Restricted Immunoglobulin Junctional Diversity in Neonatal B Cells Results from Developmental Selection Rather Than Homology-based V(D)J Joining

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Summary

The mechanism by which coding ends are joined during immunoglobulin (Ig) recombination is poorly understood. Recently, short sequence similarities (2-6 bp) observed at the ends of certain variable (V), diversity (D), and joining (J) gene segments of Ig have been correlated with limited junctional diversity observed in coding exons assembled from these elements. However, it is unclear whether these sequence homologies play any direct role in favoring coding joint formation by influencing the V(D)J recombination process. In this report, we demonstrate that coding sequence similarities do not influence the position of coding joints during V(D)J recombination in vivo. Instead, during embryonic development, B cells with certain joining products undergo progressive selection. Developmental selection is completed even before exposure to external antigens and appears to be determined by the amino acid sequence encoded by the coding joint. We conclude that the nucleotide sequences of the coding regions do not play a major role in directing V(D)J recombination. Instead, we propose that limited Ig junctional diversity results from prenatal developmental selection of B cells based on the protein sequence of their surface Ig antigen-binding site. Sequence identities at the ends of coding segments may have evolved because they increase the likelihood that a selectable antigen-binding site is created during a random recombination process.

The need to respond to a wide variety of foreign antigens requires that the vertebrate immune system produce a large repertoire of Ig and TCR. Production of a diverse set of Ig and TCR is facilitated by the fact that the genes that encode each of these receptors are composed of families of discrete germine elements (1, 2). During development, individual members of variable (V), diversity (D), and joining (J) families in the B and T cells must recombine to create a translatable antigen-binding exon for each chain of their individual antigen receptor. The developmental process by which rearrangement occurs has been termed V(D)J recombination. V(D)J recombination is a form of site-specific recombination whose specificity is determined by recombination signal sequences (RSS) consisting of a palindromic heptamer and an AT-rich nonamer element that flank the coding sequences of the individual V, D, and J elements (1). During V(D)J recombination, two RSS elements are paired so that one of the pair has a 12-bp spacer between the heptamer and nonamer elements, whereas the other has a 23-bp spacer between the heptamer and nonamer elements. Two recombination products are produced during a V(D)J recombination event (Fig. 1). One product is the precise end-to-end joining of the two recombination signal sequences. The other recombination product results from the joining of the coding sequences of the two Ig gene segments themselves (3). It takes two serial recombination events to generate an Ig H chain or a functional TCR β or δ chain. First, recombination occurs between a D and a J element followed by the joining of a V element to the previously recombined DJ element (4-6). For Ig L chain and TCR α and γ chains, a functional gene product is created directly by the recombination of individual V and J gene elements (7, 8).

The recombination of the individual coding elements of Ig and TCR creates diversity by several mechanisms. First, in many species there are families of V, D, and J gene elements encoded in the germline (1). The random recombination of individual elements results in considerable combinatorial diversity. Second, unlike the signal joints that are usually created by precise joining of the heptamer elements of the two recombination signal sequences, joining of individual V, D, and J elements is imprecise (4, 9, 10). Coding ends are subject to both base loss and addition before ligation resulting in diversity of the sequences at the joining po-

1 Abbreviation used in this paper: RSS, recombination signal sequences.
joints are brought together during V(D)J recombination (for rendering the locus susceptible to gene conversion and/or before end ligation. Coding ends are also subjected to two highly variable and has been suggested to occur by pairing of exposed coding joints there is nucleotide loss from both coding ends, and although enriched for G/C bases, are otherwise able variability in the precise position of joining. In most
positions of the individual elements (3). Finally, recombination appears to activate Ig gene transcription, and this may play a role in the generation of further diversity in some species by rendering the locus susceptible to gene conversion and/or somatic mutation (11–13).

