Chicken IgL Gene Rearrangement Involves Deletion of a Circular Episome and Addition of Single Nonrandom Nucleotides to Both Coding Segments

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Summary

Chicken immunoglobulin light chain (IgL) gene rearrangement has been characterized. Rearrangement of the single variable (V,) segment with the single joining (J,) segment within the chicken IgL locus results in the deletion of the DNA between V, and J, from the genome. This deletion is accomplished by a molecular mechanism in which a precise joining of the IgL recombination signal sequences leads to the formation of a circular episomal element. The circular episome is an unstable genetic element that fails to be propagated during B cell development. Evidence was obtained that the formation of the circular episome is accompanied by the addition of a single nonrandom base to both the V, and J, coding segments. The subsequent joining of the V, and J, segments appears to occur at random, as we observed at least 25 unique V-J junction sequences, 11 of which are out-of-frame. A novel recombination mechanism that accounts for the observed features of chicken IgL gene rearrangement is discussed.

Introduction

The chicken immunoglobulin light chain (IgL) locus consists of single functional V, (variable), J, (joining), and C, (constant) gene segments that are aligned along 5.4 kilobases (kb) of DNA in the germ line, and an upstream family of 25 V, pseudogenes (Reynaud et al., 1985, 1987). A functional IgL gene is created during B cell development by in-frame joining of the V, and J, coding sequences. Diversity of the chicken IgL gene is generated by gene conversion of the rearranged V, segment with pseudogene donor sequences during B cell development in the bursa of Fabricius (Reynaud et al., 1987; Thompson and Neiman, 1987).

The chicken IgL locus, because of its small size and unique germ-line structure, provides a novel in vivo model for the study of the V-J joining process. While the molecular mechanism of V-J joining in chicken B cells has not been characterized, the chicken IgL gene segments possess recombination signal sequences similar to those that flank the Ig and T cell receptor (TCR) gene segments in other vertebrate species (Tonegawa, 1983; Hood et al., 1985; Yancopoulos et al., 1988). The germ-line V, gene segment is followed by a heptamer and nonamer sequence separated by a 23 bp spacer region, whereas the germ-line J, segment is preceded by a heptamer and nonamer sequence separated by a 12 bp spacer (Reynaud et al., 1987). In mammals, these signal sequences appear to target lymphoid-specific recombination events (Akira et al., 1987). Mammalian Ig gene rearrangement has been hypothesized to occur by unequal sister chromatid exchange, intramolecular DNA deletion, deletion and reintegration, and/or chromosomal inversion (reviewed by Kronenberg et al., 1986).

Analysis of 48 clonal chicken B cell lines demonstrated that the DNA between V, and J, is lost from the genome during IgL gene rearrangement. After determining the complete nucleotide sequence of the germ-line V, and J, gene segments and the region between them, oligonucleotide primers were designed to rescue potential intermediates in the deletional process using the polymerase chain reaction (PCR; Saiki et al., 1988). Evidence was obtained that the DNA between V, and J, is precisely excised and circularized during rearrangement. The resultant circular episome fails to be propagated during B cell development, and is therefore absent in most, if not all, mature B cells. Rearranged IgL genes were isolated from an embryonic bursal cDNA library and cloned following PCR amplification from embryonic lymphoid tissue DNA using IgL-specific primers. Analysis of 85 cloned V-J coding joints revealed considerable V-J junctional diversity, with at least 25 distinct V-J joints identified. Surprisingly, comparison of the V-J coding joint sequences with the germ-line sequences suggests that single nonrandom nucleotides are added to the V, and J, coding ends during the deletional process and prior to V-J joining.

Results

Chicken IgL Gene Rearrangement Results in Loss of the DNA between V, and J,

The genomic organization of the IgL locus of the SC chicken and the DNA probes used in these experiments are shown in Figure 1A. Rearrangement of the chicken IgL gene results in the joining of the V, and J, coding segments. The fate of the 1.8 kb of DNA between V, and J, has not previously been determined. To address this issue, 48 clonal chicken B cell lines and tumors were examined for the presence of the DNA segment between V, and J, in all instances probes derived from the region between V, and J, detected only germ-line segments retained in unarranged alleles (data not shown). Thus, the DNA between V, and J, is deleted from the genome during V-J joining.
Precise Joining of V<sub>L</sub> and J<sub>L</sub> Signal Sequences Creates a Circular Episome

