Selection for B cells with productive IgL gene rearrangements occurs in the bursa of Fabricius during chicken embryonic development

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The vast majority of immunoglobulin-expressing mature chicken B lymphocytes contain one functionally rearranged and one unrearranged allele of the immunoglobulin light chain (Ig_L) gene. Therefore, nearly all Ig_L V-J rearrangements present in mature chickens are in-frame. In contrast, the Ig genes of mature mammalian B cells contain a high proportion of out-of-frame V-J joints. To investigate the basis for this difference, gene rearrangement at the chicken Ig_L locus was characterized during embryonic development and in mature B-cell lines. Joining of the single functional variable (V_L) segment with the single joining (J_L) segment occurs in cells in multiple tissues during a transient period of chicken embryogenesis. Only one-third of the V-J joints cloned from days 10–12 of development are in-frame. An increasing proportion of in-frame V-J joints is observed within the bursa of Fabricius at successively later stages of development. Our data suggest that the bursa of Fabricius serves during embryonic development as a site of selective amplification of cells that have undergone productive V-J joining, such that nearly all V-J joints present in postembryonic B cells are in-frame. The high frequency of rearranged alleles joined in-frame that is found in posthatching bursal cells and mature B-cell lines appears to result from a low frequency with which cells undergo Ig_L rearrangement at both alleles, rather than from an increase in the precision of V-J joining in avian species.

[*Key Words:* Allelic exclusion; bursa of Fabricius; chicken; gene rearrangement; immunoglobulin genes]

Received January 16, 1989; revised version accepted March 20, 1989.

The germ-line immunoglobulin light chain (Ig_L) locus in the chicken includes single functional variable (V_L), joining (J_L), and constant (C_L) gene segments (Reynaud et al. 1985). Therefore, all chicken light chains are encoded by the same V_L and J_L gene segments, which have been joined in-frame during Ig_L gene rearrangement. Whereas some junctional diversity may be generated by V-J joining, more extensive diversity is introduced into the rearranged V_L gene segments by somatic gene-conversion events. These gene conversions use an upstream family of 25 V_L pseudogenes as sequence donors and occur in the bursa of Fabricius during a specific stage of B-cell development (Reynaud et al. 1987; Thompson and Neiman 1987).

Joining of V_L and J_L gene-coding segments in mammals appears to be a random process. Because of the junctional diversity that results from V-J joining (Tonegawa 1983), only one-third of the V-J joints made in mammalian pre-B cells are in the correct translational reading frame (Engler and Storb 1988). In the murine system, rearrangement of the Ig loci in most B lymphocytes is be-

lieved to occur sequentially (H → κ → λ) and be regulated by feedback inhibition, such that further rearrangement of light-chain genes is halted upon synthesis of complete Ig molecules (reviewed by Yancopoulos and Alt 1986). Some Ig_κ-expressing B cells, on the other hand, do not appear to show feedback inhibition of gene rearrangements (Gollahon et al. 1988).

By analogy with mammalian B cells, only one-third of chicken B cells might be expected to produce a functionally rearranged Ig_L gene as a result of their first rearrangement. If feedback inhibition regulates chicken Ig_L gene rearrangement, those cells would cease Ig_L rearrangements. Of the remaining two-thirds, an additional one-third might be expected to create a functional Ig_L gene upon rearrangement of their second allele. A complete lack of feedback inhibition would result in rearrangement at both alleles in all chicken B cells. According to either mechanism of regulation, quantitation of the percentages of rearranged and germ-line alleles in a polyclonal population of surface Ig-expressing (sIg⁺) chicken B cells would be expected to show an excess of

rearranged alleles in the absence of a deletional mechanism to remove nonfunctional rearranged Ig_L genes. However, it was found that only $50 \pm 5\%$ of the Ig_L genes in a polyclonal sIg⁺ population of bursal lymphocytes from 4- to 6-week-old birds are rearranged (Reynaud et al. 1985; Thompson and Nieman 1987). Based on this analysis, the vast majority of sIg⁺ bursal lymphocytes appear to contain one functionally rearranged allele and one unrearranged allele, suggesting that nearly all Ig_L rearrangements present after the bursal stage of B-cell development are functional.

These observations suggested that there may be differences in the regulation of Ig gene rearrangement between chicken and mammalian B cells. One possible explanation for the observed differences may be that rearrangement of both chicken Ig_L loci is not compatible with cell viability. For example, rearrangement of both alleles might delete both copies of an essential sequence element lying between V_L and J_L. Cells in which both alleles were rearranged would be viable only if a copy of this sequence were reintegrated into the genomic DNA. Alternatively, avian V-J joining may be much more precisely controlled than mammalian V-J joining, perhaps because of the close proximity (1.8 kb) of the unique V_L and J_L elements. This increased precision might result in the production of mostly in-frame V-J joints. Finally, it is possible that Ig_L gene rearrangement may occur only during a limited period of B-cell development such that the majority of B cells would only have time to rearrange one Ig_L allele. Only cells that rearranged the Ig_L allele productively would be capable of completing B-cell differentiation.