At present, relatively little is known about how coding joints are brought together during V(D)J recombination (for review see reference 14). As noted above, there is a considerable variability in the precise position of joining. In most coding joints there is nucleotide loss from both coding ends before end ligation. Coding ends are also subjected to two forms of nucleotide addition. P nucleotides are nucleotides added only to full-length coding sequences and are complementary to the sequence at the ends of coding elements (15, 16). In contrast, N nucleotides can be added to intact or processed ends, and although enriched for G/C bases, are otherwise random in sequence composition (for review see reference 17).
The average number of N nucleotides added during V(D)J recombination increases during development (18–21). Since coding joints involve the joining of two coding sequences in a random fashion, they are frequently out-of-frame. In fact, two thirds of joints formed randomly would be expected to be out-of-frame. This high percentage of out-of-frame joints may be tolerated simply because of the high degree of diversity created through the formation of coding joints.

Despite the great variability in the generation of coding joints, several examples of limited coding joint diversity have been observed (15, 22–25). In these cases, it has been postulated that short stretches of sequence identity increase the likelihood of recombination occurring at a given position (24, 25). This has led to the proposal that the presence of complementary sequences on exposed single-stranded tails of the two coding elements are used to facilitate coding end alignment and joining (14, 24, 25). However, it seems paradoxical that a recombination mechanism that appears to have evolved to maximize heterogeneity would involve an intermediate that limits its diversity. Therefore, an alternative hypothesis for the observed limitation in junctional diversity is that there is developmental selection for specific coding joint sequences.

Recently, during our studies of B cell development in the Muscovy duck, we encountered an example of limited junctional diversity. 53 of 64 rearranged V13-JJ gene products isolated from posthatch ducks had identical sequences at the site of VJ joining. The ends of V11 and JJ contained a 9-bp region of sequence similarity in which 7 out of 9 bp were identical. Alignment of these two segments of homology could result in the creation of the preferential joint observed in B cells of adult ducks. Unlike mammals, recombination in avian B cells occurs only during a limited period of development (16). Therefore, we were able to design experiments to address whether this preferential joint was the result of the V(D)J recombination process or of developmental selection that occurred subsequent to V(D)J recombination. In the Muscovy duck, Ig L chain recombination occurs only in lymphoid progenitor cells arising in the spleen between days 20 and 30 of embryonic development. Upon sequencing the initial joints created in the spleen on day 21 of embryonic life, we found that there was no bias for the creation of V11-JJ joints as a result of the sequence homology at the V11 and JJ coding ends. Instead, we were able to document that at sequential times during embryonic development, there was a progressive increase in the proportion of B cells that had Ig L chains containing the V11-JJ joint observed in adult ducks. In fact, this joint was observed in over 94% of V11-JJ joints observed in B cells from the spleen and bursa at day 35 of embryogenesis (day of hatch). This developmental selection was not limited to the V11-JJ product. In the other functional V-region gene (V3) that we studied, two V3-JJ recombination joints were observed to increase in frequency during development, both of which encoded the same amino acid product across the V3-JJ joint. Based on these results, we conclude that sequence homology does not play a major role in promoting the formation of the coding joint during V(D)J recombination. Instead, developmental selection is the reason why cells with a particular joint predominate at birth. It appears that selection occurs as result of the protein product of V(D)J recombination rather than as a result of the process of recombination itself. This selection occurs before exposure of the embryo to exogenous antigen and therefore must be mediated by intrinsic developmental factors and/or antigens.

Materials and Methods

Cell Isolation and DNA Extraction. Muscovy ducks were killed at days 20, 21, 25, 26, and 30 of embryogenesis, at the day of hatch (day 35 of embryogenesis), and at 1, 2, and 4 wk of age. At each of these times, the bursa and spleen were removed and the lymphoid cells isolated as described previously (26). Cells were lysed
in RSB buffer (10 mM Tris, pH 7.4; 10 mM NaCl; and 5 mM MgCl₂) containing 0.5% NP-40, and nuclei were isolated. The nuclei were then solubilized in 0.5% SDS, 300 mM NaCl, 10 mM Tris, pH 7.4; and 5 mM EDTA. Proteinase K (250 mg/ml) was used to digest proteins and the DNA was precipitated in 2.5 M ammonium acetate and 50% (vol/vol) isopropanol. It was resuspended in TE (10 mM Tris, pH 7.4; and 1 mM EDTA) and then reprecipitated as above. The resulting precipitate was then recovered by centrifugation, washed twice with 70% ethanol containing 50 mM NaCl, and then resuspended into ~1 mg/ml.