Deletion of the DNA between V<sub>L</sub> and J<sub>L</sub> in a clonal B cell line could have occurred by either unequal sister chromatid exchange or intrachromosomal deletion. Okazaki et al. (1987) have shown that at least some TCR β gene rearrangements occur by a process of intrachromosomal deletion in which circular DNA molecules are created by ligation of the VB-DB or DB-JB signal sequences. To test whether a similar deletional process occurs during chicken IgL gene rearrangement, we designed oligonucleotide PCR primers (993 and 994, Figure 1B) to amplify potential signal joints from embryonic bursal cell DNA. The experimental strategy is diagrammed in Figure 2A. The PCR primers are in opposite orientations in the unrearranged germ-line locus such that no DNA amplification is possible. If the formation of a signal joint creates a circular episomal product, the PCR primers will amplify a fragment approximately 1.2 kb in size. Should this occur, further characterization of the ligation event is then possible, because perfect heptamer-to-heptamer joining without base loss or addition would result in the creation of a novel ApaLI restriction site (Figure 2A).

Southern blot analysis of undigested PCR products amplified from day 15 and day 18 bursal cell DNA and hybridized with the U3 probe revealed the expected 1.2 kb fragment (Figure 4A). Digestion of the PCR products with ApaLI resulted in a complete shift of the 1.2 kb fragment to the predicted ~600 bp size (Figure 4B), suggesting that virtually all signal joints result in the formation of the ApaLI site by precise heptamer-to-heptamer joining. As confirmation of the identity of the amplified segment, digestion with the enzyme BamHI produced the expected ~300 bp fragment. Signal joints were also cloned, and nucleotide
Figure 2. A Signal Joint Episome Is Detectable during Embryonic Development

(A) Schematic representation of the experimental strategy used to recover the signal joint from embryonic bursal lymphocyte DNA by PCR amplification. The signal sequence heptamers and nonamers are represented by triangles and squares, respectively. The position and orientation of PCR primers are indicated by arrows. The location of a known BamHI restriction site and the ApaLI site that may be formed by heptamer-to-heptamer joining are indicated.

(B) The signal joint was amplified from day 15 and day 18 embryonic bursa DNA by PCR with the primers #993 and #994. PCR products were untreated (-), or digested with ApaLI or BamHI, prior to separation on a 1% agarose gel. Southern blots of these samples were hybridized with the U3 probe, and the resulting autoradiogram is shown. Marker bands (M) are the end-labeled fragments of phage λ DNA digested with HindIII, with sizes in kb indicated.

sequences of the joints were determined. All of the signal joints sequenced had undergone the expected precise joining of heptamer-to-heptamer.

Interestingly, the relative amount of product amplified from day 18 embryonic bursa was less than that from day 15 bursa DNA. This result, which was consistently observed after amplifications of several different DNA preparations from each developmental stage, suggests that the relative abundance of the signal joint episome is lower in the day 18 than in the day 15 bursal DNA sample, and implies that a significant proportion of the bursal lymphocyte population have failed to propagate the episome during this period of development.

Junctional Diversity of Chicken Coding Joints

In view of the precision with which the signal joint is created and the high percentage of previously sequenced rearranged IgL genes that are in-frame (Reynaud et al., 1987; Parvari et al., 1987), we next sought to determine the degree of junctional diversity within V-J coding joints during early embryonic life. V-J coding joints were isolated from a day 18 embryonic bursal cDNA library and cloned from day 10-18 embryonic bursal and spleen DNA following PCR amplification with primers #1001 and #839, located 5' of VL and 3' of JL, respectively (Figure 1B). The nucleotide sequences of the V-J coding joints are shown in Figure 3. Additional clones from day 18 bursae revealed gene conversion events, which will be reported separately.

We observed extreme junctional diversity at chicken V-J joining events, with a total of at least 25 different junction sequences represented (Figure 3). Neither the position of recombination within the coding triplets nor the number of codons of VL and JL retained in the rearranged gene is conserved in the V-J joints. It is important to note that the exact junction of many clones cannot be determined because the sequence TG is repeated at the 3' end of VL and at the 5' end of JL. However, at least 6 sites within VL and 11 sites within JL were used in V-J joining events. The maximal extent of base loss before joining was 11 nucleotides from VL (clone 17) and 14 nucleotides from JL (clone 37).