Here we report a molecular analysis of chicken V-J joining events during development to address these issues. The rearrangement status of the Ig_L locus in polyclonal bursal lymphocyte populations at various stages of development and in cell lines representing mature B cells was determined by Southern blot analysis. The timing of Ig_L gene rearrangement events during embryogenesis was investigated by comparing the relative abundance of the signal joint episome deleted during V-J joining at different developmental stages. Finally, V-J junctions were sequenced to determine the proportion of productive versus nonproductive Ig_L gene rearrangements present in the bursa of Fabricius at each developmental stage. Nucleotide sequences of V-J joints at the earliest stage of embryonic development examined revealed that two thirds of the V-J joints are out-of-frame. During the expansion of B cells in the bursa of Fabricius, there was a marked increase in the percentage of rearranged alleles that are in-frame. This enrichment occurs prior to the initiation of gene conversion, suggesting that the enrichment results from a cell selection process rather than correction of out-of-frame joints by gene conversion. These experiments suggest a model for chicken B-cell differentiation in which there is a limited period of development during which Ig gene rearrangement occurs. During embryogenesis, the bursa of Fabricius serves both to selectively amplify B cells with

productive Ig gene rearrangements and to activate the gene conversion process.

Results

Rearrangement status of Ig_L loci during development

The organization of the germ-line and rearranged chicken Ig_L locus is shown in Figure 1A. Southern blot analysis of bursal lymphocyte DNA digested with the enzyme *Bcl*I and hybridized with the C_L probe distinguishes between the germ-line and rearranged Ig_L alleles. The 10.2-kb germ-line fragment is reduced to an 8.4-kb fragment during the V-J joining process. The rearranged allele is first detectable in the bursa by Southern blot analysis at day 15 of embryonic development (Fig. 1B). Cells with rearranged Ig_L alleles cannot be detected by Southern blot analysis in peripheral lymphoid organs such as the spleen until after hatching (Fig. 1B).

Between day 18 of embryogenesis and 2 weeks post-hatching, all chicken B cells undergo a period of development within the bursa of Fabricius (Ratcliffe 1985). Lydyard et al. (1976) have shown that even at 18 days, the start of the period of bursa-dependent B-cell development, virtually all bursal lymphocytes express sIg. We find that by day 18 of embryonic development and at later stages, the germ-line and rearranged *Bcl*I fragments of the Ig_L gene appear at equal hybridization intensities (Fig. 1B). As described previously for sIg⁺ bursal lymphocytes at 4–6 weeks of age, this result has been interpreted to suggest that most bursal lymphocytes rearrange only one allele (Reynaud et al. 1985; Thompson and Nieman 1987). To support this conclusion, we have analyzed 48 clonal chicken mature B-cell lines or tumors for their Ig_L rearrangement status (Table 1). Thirty sIg⁺ B-cell lymphomas derived by transformation with a *v-rel*-containing retrovirus were analyzed. Twenty-seven were found to have one germ-line and one rearranged allele. Only three *v-rel*-transformed cell lines had both Ig_L alleles rearranged. In addition, 18 sIg⁺ bursal lymphomas induced by avian leukosis virus (ALV) integration adjacent to *c-myc* were analyzed. All were found to have one rearranged and one germ-line allele at the Ig_L locus. Therefore, 94% (45/48) of the clonal sIg⁺ chicken B-cell lines or tumors examined rearranged only one Ig_L allele.

The region between V_L and J_L is nonessential

The availability of the three cell lines with two rearranged Ig_L alleles provided an opportunity to evaluate the possibility that the DNA region between V_L and J_L is required for cell viability. If this were the case, we would expect to find one copy of the intervening DNA (or a portion of it) reintegrated into the genome of cells with rearrangement of both Ig_L alleles. Such reintegration at random positions in the genome would not be detectable in DNA prepared from a polyclonal population of chicken B cells. The rearrangement status of five *v-rel*-