**PCR and Southern Blot Analysis.** PCR (27) was used to clone the coding joints and to amplify the signal joints created during V(D)J recombination as described previously (16). Oligonucleotide primers were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA). The primers for V_i and J_k segments had restriction sites for EcoRI or HindIII to allow directional cloning into pGEM-3Z.

35 cycles of amplification (1 min at 94°C, 2 min at 72°C) were performed in a thermal cycler (Perkin-Elmer Cetus Instruments, Norwalk, CT) followed by a final 10 min. extension. The PCR primers used for signal joint assays were: no. 2081-GGGGGAATTCTCCTCATCCCCACCACACTTTTCCATCTGTTGCA; no. 3544-GGGGGAATTCTGACAGGATGCGATCCCACCTGCAGCTG; no. 5299-TTGGAAGCTTGCCAGCGAGGATATCCTGAACTTGCAGCAGGAGC; and no. 7009-TGGAAGCTTGCCACAAGGATTTGCTAGTCA.

The PCR primers used to amplify rearranged IgL clones were chosen so that the rearranged joints amplified were not subject to bias by subsequent gene conversion within the primer sequence. For this purpose, the primers for various V_i gene segments were located in FR1 region or 5' of it. The primers were: for V_i: GGGGGAATTCTCCTGAGGGATGCGGAGTCTGCGAGAGGGAGC; for J_k: GGGGGAATTCTCCTGAGGGATGCGGAGTCTGCGAGAGGGAGC; and no. 7009-TGGAAGCTTGCCACAAGGATTTGCTAGTCA.

DNA Sequencing. Dideoxynucleotide sequencing reactions were performed using double-stranded DNA template with a Sequenase kit according to the supplier's protocol (U.S. Biochemical Corp., Cleveland, OH), and a primer specific for the SP6 promoter site of pGEM-3Z.

**Results**

**Bias for One V_i-J_k Joint in Muscovy Ducks.** Muscovy ducks have a single functional J_k and at least five functional and 32 pseudogene V_i segments in the IgL locus (28; Tjoelker, L. J., L. M. Carlson, and C. B. Thompson, manuscript in preparation). To activate IgL gene expression, a functional V_i gene segment must undergo rearrangement with a J_k segment. When the two coding segments are joined in-frame, subsequent transcription and processing lead to a translatable IgL transcript. Using PCR primers specific for V_i and J_k, we sequenced the productive V_i-J_k joints from 1-4-wk-old Muscovy ducks. It is surprising that one particular joint was found in 53 out of 64 (83%) total clones and 53 out of 58 (91%) clones with in-frame V_i-J_k joints (Fig. 2). This predominant joint has the nucleotides GAT TTT GGA at the V_i-J_k junction.

Southern blot analysis was carried out as described previously (12). The signal joints were confirmed by hybridizing with a radiolabeled J_k, RSS-specific fragment.
To estimate the time of peak recombination in the spleen, the same method of analysis was repeated on DNA samples from 20-, 25-, and 30-d embryonic spleen and 2-wk posthatch spleen (Fig. 5 b). In these results, which are representative of five separate experiments, we observed that recovery of signal joints by PCR was greatest at day 25. Lower levels of signal joint recovery were observed at days 20 and 30. No recovery of signal joints was observed at hatch (data not shown) or subsequent time periods. Taken together, these data suggest that recombination occurs during a brief developmental period in the spleen of the Muscovy duck. Based on our analysis, we conclude that recombination begins by day 20 and peaks around day 25 of embryonic development in the Muscovy duck.