Whereas all previously reported embryonic chicken V-J joints are in-frame, nonproductive joints were found in the V-J joints amplified from embryonic tissue by PCR (Figure 3). The percentage of clones with out-of-frame joints found between days 10-12 of embryogenesis was 66%, further suggesting that V-J joining is a random process. The lower frequency of out-of-frame joints observed at later stages of embryonic development suggests that the
bursa is a site for the selective amplification of cells with productive V-J joints (McCormack et al., submitted).

Nonrandom Nucleotides at V-J Junctions
Ten clones (four isolated by cDNA cloning and six isolated by PCR amplification) encoded V-J joints with an A nucleotide at the junction that is not found in the germ-line VL or JL segments. In each case, the V-J junction may be written such that the A nucleotide occurs at the 5' end of a full-length JL gene segment. In addition, 17 clones were found that encoded V-J joint sequences with an additional C nucleotide, which appears to be added in each case to the 3' end of a full-length VL gene segment. Of note, one sequence (clone 20) encodes full-length VL and JL segments, and contains both added nucleotides (C and A) at the junction. In order to characterize the positional nature and frequency with which we observe nucleotide addition during V-J joining further, the positions of VL and JL recombination among the 85 clones were tabulated (Figure 4). The frequency with which a
given base pair occurs at the V-J junction appears to be dependent on its distance from the end of the V, or J, coding sequence. The frequency with which we observe the additional C or A nucleotides, which we have assigned the +1 positions of V, and J, respectively, is comparable to the frequency with which neighboring nucleotides are utilized. Possible origins of the C and A nucleotides are discussed below.

Discussion

The studies described in this paper characterize the molecular events associated with V-J joining at the chicken Ig, locus. Consistent with previous reports (Reynaud et al., 1985; Thompson and Neiman, 1987), Ig, gene rearrangement results in the removal of the 1.8 kb of germ-line DNA between V, and J, The rearrangement occurs as a result of an intrachromosomal deletion that removes a circular episomal element in which the conserved heptamers 3' of V, and 5' of J, are precisely joined. No evidence for nucleotide addition or deletion at the signal joint was obtained. Previous examples of nucleotide addition or deletion at signal joints have occurred in cells in which Ig or TCR gene rearrangement is an ongoing event (Lieber et al., 1986; Okazaki et al., 1987). In these cases nucleotide addition or deletion may have been derived from imprecise excision near the heptamer borders, or may be the result of secondary rearrangements involving the signal joint. During secondary rearrangements, one half of the signal joint would be treated by the recombinase as a "coding" end, and therefore would be susceptible to nucleotide loss or addition. Despite the precision observed in the generation of the signal joint, joining of the chicken V, and J, coding segments appears to occur randomly. Just as in mammals, the coding ends appear susceptible to exonucleolytic activity prior to their ligation to form a coding joint. While the random nature of V-J joining leads to a considerable proportion of rearrangements being out-of-frame, it also serves to generate diversity within the V-J joining region of in-frame clones. The chicken V-J coding joint sequences also revealed the presence of unexpected C and/or A nucleotides at the junctions. Based on the data presented in Figure 4, it seems possible that every V, segment has a 3'C added and every J, segment a 5'A added during the rearrangement process. Alternatively, the C or CA nucleotides may originate from the dinucleotide CA immediately 3' of the germ-line V, gene segment in the signal sequence heptamer. In these cases, cleavage may occur 3' of the C or A nucleotide of the heptamer. However, the uniform presence of the ApaI restriction site in the signal joints we have isolated (Figure 2B) suggests that the C or CA is not lost from the heptamer during V-J joining; therefore cleavage 3' of C or CA would have to result in a staggered cut that is repaired during ligation. There does not appear to be a simple way of generating the A nucleotide alone from the germ-line signal sequences.

Six of the clones that contain either the C or the A nucleotide were isolated from the bursa at day 18 of embryonic development. A possible somatic origin of the added nucleotides in these clones is gene conversion, which has been initiated by this time of embryonic development (Reynaud et al., 1987). However, most of the clones with the junctional C and/or A were isolated from day 10-15 embryonic tissue, a time period during which we have failed to observe other examples of Ig, gene conversion (McCormack et al., submitted). In addition, 7 of 26 clones (26.9%) containing the extra C and/or A nucleotide were isolated from embryonic B cells in the spleen in a developmental stage prior to bursal selection and gene conversion. The percentage of splenic clones with C and/or A was comparable to the total number of clones isolated from splenic tissue (23/85 = 27.1%). These data further argue against roles for bursal selection or gene conversion in the addition of C or A nucleotide.