Figure 1. Organization of the germ-line and rearranged chicken Ig_L locus. (A) Restriction maps of the germ-line and rearranged Ig_L alleles of the SC chicken. Exons are indicated by open boxes, and flanking sequence and introns are shown as lines. *Bam*HI* and *Eco*RI* restriction site polymorphisms distinguish the two alleles. Both sites are present on one allele and absent from the other. Solid bars above the maps indicate the locations of hybridization probes. (B) DNA samples from bursal and splenic tissue of a day-12 embryo and from bursal and splenic cells of day-15, day-18, day-of-hatch, and 6-week-old SC birds were digested with the enzyme *Bcl*II. Southern blots of these samples were hybridized with the C_L probe. The resulting autoradiograms are shown. Marker bands (M) are the end-labeled fragments of phage λ DNA digested with *Hind*III (top to bottom): 23.1, 9.4, 6.7, and 4.4 kb.

induced bursal lymphomas is shown in Figure 2A. The three cell lines that rearranged both Ig_L alleles are shown in lanes 2, 3, and 5. Two cell lines that rearranged one Ig_L allele (lanes 1 and 4) and bursal lymphocyte DNA from 6-week-old birds (lane 6) were included for comparison. When the Southern blot was reprobed with probes span-

ning the region of DNA between V_L and J_L , hybridization was observed to the germ-line fragment of the cell lines with one rearranged allele (Fig. 2B). However, these probes did not hybridize to DNA from the three cell lines that rearranged both Ig_L alleles (lanes 2, 3, and 5), indicating that the region between V_L and J_L is deleted from the genome during V-J joining.

Table 1. Ig_L rearrangement status of chicken B-cell lines

Cell lines	GR	RR
REV-T	90% (27/30)	10% (3/30)
ALV	100% (18/18)	0% (0/18)
Total	94% (45/48)	6% (3/48)

The Ig_L rearrangement status of chicken B-cell lines generated by reticuloendotheliosis virus strain T infection (REV-T; Barth and Humphries 1988) and chicken B-cell lymphomas caused by avian leukosis virus integration (ALV; Thompson et al. 1987) was determined as described in Fig. 1. The percentages of cell lines with one germ-line and one rearranged (GR) and with two rearranged (RR) Ig_L alleles are shown. The number of cell lines analyzed is given in parentheses.

Signal joints as a marker for V-J joining during development

The rearrangement of the chicken V_L and J_L gene segments occurs by deletion of the region between V_L and J_L , and formation of a circular signal joint episome that is detectable after DNA amplification using the polymerase chain reaction (PCR; McCormack et al. 1989). This episome is an unstable genetic element that is not propagated during B-cell development and is not reintegrated into the genome, as demonstrated for the cell lines shown in Figure 2. The presence of the deleted circular episome may therefore be used as a marker for recent gene rearrangements.

Bursal selection of rearranged Ig_L genes

Figure 2. Rearrangement of the Ig_L locus in bursal lymphoma cell lines. DNA samples from bursal lymphoma cell lines (lanes 1–5) and from bursal lymphocyte DNA of 6-week-old SC chickens (lane 6) were digested with the enzyme *Bcl*I. Cell lines used were 27L1 (lane 1), 28L1 (lane 2), 30L1 (lane 3), 30B3 (lane 4), and 41L1 (lane 5). Identical Southern blots of these samples were hybridized with the C_L probe (A) and the U₃ probe (B), and the resulting autoradiograms are shown. Hybridization with the U₂ probe produced identical results to B (not shown). Size markers (top to bottom: 23.1, 9.4 6.7, 4.4, 2.3, and 2.0 kb) were prepared as in Fig. 1.

The bursa and spleen DNA samples from different stages of development that were used for Southern blot analysis of the chicken Ig_L locus (Fig. 1B) were also analyzed for the presence of the signal joint episome. The DNA samples were subjected to PCR amplification using primers 993 and 994, and the presence of signal joints was confirmed by specific hybridization of the U₃ probe to equal aliquots of the amplified products (Fig. 3A). As shown in Figure 3B, significant hybridization occurred in both splenic- and bursal-amplified DNA at day 15 of embryogenesis, but little hybridization was detected in either tissue at later times during development. Whereas the signal joint episome was detected in additional DNA preparations at later stages (not shown), the high relative abundance of the signal joint episome at day 15 was consistently observed. Despite the differences observed in signal joint products during development, amplification of the same DNAs with primers specific for the histone H2B-IV gene resulted in similar levels of PCR-amplified product (Fig. 3C). Similar results were also obtained with the α -tubulin α 5 gene-specific primers (data not shown). In additional experiments, DNA samples were subjected to two separate rounds of PCR amplification to increase the sensitivity for the detection of signal joints. Under these conditions, we were able to detect signal joints in 10- to 12-day embryo tissues from a variety of sources, including liver/spleen, bursa, and bone marrow (data not shown).

