and in the V_{L1} gene encoded by 14S4, the *KpnI* site spanning codons 29–31 has been destroyed by an internal nucleotide substitution.

transformed B-cell lines obtained from bursal lymphocytes at the day of hatching and at 6 weeks of age (Fig. 5). The map of confirmed *KpnI* and *ScaI* sites present within the ψV_L segments of both the G4 and S3 alleles is shown in Figure 1. Restriction digests of cell line DNA samples were probed with a V_L -specific fragment obtained from digestion of a germ line V_L gene with *KpnI* and *ScaI*. In no cell line was there any evidence for acquisition of additional *KpnI* or *ScaI* sites not present in the germ line parental strains (Fig. 5 and data not shown). In addition, we have examined six additional restriction endonucleases known to occur in two or more

Figure 5. Analysis of restriction endonuclease sites in the ψV_L cluster. DNA isolated from seven *v-rel*-transformed B-cell lines (Lanes 1–7) isolated from 6-week-old chickens were digested with (A) *Bcl*II, (B) *Kpn*I, and (C) *Sca*I restriction endonucleases, and Southern blots of these digests were hybridized with a V_L -specific probe. The germ line DNA from the F₁ SC bird (G4 $\times$ S3) and the G4 and S3 parental strains are provided for comparison. Marker lanes (M) show end-labeled *Hind*III fragments of λ DNA (sizes from top to bottom: 23.1, 9.4, 6.7, 4.4, 2.3, 2.0, and 0.6 kb). The positions of the germ line (G) and rearranged (R) bands for V_{L1} are indicated in all three panels. Because both *Kpn*I and *Sca*I sites are within the diversifying V_{L1} gene, some clones will have undergone sequence modification at these restriction sites to produce the unique fragments labeled M. Schematic representations of the germ line, rearranged, and modified V_{L1} -specific bands are depicted below each panel. In each digest, the multiple additional bands observed with the V_L -specific probe are the result of hybridization to the ψV_L segments (a restriction map of *Kpn*I and *Sca*I sites located within ψV_L segments is shown in Fig. 1A). Notes: (A) For *Bcl*II, V_{L1} – J_L rearrangement results in a 1.8-kb deletion. A polymorphism within the ψV_L regions of the parental strains that does not map within a ψV_L segment is present. Lane 6 contains a cell line that has undergone rearrangement on both alleles. (B) The *Kpn*I polymorphism observed between the parental strain maps within the ψV_L region is not within a ψV_L segment. (C) Rearrangement is not detected in the *Sca*I digest using this probe, and therefore, germ line and rearranged bands are the same. In the *Sca*I digests, the G and R bands are superimposed on a restriction fragment from the 5' ψV_L gene region, accounting for the persistence of this band in clone 6 in which both alleles have undergone rearrangement and loss of *Sca*I in V_{L1} . In none of the tumors is there any evidence for acquisition, loss, or rearrangement of *Bcl*II, *Kpn*I, or *Sca*I restriction endonuclease sites for the ψV_L region cluster. The cell line data are representative of data obtained for cell lines derived at day of hatch or 6 weeks of age as described in Table 1.

of the ψV_L segments (*Bam*HI, *Bcl*I, *Pvu*II, *Pst*I, *Nco*I, *Sac*I). In all, 23 of the 25 ψV_L segments contain one or more of the restriction sites tested. In no instance have we detected evidence for an alteration of the restriction map of any of these pseudogenes within the ψV_L region. In contrast, we were able to detect the acquisition of a *Bam*HI site within the rearranged V_{L1} gene of one clone as a result of unidirectional transfer of sequence information between the ψV_L segments and the rearranged V_{L1} gene (data not shown). In addition, we have been able to detect polymorphisms in the ψV_L region between the parental strains both within ψV_L segments (for example *Bam*HI in ψV_{L2} in Fig. 1) and adjacent to ψV_L segments (for example *Bcl*I and *Kpn*I polymorphism observed in Fig. 5), which are inherited and maintained in a Mendelian manner. These data suggest that double homologous recombination between sister chromatids does not contribute significantly to the sequence diversification of rearranged V_{L1} genes during bursal development within the bursa of Fabricius.