In mammals, additional non-germ line base pairs have been observed frequently in rearranged Ig heavy chain genes. This nucleotide addition is thought to be due to the action of terminal deoxynucleotidyl transferase (TdT), and usually results in the addition of G-rich sequences termed "N segments" (Alt and Baltimore, 1982; Alt et al., 1986). Whereas N segments are generally associated only with Ig, gene rearrangement (Yancopoulos and Alt, 1986), at least two examples of Ig, gene rearrangement with base insertion have been reported (Max et al., 1980; Klobeck et al., 1987), and N segments are added to coding joints of recombination constructs in vitro (Lieber et al., 1988a). The nucleotides added to the chicken V-J joints do not resemble random N segments for two reasons. First, the extra nucleotides are always C and/or A rather than a G-rich polynucleotide region. Second, the added nucleotides are restricted in position, i.e., an extra C nucleotide appears only at V-J junctions in which there has been no apparent base loss from the V, gene segment, and an extra A nucleotide appears only when there has been no base loss from the J, gene segment.

Some reports of N segment addition suggest that specific codons may be added at junctions by a complex sequence-specific and/or template-free mechanism. For example, the serine residue at position 99 of anti-arsonate Ig chains, which is selected at the protein level for binding to the hapten arsonate, is encoded by an N segment (Miller et al., 1986; Wysocki et al., 1986). It could be argued that some instances of A addition at in-frame chicken V-J junctions produce a specific codon (e.g., the aspartic acid codon GAT in clones 50, 8, and 99) which could be selected at the amino acid level. However, this selection would have to occur prior to exposure to exogenous antigen or bursal selection, because two of these sequences were identified in clones isolated from splenic tissue at day 12 of embryonic development. Additional A nucleotides were also observed in an out-of-frame joint and in a joint codon encoding asparagine.

Our findings concerning the rearrangement of the chicken Ig, genes have led us to consider various models to account for the observed features of Ig gene rearrangement. Any model of molecular recombination must account for the observation that during rearrangement the
V and J coding ends appear to be susceptible to exonucleases and/or TdT, whereas the signal ends are protected from these activities. To account for these observations, it has been suggested that the DNA recombination events involved in V–J joining occur sequentially, with the signal joints being made first and the coding ends being joined later (Alt and Baltimore, 1982). During the time between creation of the signal joint and the coding joint, the coding ends would be susceptible to base loss or addition. However, a sequential model no longer seems likely because it has recently been observed in a mammalian in vitro recombination system that coding ends can be separated from and then rejoined to signal ends ("open-and-shut" joints, S. Lewis, personal communication). During these events the coding ends still display sensitivity to base pair addition and loss, while the signal ends are protected from these processes. These data suggest that all four DNA ends are available for ligation at the same time, rather than ligated sequentially (Lieber et al., 1988a).

The above data and the data presented in this paper could be accounted for by a novel mechanism of gene rearrangement. In this model, we propose that the "recombinase" complex exchanges a C nucleotide for the signal sequence 3' of the V\(_i\) segment. The result of this process would be that the signal sequence would become bound to the "recombinase" complex and the V\(_i\) coding sequence would now end in a 3' non-germ-line C. A similar exchange would occur between the "recombinase" complex and the 5' end of the J\(_k\) segment. In this case the signal sequence would be exchanged for an A nucleotide. The generation of these molecular intermediates would leave the coding sequences susceptible to base addition and/or deletion while protecting the signal sequences from these processes. The entire exchange process may occur on a single strand. The second strand could then be resolved by either DNA replication or template-directed repair. This model may help to explain why mice, which are deficient in ligation activity associated with Ig "recombinase," can still form some signal joints but not V–J coding joints (Lieber et al., 1988b). If the signal sequences are incorporated into and protected by the "recombinase" complex, they may be more likely to be resolved over time by whatever residual ligase activity the cells retain or joined by homologous recombination. The validity of this alternative model of Ig gene rearrangement should be testable in the future in a variety of experimental systems.