The decrease in abundance of DNA encoding an episomal signal joint within the bursa between days 15 and 18 of embryogenesis coincides with a large increase in the number of bursal cells containing an Ig_L rearrangement (see Fig. 1B). These observations strongly suggest that the increase in Ig-expressing cells during this time

Figure 3. Presence of the signal joint episome during development. (A) Cartoon illustrating detection of the signal joint episome by PCR amplification. The V_L and J_L-coding segments are indicated by open boxes, and signal sequences are indicated by triangles. The orientation of PCR primers 993 and 994 is shown by arrows, and the U₃ hybridization probe is indicated by the solid bar. (B) The DNA samples isolated from embryonic tissues at different stages of development (Fig. 1) were used to amplify signal joints by PCR. Southern blots of equal aliquots of the PCR products were prepared and hybridized with the U₃ probe, and the resulting autoradiogram is shown. Size markers (top to bottom: 23.1, 9.4 6.7, 4.4, 2.3, and 2.0 kb) were prepared as in Fig. 1. (C) The same DNA samples used to amplify signal joints in B were used to amplify the histone H2B-IV gene. PCR was performed using unique flanking sequence primers. Ten percent of the reaction mix was run on a 1% agarose gel, stained with ethidium bromide, and photographed. Lanes are as indicated in B. In each lane, the upper band is the amplified H2B-IV gene, and the lower band is composed of the remaining nucleotides and primers.

period results from migration to the bursa and/or clonal expansion of cells with productive Ig_L rearrangements, rather than further Ig_L rearrangement within the bursa.

Coding joints of cDNA clones from the bursa at day 18

Twelve Ig_L cDNA clones were isolated from a cDNA library made from bursal lymphocytes of day-18 embryos. Nucleotide sequencing of the V and J regions of the cDNA clones revealed seven unique sequences, all with in-frame V-J joints. These V-J junctions are compared to the germ-line sequences of V_L and J_L in Figure 4A. Germ-line V_L and J_L sequences were determined after PCR amplification of the Ig_L alleles from both parental strains of the SC chicken.

At least four of the seven cDNA clones encode V-J joints that had not undergone gene-conversion events. All of these cDNA clones encoded gene-conversion events in other regions of the V_L gene segment similar to those described previously by Reynaud et al. (1987). An analysis of the gene-conversion events will be reported separately. Six different V-J junction sequences were found in these cDNAs. The exact joining event that created some V-J junctions is impossible to determine, however, due to the repeated TG sequence at the 3' end of V_L and at the 5' end of J_L.

Coding joints of PCR clones from the bursa during development

To extend the sequence analysis of chicken V-J joining to earlier stages of embryonic development, we amplified rearranged Ig_L genes by PCR using primers 1001 and 839, located 5' of V_L and 3' of J_L, respectively. V-J joints were cloned from bursal and spleen DNA isolated at days 10–12 of development and from bursal DNA of day-15 and day-18 embryos. Thirty or more clones were sequenced from each stage of development, and the V-J joints of these clones are shown in Figure 4, B–D. Although several of the clones obtained at 18 days of development contain gene-conversion events involving codons 84 and 86, the sequence data confirm that both Ig_L alleles are involved in V-J joining at approximately equal frequencies, as indicated by the sequence polymorphisms between the parental strains of the heterozygous SC chicken at codons 84 (C/G) and 86 (A/G).

A large proportion (24 of 44) of the clones obtained at day 18 had gene-conversion events at the V-J junction (data not shown) and will be reported separately. Although gene conversion of clone B18-63 resulted in the insertion of 4 nucleotides and a reading frame shift (not shown), it is listed with the other in-frame sequences because the V-J joining event itself was productive. Definitive gene-conversion events were absent from the PCR clones isolated from day-15 bursa and day-10 to day-12 tissues. This result agrees with earlier Southern blot results that indicated significant modification of the rearranged V_L gene segment by day 18 of development, but not earlier (Thompson and Neiman 1987).

At least 28 different joining events are represented in the PCR clones, revealing a higher degree of chicken Ig_L junctional diversity than described previously. The extent of nucleotide deletion from the V_L and J_L gene segments prior to ligation of the coding joints was as high as 11 nucleotides from V_L (in clone B15-17) and 14 nucleotides from J_L (in clone B15-37). Such extreme base loss is not observed in day-18 cDNA or PCR sequences, possibly because too many codons are deleted to encode a functional light-chain protein.

A somewhat surprising feature of the V-J junction sequences is the occurrence of an A nucleotide at the 5' end of some J_L segments that is not found in the germ-line sequence (e.g., cDNA 99, B15-28) and a C nucleotide at the 3' end of some V_L segments (e.g., B18-71, B15-13). One sequence has both nucleotides (B15-20). We have speculated that these nucleotides are added to the coding segments during the rearrangement process (McCormack et al. 1989).