Unmodified rearranged V_{L1} genes are not maintained within the bursal lymphocyte population during B-cell development

On the basis of the above analyses, only two potential mechanisms for sequence diversification of rearranged V_{L1} genes remain: intrachromosomal gene conversion and gene conversion between sister chromatids. These two mechanisms predict two distinct fates for the unmodified rearranged V_{L1} gene segment during B-cell development within the bursa of Fabricius. If diversification occurs by intrachromosomal gene conversion prior to DNA replication, each event would lead to the elimination of a rearranged unmodified V_{L1} gene segment from the bursal lymphocyte population. In contrast, if gene conversion occurs subsequent to DNA replication, each gene conversion between sister chromatids would lead to the production of one daughter cell that had undergone sequence diversification within the rearranged V_{L1} gene and one daughter cell that retained the parental configuration of an unmodified rearranged V_{L1} gene (Fig. 2). Such a mechanism of diversification would lead to the maintenance of cells retaining an unmodified rearranged V_{L1} gene segment and thus provide for the maintenance of cells that can be used to regenerate somatic diversity within the environment of the bursa of Fabricius. Some evidence against the maintenance of such a stem-cell activity already exists. Bursal transfer studies have shown that the frequency with which a cell can be obtained that is capable of reconstituting the B-cell immune repertoire of a chicken following transplantation is high in bursal lymphoid populations between days 15 and 18 of embryogenesis and then falls off rapidly soon after birth (Toivanen and Toivanen 1973). Bursal stem-cell activity decreases from 1 cell in 100 at day 18 of embryogenesis to less than 1 cell in 1000 by four days post-hatching (Pink et al. 1985). This decrease in stem-cell activity is not the result of a developmentally programmed alteration in the stromal environment that se-

lects against cells with undiversified V_{L1} gene segments. Post-hatching, lymphoid-depleted bursal stroma can serve as a substrate for lymphoid reconstitution using embryonic Ig⁺ cells (Toivanen et al. 1972; Toivanen and Toivanen 1973; Thompson et al. 1987). The Ig⁺ cells that normally migrate to the embryonic bursa between days 10 and 15 of embryogenesis lack diversification of their rearranged Ig_L allele (McCormack et al. 1989b).

To determine whether the frequency of the unmodified rearranged V_{L1} gene is maintained at a significant level during B-cell development in the bursa of Fabricius as predicted by gene conversion between sister chromatids, we isolated and analyzed V_{L1} -J_L rearrangements at three different developmental time points (Table 2). At day 15 of embryogenesis during the initial phases of B-cell clonal expansion within the bursa of Fabricius, a high percentage of the rearranged alleles (7 of 10) contained an unmodified V_{L1} gene segment. In contrast, by day 18 of embryogenesis only 1 clone in 42 retained an unmodified V_{L1} gene. In 62 post-hatching clones analyzed to date, we have yet to be able to identify an unmodified V_{L1} gene. These data confirm that cells with the parental configuration of the rearranged V_{L1} gene are not maintained as a result of the diversification mechanism, and suggest that V_{L1} gene diversification does not result from gene conversion between sister chromatids following DNA replication in S phase. Although the same result could be obtained if undiversified cells were selectively killed, the ability of the post-hatching bursal stroma to support the clonal expansion of embryonic stem cells argues against this possibility (Toivanen et al. 1972; Toivanen and Toivanen 1973; Thompson et al. 1987). In addition, the observation that pseudogenes in the antisense orientation relative to the V_{L1} gene segment are used statistically more frequently as templates of sequence donation than pseudogenes in the sense orientation (McCormack and Thompson 1990) suggests an intrachromosomal mechanism of gene conversion. Although simple folding allows for such a sequence alignment in an intrachromosomal gene conversion, it is difficult to explain the antisense orientation bias by an intermolecular process.