**V_{L1}-J_{L} Joints at Day 21 in the Spleen.** We wanted to look at the V_{L1}-J_{L} joints before a time at which selection could have occurred. Thus we sequenced V_{L1}-J_{L} rearrangements from 21-d spleen. At this time, no gene conversion had occurred and hence it was impossible to predict if two identical clones out of one PCR reaction were independent sequences or amplified products of one DNA sequence. This problem was circumvented by performing seven separate PCR reactions on 21-d spleen DNA samples. We found that 24 out

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**Figure 3.** Sequence of the preferred joint in adult Muscovy ducks. (A) Germline sequence of V_{L1} and J_{L} genes in the Muscovy duck IgL locus is shown. (B) Possible alignment of complementary sequences in V_{L1} and J_{L} during the process of recombination.

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**Figure 4.** Detection of V(D)J recombination in the spleen. Illustration of the strategy used to detect signal joints by PCR amplification. A schematic representation of the 3' end of the V region cluster and J region of the Muscovy duck IgL locus. (**) V_{L} and J_{L} coding segments. (v-4) V region recombination signal sequences (RSS). (**4) J region RS. (Arrows) Orientation of the PCR primers used. V2, V3, and V4 are pseudogenes.
of 51 (46%) joints were in-frame, indicating that little if any selection had occurred. We found 25 different types of joints (Fig. 6). As seen in other species, several of these joints contained one or two basepair P elements but none contained N nucleotide addition. However, only 4 out of 51 (8%) of these joints were of the type GAT TTT GGA that predominate in adult ducks (Fig. 6, arrow). Ignoring identical joints from a single PCR reaction essentially gives similar results. 14 out of 36 (39%) were in-frame and 4 out of 36 (11%) were of the type GAT TTT GGA. Thus, by either method of analysis it is clear that no single joint predominates at day 21.

The Predominant V11-J3 Joint Is Due to Selection. To determine how a predominant V11-J3 recombination joint arises, we next examined the relative numbers of individual V11-J3 joints at different times during development. To this end, we sequenced V11-J3 joints from days 21, 26, and 35 (hatch) bursa and spleen using PCR. The results are tabulated in Fig. 7. A clear increase in the presence of the GAT TTT GGA joint occurs throughout embryonic development. For example, at day 21, there were 25 types of joints in the spleen, whereas four types were found at day 26, and only two types were found at day 35. Concomitantly, the proportion of joints with GAT TTT GGA increased from 4/51 (8%) at day 21, to 19/28 (68%) at day 26, and to 16/17 (94%) at hatch. As we have previously described, most avian B cells undergo VJ rearrangement of only one of their IgL alleles (16). Thus, cells that produce an out-of-frame joint cannot proceed with further B cell development. As a result, it might be expected that an increase in GAT TTT GGA joints which are in-frame might
Figure 6. Nucleotide sequences of V₁<sub>L</sub>-J<sub>L</sub> joints from 21-d embryonic spleen. The nucleotide sequences of rearranged V₁<sub>L</sub>-J<sub>L</sub> genes are compared with the germline sequence. (Dashes) Identity to the germline sequence. Several clones have P nucleotides present at the coding joint, but no N nucleotides were observed. The other base pair changes are consistent with PCR incorporation errors. Rearranged IgL genes were cloned from the spleen of 21-d-old Muscovy duck embryos after PCR amplification. (n) Number of clones isolated with a particular V₁<sub>L</sub>-J<sub>L</sub> joint. (Right) Histogram showing the frequency of the joints as a percentage of total joints cloned. (+ -) Joint predominant in adult ducks.

result just from loss of cells with out-of-frame joints. However, developmental selection of the GAT TTT GGA is just as apparent when the data are expressed as a percentage of in-frame joints. The number of joints with GAT TTT GGA as a percentage of all in-frame joints in the spleen increased from 4/24 (17%) at day 21, to 19/26 (73%) at day 26, and to 16/16 (100%) at hatch. Essentially the same phenomenon was observed in the bursa.