Selection of productive V-J joints in the bursa during development

Whereas few out-of-frame V-J joints are observed in sequences derived from day 18 of development, many nonproductive V-J joints were isolated from the earlier stages (Fig. 4). The percentages of productive versus nonproductive rearrangements cloned from the bursa at different developmental stages are summarized in Table 2. Independent joining events were identified by unique V-J junctions and by gene-conversion events. These numbers may underestimate the actual number of independent joints represented, because many of the junction sequences may be formed by more than one joining event (e.g., B18-06 and B18-65) but are counted only once. The percentages calculated from the total set of V-J joints assume that the amplification and cloning efficiencies were equal and that a large number of random events were cloned.

The data summarized in Table 2 show that the proportion of nonproductive V-J joints at the earliest bursal stage examined, days 10–12, approaches the two-thirds expected if V-J joining is random for the nucleotide position of ligation of the V_L and J_L gene segments. Similar percentages of out-of-frame joints were also identified in clones isolated from splenic tissue at this developmental stage (Fig. 4D). At successively later stages of bursal development, the percentage of nonproductive joints decreases to 21% of all joints at day 15 and to 6% at day 18. These results are consistent with the selection of cells expressing a productive Ig_L rearrangement for clonal expansion within the bursa of Fabricius during this period of embryonic development.

V-J joints of cell lines with two rearranged Ig_L alleles

The V-J joints made by B-cell lymphomas that rearranged both Ig_L alleles were also investigated to determine the functional status of their Ig_L gene rearrange-

Table 2. Percentage of productive versus nonproductive V-J joints cloned from the bursa

Day	Independent V-J joints		All V-J joints sequenced	
	productive	nonproductive	productive	nonproductive
10–12	38% (3/8)	62% (5/8)	33% (10/30)	67% (20/30)
15	63% (15/24)	37% (9/24)	79% (34/43)	21% (9/43)
18	91% (21/23)	9% (2/23)	94% (30/32)	6% (2/32)

The numbers of productive and nonproductive V-J joints are shown in parentheses. Bursal cDNA clones were included with the PCR clones in the calculations for the day-18 V-J joints.

ments. Rearranged Ig_L genes were amplified from cell line DNA by PCR and cloned. Clones representing each of the two alleles were distinguished by the presence or absence of the *ScaI* site at the 3' end of the V_L gene segment, as determined by restriction enzyme analysis, and pooled colonies for each allele were then used for sequencing. The nucleotide sequences of the V-J junctions are summarized in Figure 5.

The cell line 30L1 has one productive and one nonproductive Ig_L rearrangement. The productively rearranged allele has a non-germ-line G nucleotide in codon 90 of V_L that may be the result of a gene-conversion event and a second gene-conversion event elsewhere in the V region (not shown). The other allele has the V_L -terminal C nucleotide at an out-of-frame V-J junction. Additional non-germ-line nucleotides elsewhere in the V_L region of this allele (not shown) may be the result of gene conversions, demonstrating that gene conversion may occur in both productively and nonproductively rearranged Ig_L alleles. These data also rule out the possibility that an out-of-frame V-J joining event is a lethal event by itself.

The cell line 41L1 has distinct productive rearrangements at both Ig_L alleles. It is not known whether both Ig_L alleles are expressed by 41L1. Productive rearrangements of nonexpressed alleles of some mammalian Ig loci have been reported (Nottenburg et al. 1987). Both rearranged alleles of 41L1 have also undergone gene conversion within V_L (Fig. 5, and data not shown).

Discussion

The analysis of Ig_L rearrangement during B-cell development in the chicken has allowed a detailed characterization of the early stages in avian B-cell differentiation. The exact location and period of active chicken Ig gene rearrangement has not been characterized previously.

Our data demonstrate that most Ig_L rearrangements occur at or just before day 15 of development, because the amount of the signal joint episome that is detectable during development in bursal and spleen tissue peaks at day 15 (Fig. 3). The fact that both rearranged V-J joints and signal joints can be isolated from extrabursal tissue between days 10 and 15 of development suggests that the bursa is not required for Ig_L gene rearrangement. These data are consistent with previous studies showing that removal of the bursa anlage at 60 hr of development does not prevent the expression of normal levels of serum Ig or the appearance of sIg^+ B cells in peripheral lymphoid organs (Jalkanen et al. 1983). However, the serum Ig of bursectomized chickens displays extremely limited Ig_L diversity, as demonstrated on isoelectric focusing gels (Jalkanen et al. 1984). Several recent studies have suggested that this is because the bursa of Fabricius is the site where diversity is generated within rearranged Ig_L genes by gene conversion (Reynaud et al. 1987; Thompson and Neiman 1987). The sequence data presented in this paper are consistent with this hypothesis. The Ig_L cDNA and PCR clones isolated from day-18 embryonic bursae demonstrate a high rate of base pair substitution within the V_L segments.