Discussion

The studies described in this report characterize the molecular events associated with diversification of the rearranged V_{L1} gene during B-cell development within the bursa of Fabricius. Consistent with the work of Reynaud et al. (1987), we have demonstrated that se-

Table 2. Frequency of randomly cloned V-J segments that retain unmodified V_{L1} sequence at different times in development

Day 15	Day 18	Post-hatching
7 of 10	1 of 42	0 of 62

Data expressed as the number of clones with an unmodified rearranged V_{L1} gene segment over the number of clones sequenced.

sequence diversification results from the transfer of sequence information from the ψV_L segments to the functionally rearranged V_{L1} gene. This sequence transfer occurs primarily from sequences in *cis* with the rearranged V_{L1} gene. These sequence substitutions do not lead to the reciprocal transfer of information from the rearranged V_{L1} gene to the ψV_L segment either intrachromosomally or in *trans* to a sister chromatid. The lack of maintenance of cells with undiversified rearranged V_{L1} genes during B-cell development in the bursa of Fabricius suggests that most if not all gene conversions occur prior to DNA replication in S phase. The frequency with which unmodified rearranged V_{L1} genes are found within developing bursal population is proportional to the frequency with which bursal stem-cell activity has been detected in a bursal transfer assay (Pink et al. 1985). Together, these data demonstrate that the mechanism for the diversification of rearranged V_{L1} genes during B-cell development in the bursa of Fabricius is intrachromosomal gene conversion.

On the basis of our studies, there are a number of striking similarities between chicken Ig_L gene diversification and the regulation of mating type switching in haploid yeast (for review, see Strathern 1988; Klar 1989). In *Saccharomyces cerevisiae*, mating type switching occurs as an intrachromosomal gene conversion event. Gene conversion occurs in a directional manner from the nontranscribed loci HML- α and HMR-*a* to the transcribed MAT locus, which determines the mating type. HML- α and HMR-*a* contain the genetic information required to determine either the α or the *a* mating types, respectively, and the presence of either an α or an *a* allele in the MAT locus determines the mating type of a haploid yeast cell. Switching between *a* and α occurs prior to DNA replication in the G₁ phase of the cell cycle. This results in the change of the mating type of both progeny of the cell undergoing a switch, in a manner analogous to the way we would suggest that unmodified rearranged V genes are lost during B-cell development in the bursa of Fabricius. Yeast mating type switching appears to be initiated by a double-strand break in the sequence flanking the 3' side of the segment to be converted. Such a mechanism in the avian Ig_L locus may account for the restriction of conversion to the rearranged V_{L1} gene (Thompson and Neiman 1987) if the initiating double-strand cut occurred in the J_L segment and leads to conversion only if the V_{L1} region resided immediately adjacent to the break. Efforts to detect double-strand breaks within the rearranged V_{L1} - J_L region of B cells in the bursa of Fabricius have been unsuccessful (C.B. Thompson, unpubl.). Although it appears that the HO endonuclease initiates the mating type switching event in yeast, the endonuclease site does not apparently define the border(s) of a conversion event. Similarly, in chicken B cells, existing sequence information suggests that multiple positions within V_{L1} can be used as sites of resolution of individual gene conversion events (e.g., Fig. 1B).

Partial characterization of several other systems has suggested that phenotypic variation in pathogenic or-

ganisms can be induced by a similar process of somatic diversification of antigenic domains. Antigenic variation of trypanosome variable surface glycoprotein genes (for review, see Donelson 1989), *Neisseria gonorrhoeae* pilin genes (Swanson and Koomey 1989), and *Borrelia* variable major protein genes (Plasterk et al. 1985) have been suggested to result from duplicative transposition of copies of pseudogene or silent gene copies into a transcribed locus, thus generating antigenic variations and allowing the organism to escape the immune surveillance of their host. A number of the features in each of these organisms suggest the transfer of sequence information occurs between a nontranscribed and a transcribed locus by a gene conversion event. However, it has been suggested that in trypanosomes, some of the antigenic variation may also be due to homologous recombination (Thon et al. 1989). In addition, it has recently been suggested that *Neisseria* antigenic variation may be accounted for at least partially by homologous recombination of exogenous DNA taken up by the naturally competent *Neisseria* organisms at times of high cell death in the population (Gibbs et al. 1989).