Similar Selection Occurs in V₁<sub>S</sub>-J<sub>L</sub> Joints. We were interested in addressing whether this process of selection was limited to V₁<sub>L</sub> or if it also involved other functional V<sub>L</sub> gene rearrangements. Therefore, we sequenced V₁<sub>S</sub>-J<sub>L</sub> joints from the bursa and spleen of 4-wk-old Muscovy ducks. As shown in Fig. 8, two types of joints predominated. These two joints

Figure 7. Summary of V₁<sub>L</sub>-J<sub>L</sub> joints cloned from the bursa and spleen of Muscovy ducks at different developmental times. The number of GAT TTT GGA joints and in-frame joints are expressed as a proportion of the total or independent joints. Joints were characterized as independent on the basis of unique sequence with the V₁<sub>L</sub>-J<sub>L</sub> joint, distinct gene conversion tracts within the V₁<sub>L</sub> region, or derivation from separate PCR reactions.

Figure 8. Nucleotide sequences of in-frame V₁<sub>S</sub>-J<sub>L</sub> joints from bursa and spleen of 4-wk-old Muscovy ducks. (Dashes) Identity to the germline sequence. Together, the first two joints comprise 83% of all joints in this data set. The top sequence includes a P nucleotide within the coding joint.
together formed 83% of all in-frame joints in the 4-wk-old ducks. However, these two joints comprised only 16% of independent joints isolated from spleen cells at day 21 of development. Both of these joints code for the same amino acids and hence for the same protein product.

Discussion

The process of coding joint formation results in the generation of considerable diversity (3, 8). The junctional diversity created by V(D)J recombination occurs in the CDR3 of the TCR and Ig genes (1). Analysis of the crystal structure of Ig has shown that the CDR3 region forms a major portion of the antigen-binding pocket (8, 29). Thus, junctional diversity results in variations in the CDR3 region of the antigen-binding pocket of both Ig and, by analogy, TCR (9). Therefore, it was surprising when a number of examples of limitations in junctional diversity were reported. Limited junctional diversity has been observed primarily in the neonatal immunologic repertoire. The Ly-1 B cell lineage and y/5 T cell lineage, both of which develop before birth, have been shown to have V(D)J recombination products with restricted junctional diversity (14, 24, 30, 31). Gu et al. (24) proposed that sequence homologies present at the coding ends are utilized during V(D)J recombination to align the coding sequences before ligation. They suggested that V(D)J coding joint formation occurred in a manner similar to the mechanism of illegitimate recombination proposed by Roth and Wilson (32). In this model, illegitimate recombination of coding joints is initiated by exonuclease digestion of both 5' to 3' or both 3' to 5' strands of the coding ends followed by alignment of the single-strand protrusions. Joining of ends is then mediated by stretches of sequence homology. Gu et al. (24) noted that the coding joints of y/5 T cells or Ly-1 B cells observed early during ontogeny all involve potential alignment of the most appropriate sequence similarities at the ends of the recombining segments.

It has recently been noted that N segment addition is developmentally regulated with N segment addition beginning before birth and increasing until maturity in a variety of vertebrate species (13, 19-21). Based on these observations, several authors have suggested that homologous joining is a generalized rule for the formation of coding joints (14, 24, 25). They argue that the only reason junctional restriction is limited to neonatal cells is that with the increase in N segment addition observed after birth, the sequence homologies used for alignment are shifted to the N segments, and therefore, are not apparent in the final product. This suggests that the use of homology in the alignment of coding joints is not restricted to neonatal development but is a more general rule of V(D)J recombination. These observations have led to the widely held assumption that homology-mediated pairing provides an important means for the alignment of coding ends for coding joint formation.