The data we have obtained concerning chicken Ig_L rearrangement are consistent with a model in which Ig_L genes are activated to rearrange during a limited stage of B-cell differentiation. Our data do not support a significant role for feedback mechanisms to prevent rearrangement of both chicken Ig_L alleles, as proposed for mammalian allelic and isotypic exclusion (Yancopoulos and Alt 1986). Instead, it appears more likely that in developing chicken B cells, the V-J 'recombinase' complex may be present for such a short time (e.g., only one or a few cell cycles) that most prebursal stem cells either fail to rearrange or only rearrange one Ig_L allele. Only a few

Figure 4. V-J coding joint nucleotide sequences. The nucleotide sequences of the V-J joints (V_L codons 84–92 through J_L) of rearranged genes are compared to the germ-line sequences obtained from the SC chicken. Two nucleotide sequence polymorphisms (C/G in codon 84 and A/G in codon 86) distinguish the two germ-line alleles in this region. Identity to the top germ-line sequence is indicated by a dash. Rearranged Ig_L genes were cloned from a day-18 bursal lymphocyte cDNA library (A) and after PCR amplification from embryonic tissues at different stages with the primers 1001 and 839 (B–D). PCR clones are designated by the tissue of origin (B, bursa; S, spleen), day of development, and clone number. The total number of clones with each sequence shown is given in parentheses. Previous work has suggested that Taq polymerase does not cause base addition or deletion, but the misincorporation rate has been estimated to be 2×10^{-4} per nucleotide per cycle (Saiki et al. 1988). Consistent with this, we observed several instances of possible misincorporation (e.g., the G substitutions in clones B10-17, S12-39, and S12-07). Misincorporation would be expected to be more common in the day-10 to day-12 clones because of the additional amplification required to isolate them.

	V_L										J_L						Reading frame	
germline	G _G ^C	AGT	A _G ^{CA}	GAC	AGC	AGC	AGT	ACT	GCT	G	T	GGT	ATA	TTT	GGG	GCC	GGG	
30L1	-	-	-	-	-	-	-	-	-	-C	-	-	-	-	-	-	-	-
30L1	-	G-	-	G-	-	-	-	G-	-	-	-	-	-	-	-	-	-	+
41L1	-G-	-C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+
41L1	-G-	-C	TAC	-A	GA-	-	-	-	-	-	-	-	-	-	-	-	-	+

Figure 5. Nucleotide sequences of bursal lymphoma V-J joints. Both rearranged Ig_L alleles were cloned from the bursal lymphoma cell lines 30L1 and 41L1, following PCR amplification of total cell line DNA with the primers 1001 and 839. The nucleotide sequences of the V-J joints (V_L codons 84–92 through J_L) of the rearranged genes are compared to the SC chicken germ-line sequences. Identity to the top germ-line sequence is indicated by a dash. (Right) A productive V-J joint is indicated by a plus sign (+), and a nonproductive joint is indicated by a minus sign (–).

cells would have time to rearrange both alleles. In chickens, rearrangement of the Ig_L gene serves primarily to activate gene expression. A large number of cells with productive rearrangements is not required because Ig_L gene diversity is created by gene conversion subsequent to rearrangement. Consistent with this hypothesis, several experimental approaches have demonstrated that only a few stem cells are required to colonize each of the 10⁴ bursal follicles (Le Douarin et al. 1975; Pink et al. 1987; Weill et al. 1986). The presence of two rearranged Ig_L alleles could potentially complicate the regulation of Ig_L diversification, because both rearranged alleles appear to be susceptible to gene conversion whether they are in-frame or not. Alternatively, during early lymphoid development, it is possible that one Ig allele in each cell is randomly chosen to undergo rearrangement. One mechanism for such selection would be random inactivation of one Ig_L allele prior to the initiation of rearrangement. This inactivation might be analogous to the way mammalian X chromosomes undergo inactivation early in development.