A number of the features of Ig_L gene conversion suggest that this somatic process is distinct from meiotic gene conversion. During somatic diversification of the rearranged V_{L1} gene segment, we failed to detect any evidence of interconversion among pseudogenes. In contrast, we obtained evidence of frequent interconversion among pseudogenes during meiosis as demonstrated by the sequence comparison of ψV_L gene segments of the two parental chicken strains G4 and S3 that have been maintained independently as inbred lines for 50 years (W. McCormack, C. Postema, and C.B. Thompson, unpubl.). Furthermore, meiotic gene conversion can occur both in *trans* and in *cis*, and leads to frequent allelic recombination downstream of converted segments (Hastings 1988). In contrast, the somatic gene conversion of the rearranged Ig_L V gene segment appears to occur exclusively in *cis* and fails to demonstrate a significant rate of recombination between alleles. Although several existing models of gene conversion have been suggested that can account for all of the features of meiotic gene conversion (Radding et al. 1982; Szostak et al. 1983), it is not yet clear how the more specific and developmentally regulated characteristics of immunoglobulin gene conversion can be determined by existing models of gene conversion. To this purpose, studies in the accompanying manuscript (McCormack and Thompson 1990) have been performed to characterize more completely the individual gene conversions observed within the avian system. These and further studies should serve to refine our understanding of this developmentally regulated mechanism for generating antibody diversity.

Methods

Chicken strains

Chickens used in these experiments were Hyline SC birds, an F₁ cross between two inbred B² chicken strains, designated G4

and S3. Blood samples from the parental strains were obtained from Hyline. The parental strains differ in a number of sequence polymorphisms around the Ig_L locus, including restriction endonuclease sites 5' and 3' of the V_{L1} and J_L gene segments and nucleotide sequence polymorphisms within the leader intron, V_{L1} exon, and several ψV_L segments (Thompson and Neiman 1987; McCormack and Thompson 1990; and this paper).

Cell lines

The cell lines derived from B cells at the time of hatch were established directly from tumor tissue of REV-T (CSV)-infected chickens as described (Barth and Humphries 1989). Cell lines prepared from B cells isolated from 6-week-old chicks were established by in vitro transformation with REV-T (CSV). After isolation of B cells by centrifugation through Ficoll-hypaque, the cells were washed twice and infected with different dilutions of REV-T (CSV). Infected cells were plated in 96-well microtiter trays and incubated in Hahn's medium at 38°C. Wells positive for growth were selected at terminal dilutions producing clonal populations and were adapted slowly to Dulbecco's modified Eagle medium with added calf and chick sera.

Nucleotide sequencing of rearranged Ig_L alleles from the v-rel-induced cell lines

Isolation of rearranged Ig_L genes from clonal v-rel-induced cell lines was performed by amplification of the rearranged V_{L1} gene using PCR as described previously (McCormack et al. 1989a). Dideoxynucleotide sequencing was performed using double-stranded plasmid template DNA with a commercially available kit (U.S. Biochemical). Each clone was sequenced completely on both strands. In some experiments, 8–20 individual PCR-amplified clones were sequenced as a pool to rule out the possibility of heterogeneity in the rearranged variable gene segments of these clonal cell lines.

Sequence analysis of ψV_L segments from parental strains

Individual ψV_L segments from the parental strains were determined after PCR-amplification cloning of the segments from parental erythrocyte DNA using PCR primers defined by ψV_L -specific flanking sequences (Reynaud et al. 1987).

Identification of ψV_L -specific gene conversion events

Recombinant libraries prepared from rearranged V_{L1} – J_L gene segments were prepared for bursal DNA samples from days 15 and 18 of embryogenesis, the day of hatch, and 4 and 6 weeks of age, after PCR amplification of rearranged V_{L1} – J_L loci using primers 5' of the start of Ig_L transcription and 3' of the J_L segment (McCormack et al. 1989a). PCR products were cloned into the plasmid vector pGEM-3Z (Promega). Oligonucleotide probes (20-mers) specific for the G4 and S3 alleles of ψV_{L12} and ψV_{L11} were used to screen approximately 2000 independent V_{L1} – J_L clones as described previously (McCormack et al. 1989c). Clones displaying sequence-specific hybridization to the oligonucleotides were recovered and plasmid inserts were sequenced on both strands.