### Experimental Procedures

#### Chicken Strains

Chickens used in these experiments were Hyline SC birds, an F\(_1\) cross between two inbred \(B^\times\) chicken strains that differ in several restriction enzyme sites around the IgL locus (Thompson and Neiman, 1987). Blood samples from the parental strains were obtained from Hy-line Incorporated.

#### DNA Preparation and Hybridizations

DNA preparation and hybridization methods have been described (Thompson and Neiman, 1987). Hybridization probes are illustrated in Figure 1A. A 964 bp Scal-BamHI genomic fragment (U\(_i\)) between V\(_i\) and J\(_k\) was used as a hybridization probe to identify the signal joint product. Rearranged V\(_i\) gene cDNA clones were identified by hybridization with a V\(_i\) probe (180 bp KpnI–Scal fragment) and a C\(_\lambda\) probe (1.2 kb EcoRl–Sall fragment).

#### DNA Libraries

A Hyline SC chicken bursa cDNA library was prepared from day 18 embryonic bursa mRNA and cloned into the vector λ345 (T. St. John, personal communication). Selected cDNA clones were recovered from the phage vector as plasmids by digestion with StuI and self-ligation. A chicken genomic DNA library, prepared by partial Sau3A digestion of erythrocyte DNA from an outbred White Leghorn chicken and cloned into X-FIX (Stratagene), was kindly provided by K. Conklin (University of Minnesota). Genomic fragments were subcloned into the plasmid vector pGEM-3Z (Promega).

#### Polymerase Chain Reaction (PCR)

PCR (Sakai et al., 1988) was used to rapidly clone the reciprocal signal joint created during V–J joining; multiple joining events that occurred between the chickens V\(_i\) and J\(_k\) gene segments; and the two germ-line Ig\(_\lambda\) alleles from the SC chicken. The oligonucleotide primers (Figure 1B) were designed with restriction sites (EcoRl or HindII) at their 5' ends to allow directional cloning into pGEM-3Z, and were synthesized on an Applied Biosystems Model 380B DNA synthesizer.

Typical PCR reactions consisted of 0.2–1.0 \(\mu\)g of DNA template, 1 \(\mu\)g of each primer, 0.1 mM of each dNTP, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\(_2\), 0.01% gelatin, and 2 mM dithiothreitol. PCR reactions were performed with 2.5 \(\mu\)U of Taq polymerase (Cetus) in a Perkin-Elmer thermal cycler for 30 cycles, followed by a final 10 min extension reaction. Each cycle for the amplification of germ-line and rearranged Ig\(_\lambda\) genes consisted of 1.25 min at 94°C and 3 min at 72°C for 5–6 min. Amplification of signal joints was performed with the addition of a 55°C annealing step for 1 min. Following digestion of PCR products with EcoRl and HindIII and cloning into pGEM-3Z, colonies positive for hybridization with the V\(_i\) probe were randomly selected for sequencing. Individual colonies were used for the analysis of V–J coding and signal joints. To randomize Taq polymerase errors and allow reading of a consensus sequence for the two germ-line Ig\(_\lambda\) alleles, colonies were pooled for plasmid DNA preparation.

#### DNA Sequencing

Dideoxynucleotide sequencing reactions were performed using double-stranded plasmid template DNA with a Sequenase kit according to the supplier's protocols (U. S. Biochemical Corp.), and separated on 50 cm long, 8% polyacrylamide gels (0.2–0.8 mm) using a buffer gradient. All sequences were analyzed using the DNASTAR software package.

#### Acknowledgments

We would like to thank Kathy Conklin for providing the chicken genomic library, and Sheila Norton for oligonucleotide syntheses. The cDNA library was produced in the laboratory of Paul Neiman, with the assistance of Tom St. John. We would like to thank Paul Neiman, Ken Roux, Brian Van Ness, Wesley Dunnick, J. Latham Claffin, Jeff Bona-dio, John Lowe, and Jeff Leiden for thoughtful review of the manuscript and helpful discussions. We also thank Jeanelle Pickett for assistance in preparing the manuscript and Beverly Burck for the preparation of figures. This work was supported in part by Public Health Service Award CA41450 from the National Cancer Institute to E. H. H.

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Received November 22, 1988; revised December 21, 1988.

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tion signals are sufficient to cause immunoglobulin \(V-(D)-J\) joining. Science 238, 1134–1138.


Note Added in Proof

The data referred to as O. Lewis, personal communication, have been published: Lewis et al., Cell 55, 1099–1107.