As discussed above, previous work has suggested that a major role of the bursa is to provide the necessary microenvironment for the somatic diversification of rearranged V genes (Ratcliffe 1985). Our results provide evidence for an additional role of the bursa during B-cell development, i.e., the selective amplification of lymphocytes with a productive V-J joint at the Ig_L locus. Between days 15 and 18 of embryogenesis, we observe a large increase in cells containing rearranged Ig_L genes in the bursa (Fig. 1B). During this period, we also observe a decrease in the relative abundance of the signal joint episome (Fig. 3). These data argue that the increase in bursal lymphoid cells with rearranged Ig_L genes between days 15 and 18 results from the migration and/or proliferation of cells with rearranged Ig_L genes, rather than continued Ig_L gene rearrangement within the bursa. Consistent with this, the failure to detect either signal joint episomes (Fig. 3) or V-J rearrangements (Fig. 1B) within the spleen at day 18 supports the hypothesis that cells that rearrange their Ig_L gene migrate to the bursa over this time period of development (Houssaint et al. 1976). However, it seems unlikely that selection occurs solely because cells with unproductive rearrangements fail to migrate to the bursa, because significant numbers of cells with out-of-frame rearrangements are found in the bursa between days 10 and 15 of embryogenesis.

By day 18 of embryogenesis, virtually every bursal lymphocyte contains an in-frame V-J joint. Because the somatic diversification of the rearranged V_L gene segment has been shown to begin between days 15 and 18 of embryogenesis (Thompson and Neiman 1987), part of the increase in the frequency of productive V-J joints during this period could be the result of 'correction' of out-of-frame joints by gene conversions. However, gene conversion was not observed in any of the V-J joints isolated at days 10–15 of embryogenesis. The dramatic increase in the proportion of productive V-J joints during this time period must therefore be due to a bursal selection process. Furthermore, gene conversion may place V-J joints out-of-frame just as frequently as in-frame. Gene-conversion events have been characterized that result in the loss of a productive rearrangement by shifting the reading frame (e.g., PCR clone B18-63, Fig. 4B; cDNA clone 3W-2, Reynaud et al. 1987).

Our data demonstrate that the phenotype of mature chicken B cells does not result from an increase in the precision of V-J rearrangement but from the selection and clonal expansion of cells with productive Ig_L gene rearrangements. The selective expansion of B cells with in-frame V-J joints within the bursa could be explained by a novel model, which we present for consideration. In this model, the target structure for positive selection of cells that have made an in-frame V-J joint (and a productive Ig_H rearrangement) is the sIg molecule. If this were the case, selective amplification of cells with productive gene rearrangements could result from antigen-induced proliferation of sIg⁺ cells within the bursa, which is mediated by interaction of germ-line-encoded sIg molecules with a bursal-specific antigen(s). The observation that less junctional diversity is present at later stages of development suggests that specific junctional sequences may also be selected at the protein level by this process because certain amino acids encoded at the V-J junction may be required for antigen recognition. Bursal antigen-induced proliferation would account for the observed exponential growth of embryonic sIg⁺ bursal cells (Lydyard et al. 1976) and might also activate the gene-conversion process within the rearranged Ig_L genes. Exponential growth and gene conversion would continue for a given cell until its sIg receptor no longer reacts with antigen(s) in the embryonic bursal microenvironment. Such a model allows for the selection of B cell tolerance to self-antigens expressed within the bursa

of Fabricius during the somatic generation of the immune repertoire by gene conversion. Consistent with this model, we have observed that an increasing proportion of slg⁺ bursal cells cease exponential growth and enter a quiescent state between day 21 of embryogenesis and 2–4 weeks of age, the time at which the mature repertoire is being completed by gene conversion (Thompson et al. 1987).

In summary, we have been able to characterize Ig_L rearrangement in the chicken during embryonic development. These data have allowed us to characterize further the role of the bursa of Fabricius in avian B-cell differentiation. Ig_L gene rearrangements occur in many tissues during a limited time period of development, resulting in the generation of Ig_L junctional diversity. The bursal microenvironment selects B cells with a productive Ig_L (and Ig_H) gene rearrangement and then promotes the gene-conversion process to create the diversity required in the adult immune repertoire.

Methods

Chicken strains and cell lines

The Hyline SC chickens used in these studies result from an F₁ cross between two inbred B² chicken strains that differ in several restriction enzyme sites around the Ig_L locus (Thompson and Neiman 1987). Blood samples from both parental strains were obtained from Hyline Incorporated. *v-rel*-transformed B-cell lines were derived from Hyline SC chickens by infection with reticuloendotheliosis virus strain T and single-cell cloning (Barth and Humphries 1988). ALV-induced bursal lymphomas were derived as previously described (Thompson et al. 1987).