DNA preparation and Southern blotting

DNA was isolated from cell lines and polyclonal bursal lymphocytes as previously described (Thompson and Neiman 1987). Techniques used for Southern blot analysis were per-

formed as reported by Thompson and Neiman (1987) without modification. The 170-bp fragment of V_{L1} between the *KpnI* site at the 3' end of CDR1 and the *ScaI* site at the 3' end of CDR3 was used as a V_L -specific hybridization probe.

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References

- Barth, C.F. and E.H. Humphries. 1988a. A nonimmunosuppressive helper virus allows high efficiency induction of B-cell lymphomas by reticuloendotheliosis virus strain T. *J. Exp. Med.* **167**: 89–108.
- Barth, C.F. and E.H. Humphries. 1988b. Expression of v-rel induces mature B-cell lines that reflect the diversity of avian immunoglobulin heavy- and light-chain rearrangements. *Mol. Cell. Biol.* **8**: 5358–5368.
- Brenner, D.A., A.C. Smigocki, and R.D. Camerini-Otero. 1985. Effect of insertions, deletions, and double-strand breaks on homologous recombination in mouse L cells. *Mol. Cell. Biol.* **5**: 684–691.
- Donelson, J.E. 1989. DNA rearrangements and antigenic variation in African trypanosomes. In *Mobile DNA* (ed. D.E. Berg and M.M. Howe), pp. 763–781. American Society of Microbiology, Washington, D.C.
- Gibbs, C.P., B.-Y. Reimann, E. Schultz, A. Kaufmann, R. Hass, and T.F. Meyer. 1989. Reassortment of pilin genes in *Neisseria gonorrhoeae* occurs by two distinct mechanisms. *Nature* **338**: 651–652.
- Hastings, P.J. 1988. Conversion events in fungi. In *Genetic recombination* (ed. R. Kucherlapati and G.R. Smith), pp. 397–428. American Society of Microbiology, Washington, D.C.
- Johnson, R.S., M. Sheng, M.E. Greenberg, R.D. Kolodner, V.E. Papaioannou, and B.M. Spiegelman. 1989. Targeting of non-expressed genes in embryonic stem cells via homologous recombination. *Science* **245**: 1234–1236.
- Klar, A.J.S. 1989. The interconversion of yeast mating type: *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. In *Mobile DNA* (ed. D.E. Berg and M.M. Howe), pp. 671–691. American Society of Microbiology, Washington, D.C.
- Kourilsky, P. 1986. Molecular mechanisms for gene conversion in higher cells. *Trends Genet.* **2**: 60–63.
- Kucherlapati, R.S., E.M. Eves, K.-Y. Song, B.S. Morse, and O. Smithies. 1984. Homologous recombination between plasmids in mammalian cells can be enhanced by treatment of input DNA. *Proc. Natl. Acad. Sci.* **81**: 3153–3157.
- Liskay, R.M. and J.L. Stachelek. 1983. Evidence for intrachromosomal gene conversion in cultured mouse cells. *Cell* **35**: 157–165.
- Liskay, R.M. and J.L. Stachelek. 1986. Information transfer between duplicated chromosomal sequences in mammalian cells involves contiguous regions of DNA. *Proc. Natl. Acad. Sci.* **83**: 1802–1806.
- McCormack, W.T., L.W. Tjoelker, L.M. Carlson, B. Petryniak,