During our studies of Muscovy duck B cell development, we came across an example of extreme junctional limitation. This restriction in junctional diversity appeared to be predicted by sequence similarities at the ends of the V11 and J1 coding sequences. Therefore, we designed experiments to test whether alignment of coding joints using regions of sequence similarity could account for restricted joint formation. Avian species are an excellent system in which to test this hypothesis. All the rules of avian V(D)J recombination established to date are the same as those of other vertebrate species. However, unlike mammals, studies in the chicken showed that V(D)J recombination is restricted to a very short developmental period during early embryogenesis, allowing us to separate events that occur during recombination from those that occur during subsequent B cell development (16). In the present studies, we were able to demonstrate that the duck has a similarly restricted period of V(D)J recombination during embryogenesis. Unlike other avian species, the Muscovy duck has five functional V gene segments. We have previously characterized two of these as a part of our initial identification of the Muscovy duck locus (28). One of these (V11) was noted to have sequence similarities to the J1 gene segment which were not observed in the other functional V1 region (V13). We reasoned that if a homology-based joining process is involved in V(D)J recombination, one would expect to observe a restricted set of coding joints in the case of V11, whereas coding joints containing V13 would be diverse at the time recombination is initiated. As documented in our results, this is not the case. Initial V(D)J recombination of V11 is random. Instead, B cells with Ig L chains encoded by V11 segments undergo developmental selection which rapidly restricts junctional diversity. This selection in V1J coding joints appears to occur in the spleen or during migration to the bursa of Fabricius as virtually all of the functional V11-J1 recombination events observed in the bursa are of the GAT TTT GGA type. Selection for the GAT TTT GGA joint occurs very rapidly during development. Although the GAT TTT GGA joint accounts for only 8% of V11 joints at day 21, the GAT TTT GGA joint accounts for 94% of the V11 joints at birth. V13-containing joints in neonatal birds also show limited junctional diversity. In this case, two distinct joints together account for 83% of joints observed in postnatal ducks. Although these two joints are different in position, they result in the same amino acid sequence in the CDR3 domain, suggesting that selection is occurring at the level of the encoded protein.

Based on our data, we conclude that homology plays no significant role in V(D)J recombination. The frequent occurrence of short sequence similarities in the coding ends of recombination events that display junctional limitation does not imply a causal role. Rather, we suggest that the sequence similarities have been selected during evolution to increase the likelihood of a particular protein being formed during an essentially random recombination process. Junctional restriction in the neonatal repertoire suggests that junctional selection occurs before exposure to external antigens. This selection appears to be based on protein sequences in the CDR3 domain of the Ig L chain and suggests that such selection may involve the antigen-binding pocket. This embryonic selection process is not restricted to avian B cells since Ig genes of neonatal murine B cells display similar junctional restriction (20, 24).
The immunologic repertoire of neonatal γ/δ T cells, neonatal Ly-1 B cells, and primary avian B cells have all been suggested to be directed against self-antigens (31, 33–35). Based on our studies, it seems possible that the development of these neonatal cell lineages requires selection by self-antigen in a manner analogous to the way α/β T cells undergo selection by self-encoded MHC molecules during thymic development (36, 37). This hypothesis is supported by several observations. The fact that murine dendritic epidermal T cell clones expressed identical rearranged γ and δ genes suggests that these cells undergo significant selection during neonatal development (15, 22). Similarly, Feeney (30) has shown that there is preferential development of a particular V, D, C, J, δ1 junctional sequence during the neonatal period.

Considerable evidence suggests that the B cells that develop during embryogenesis are long-lived cells which are not constantly regenerated from stem cell precursors (for review see reference 13). One mechanism for the maintenance of such cells is constant reselection by exposure to self-antigens. In contrast, in mammalian B cells that develop postnatally, N nucleotide addition during V(D)J recombination prevents creation of the recombination products that undergo selection during the prenatal period. Thus, B cells developing postnatally do not display junctional restriction in the absence of exogenous antigens. In the absence of antigenic stimulation, these adult B cells undergo fairly rapid turnover. This suggests that primary B cells with Ig receptors directed against external antigens must be constantly regenerated in the absence of intervening antigen exposure. Thus, the adult or non-Ly-1 B cells may represent an adaptation of the immune system to allow the generation of Ig responses to external antigens. In contrast, the neonatal B cell repertoire is generated in a manner which is dependent to some degree on the recognition of self-antigens. Potentially these self-antigens may be involved in the induction of stress responses, and thus, serve as signals to the primitive immune system to initiate an inflammatory response. Our data suggest that a search for the endogenous ligands of avian B cells, mammalian Ly-1 B cells, and γ/δ T cells may provide significant insights into our understanding of the processes that shape the neonatal immune repertoire.

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