DNA preparation and Southern blots

DNA was extracted from single-cell suspensions of bursal and splenic cells at day 15 of embryogenesis and later developmental time points. Due to the small size of the bursa and spleen before day 15 of embryogenesis, DNA samples were prepared from larger tissue fragments. Cell isolation, DNA extraction, and Southern blot methods have been described (Thompson and Neiman 1987). Hybridization probes (Fig. 1A) include the 577-bp *ScaI*–*ScaI* (U₂) and 964-bp *ScaI*–*Bam*HI (U₃) genomic fragments between V_L and J_L, and a C_L probe (1.2-kb *Eco*RI–*Sal*I genomic fragment). The V_L probe is a 180-bp *Kpn*I–*ScaI* restriction fragment derived from cDNA clone 99.

cDNA library

A cDNA library was prepared from bursa mRNA of day-18 embryonic Hyline SC chickens in the phage vector λ345 (T. St. John, pers. comm.). This vector allows recovery of selected cDNA clones as plasmids by digestion of the phage DNA with *Sfi*I and self-ligation of the released plasmid.

PCR

PCR with the thermostable Taq polymerase (Saiki et al. 1988) was used to rapidly clone multiple independent V–J coding joints from SC chicken splenic and bursal DNA and the two germ-line Ig_L alleles from erythrocyte DNA of the parental strains, using the following primers, located 5' of V_L and 3' of J_L, respectively:

1001, 5'-GCCCGAATTTCGGCAGGAGTGGGCAGGGAGGTGTGGGAGA-3'
839, 5'-TGGAAAGCTTCGGAAGAAAGACCGAGACGAGGTACGCGACT-3'

*Eco*RI and *Hind*III restriction sites at the 5' ends of the oligonucleotide PCR primers (underlined) allowed directional cloning into pGEM-3Z. PCR was used to identify the signal joint created during V–J joining, using the following primers, located between V_L and J_L (Fig. 3A):

993, 5'-GCCCGAATTTCGTCTCTCCCGAGCAGTAAGTGGGTGCTCAGG-3'
994, 5'-ATGGAAGCTTGAGTTGGTGAATCTCCTGAGTCACTAGCAA-3'

As controls for single-copy DNA sequences, PCR was performed using primers specific for the unique flanking region of either the H2B-IV gene (Grandy and Dodgson 1987) or the α-tubulin *ca5* gene (Pratt and Cleveland 1988). In each case, the specificity of the product was confirmed by digestion at internal restriction endonuclease sites (K. Lee and C. Thompson, unpubl.). Oligonucleotides were synthesized on an Applied Biosystems Model 380B DNA synthesizer.

PCR reactions contained 0.2–1 μg of DNA template, 1 μg of each primer, 0.1 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, and 2 mM dithiothreitol. PCR reactions were performed with 2.5 units of Taq polymerase (Cetus) in a Perkin–Elmer thermal cycler for 30 cycles, followed by a final 10-min extension reaction. For the amplification of rearranged Ig_L genes, each cycle included a 1.25-min denaturation step at 94°C and a 3-min primer extension step at 72°C. Whereas 30 cycles of PCR were used to isolate V–J joints from day-15 and day-18 tissue, a second round of amplification (total 60 cycles) was required to isolate V–J joints from day-10 to day-12 tissue. For amplification of the germ-line Ig_L genes from erythrocyte DNA of the SC parental strains, the primer extension step was increased to 5 min. Amplification from the signal joint episome was performed with the addition of a 1-min annealing step at 55°C to each cycle before the primer extension reaction. PCR products for germ-line and rearranged Ig_L genes were digested with *Eco*RI and *Hind*III, cloned into pGEM-3Z, and selected by colony hybridization with the V_L probe. Random individual colonies were used for the analysis of multiple V–J coding joints. Colonies were pooled for the cloned germ-line Ig_L alleles and rearranged alleles of B-cell lymphomas to obtain a consensus sequence free of Taq polymerase errors.

DNA sequencing

Double-stranded DNA sequencing of both strands was performed using a Sequenase kit according to the supplier's protocols (U.S. Biochemical Corp.). The DNASTAR software package was used for sequence data analysis.

Acknowledgments

We thank Tom St. John for assistance in preparing the cDNA library in the laboratory of Paul Neiman. We also thank our colleagues Jeff Leiden and Ken Roux for thoughtful review of the manuscript and Paul Neiman, Brian Van Ness, Wesley Dunnick, J. Latham Claflin, Jeff Bonadio, and John Lowe for helpful discussions. We are grateful to Sheila Norton for oligonucleotide syntheses, Jeanelle Pickett for assistance in preparing the manuscript, and Beverly Burck for the preparation of figures. This work was supported, in part, by U.S. Public Health Service Award CA44016 from the National Cancer Institute to E.H.H.

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Selection for B cells with productive IgL gene rearrangements occurs in the bursa of Fabricius during chicken embryonic development.

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Genes Dev. 1989, **3**:

Access the most recent version at doi:[10.1101/gad.3.6.838](https://doi.org/10.1101/gad.3.6.838)

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