- C.F. Barth, E.H. Humphries, and C.B. Thompson. 1989a. Chicken Ig_L gene rearrangement involves deletion of a circular episome and addition of single nonrandom nucleotides to both coding segments. *Cell* **56**: 785–791.
- McCormack, W.T., L.W. Tjoelker, C.F. Barth, L.M. Carlson, B. Petryniak, E.H. Humphries, and C.B. Thompson. 1989b. Selection for B cells with productive Ig_L gene rearrangements occurs in the bursa of Fabricius during chicken embryonic development. *Genes Dev.* **3**: 838–847.
- McCormack, W.T., L.M. Carlson, L.W. Tjoelker, and C.B. Thompson. 1989c. Evolutionary comparison of the avian Ig_L locus: Combinatorial diversity plays a role in the generation of the antibody repertoire in some avian species. *Internat. Immunol.* **1**: 332–341.
- McCormack, W.T. and C.B. Thompson. 1990. Chicken Ig_L variable region gene conversions display pseudogene donor preference and 5' to 3' polarity. *Genes Dev.* **4**: 548–558.
- Pink, J.R.L., O. Vainio, and A.-M. Rijnbeek. 1985. Clones of B lymphocytes in individual follicles of the bursa of Fabricius. *Eur. J. Immunol.* **15**: 83–87.
- Plasterk, R.H.A., M.I. Simon, and A.G. Barbour. 1985. Transposition of structural genes to an expression sequence on a linear plasmid causes antigenic variation in the bacterium *Borrelia hermsii*. *Nature* **318**: 257–263.
- Radding, C.M., J. Flory, A. Wu, R. Kahn, C. DasGupta, D. Gonda, M. Bianchi, and S.S. Tsang. 1982. Three phases in homologous pairing: Polymerization of *recA* protein on single-stranded DNA, synapsis, and polar strand exchange. *Cold Spring Harbor Symp. Quant. Biol.* **47**: 821–828.
- Reynaud, C.A., V. Anquez, A. Dahan, and J.-C. Weill. 1985. A single rearrangement event generates most of the chicken immunoglobulin light-chain diversity. *Cell* **40**: 283–291.
- Reynaud, C.-A., V. Anquez, H. Grimal, and J.-C. Weill. 1987. A hyperconversion mechanism generates the chicken light-chain preimmune repertoire. *Cell* **48**: 379–388.
- Reynaud, C.-A., A. Dahan, V. Anquez, and J.-C. Weill. 1989. Somatic hyperconversion diversifies the single V_H gene of the chicken with a high incidence in the D region. *Cell* **59**: 171–183.
- Strathern, J.N. 1988. Control and execution of homothallic switching in *Saccharomyces cerevisiae*. In *Genetic recombination* (ed. R. Kucherlapati and G.R. Smith), pp. 445–464. American Society of Microbiology, Washington, D.C.
- Subramani, S. and J. Rubnitz. 1985. Recombination events after transient infection and stable integration of DNA into mouse cells. *Mol. Cell. Biol.* **5**: 659–666.
- Swanson, J. and J.M. Koomey. 1989. Mechanisms for variation of pili and outer membrane protein II in *Neisseria gonorrhoeae*. In *Mobile DNA* (ed. D.E. Berg and M.M. Howe), pp. 743–761. American Society of Microbiology, Washington, 200D.C.
- Szostak, J.W., T.L. Orr-Weaver, and R.J. Rothstein. 1983. The double-strand-break repair model for recombination. *Cell* **33**: 25–35.
- Thompson, C.B. and P.E. Neiman. 1987. Somatic diversification of the chicken immunoglobulin light-chain gene is limited to the rearranged variable gene segment. *Cell* **48**: 369–378.
- Thompson, C.B., E.H. Humphries, L.M. Carlson, C.-L.H. Chen, and P.E. Neiman. 1987. The effect of alterations in *myc* gene expression on B cell development in the bursa of Fabricius. *Cell* **51**: 371–381.
- Thon, G., T. Baltz, and H. Eisen. 1989. Antigenic diversity by the recombination of pseudogenes. *Genes Dev.* **3**: 1247–1254.
- Toivanen, P., A. Toivanen, and R.A. Good. 1972. Ontogeny of bursal function in chicken. I. Embryonic stem cell for humoral immunity. *J. Immunol.* **109**: 1058–1070.
- Toivanen, P. and A. Toivanen. 1973. Bursal and postbursal stem cells in chicken. Functional characteristics. *Eur. J. Immunol.* **3**: 585–595.

Templated insertions in the rearranged chicken IgL V gene segment arise by intrachromosomal gene conversion.

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References

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