Preferential Rearrangement of \( \text{VK}4 \) Gene Segments in Pre-B Cell Lines

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Summary

Examination of the in vitro \( \text{VK} \) gene rearrangements of murine adult bone marrow–derived pre-B cell lines reveals that 21 of 25 (84%) cell lines have rearranged a member of the \( \text{VK}4 \) family. In contrast, analysis of two \( \text{VK} \) cDNA libraries prepared from LPS-stimulated adult spleen cells indicates that only 17% of the \( \text{IgK} \) cDNAs contain sequences belonging to the \( \text{VK}4 \) family. Half of the pre-B cell lines examined also share an 8-kbp BamHI reciprocal product (rp). However, these rps do not involve the same \( \text{VK} \) gene, indicating that conserved BamHI sites exist 3' of some \( \text{VK} \) genes. This rp is also readily detected in DNA from normal adult spleen cells, suggesting that the in vitro rearrangements examined in this study are representative of \( \kappa \) rearrangements that occur in vivo. We suggest that, unlike the diverse \( \text{VK} \) repertoire expressed by mature B cells, the germline \( \text{VK} \) segments involved in initial rearrangements of the \( \text{IgK} \) locus are highly restricted, and that an initial \( \text{VK}4 \) rearrangement is probably followed by other, more random recombination events.

The development of the B cell repertoire relies on the rearrangement and expression of both heavy and light chain Ig variable region genes. These well-studied rearrangement events are the basis of the enormous diversity of antigen binding sites found among antibodies. Defining the numerous genetic and cellular processes and interactions that influence the somatic evolution of the primary B cell repertoire is fundamental to a full understanding of immunocompetence and the apparently programmed fashion in which the adult primary repertoire is formed (1).

Several groups have investigated the expression of Ig V region genes in adult and early B cell populations. It has been observed that B lineage cells derived from mouse fetal liver preferentially rearrange D-proximal \( \text{V}_\mu \) segments (2-4), and studies of human leukemic and fetal B cells have also demonstrated a nonrandom usage of \( \text{V}_\mu \) gene families (5-7). In contrast, the pattern of \( \text{V}_\mu \) gene expression among normal adult murine splenic B cells is a more faithful reflection of the germline library of \( \text{V}_\mu \) segments (8-11).

To gain insight into the mechanism and function of preferential V gene rearrangements, it is important to determine whether initial rearrangements on other Ig loci are also restricted to particular sets of V genes. Recent advances in the classification of mouse \( \text{VK} \) gene families (12, 13) have permitted several groups to begin to examine \( \text{VK} \) gene utilization in systems analogous to those used to study \( \text{V}_\mu \) genes (14-16).

The Ig \( \kappa \) light chain locus of the mouse is comprised of 100-300 variable and 4 functional joining region (\( \text{J}_\kappa \)) gene segments (reviewed in reference 17). By amino acid sequence criteria there are 24 known groups (18), and hybridization with V\( \kappa \) probes has defined at least 16 \( \text{VK} \) gene families, some of which encompass more than one of the previously described \( \text{VK} \) groups (12, 13).

In this study we have examined the \( \text{VK} \) genes involved in early rearrangement events that occur in A-MuLV-transformed pre-B cell lines during in vitro propagation. We show that, in contrast to the primary \( \text{VK} \) splenic B cell repertoire, most (21/25) cell lines have rearranged at least one member of the \( \text{VK}4 \) family. We suggest that initial \( \text{IgK} \) locus V gene rearrangements are highly restricted and that the B cell repertoire is likely to be significantly influenced by subsequent recombination events.

Materials and Methods

Cell Lines. Cell lines described in this study are A-MuLV–transformed pre-B cell lines derived from adult bone marrow. A total of 25 lines were analyzed, 9 of which are either primary or secondary subclones of the BALB/c cell line BM18-4. BM18-4 was kindly provided by Dr. Naomi Rosenberg (Tufts Medical School), and has been previously shown to rearrange its \( \kappa \) loci during propagation in vitro (19, 20). BM18-4 was cloned by plating the cells at a density of 0.3 cells/well in 96-well plates. Primary subclones K3, K6, K10, and K23 were further subcloned to obtain the secondary subclones used in this study. All plates contained fewer than 17 positive wells after 10 d. Cell lines were grown as described previously (21). The remaining 16 lines were each independently derived; 8 were obtained from a panel of CXXB cell lines of
cell lines derived from adult bone marrow were chosen for study based on the detection of a rearranged Igk locus, as demonstrated by Southern blot hybridization of a Cx probe to BamHI-digested DNA (Fig. 1). Six cell lines (K3-2, N4, N15, N20, M46, E5) have multiple rearranged bands of varying intensities, suggesting active rearrangement, while the remainder appear to have stably rearranged Igk loci.

A striking feature of the Igk rearrangements in these cell lines (Fig. 1, Table 1) is the frequent occurrence of a Cx-hybridizing 4.3-kbp BamHI fragment (14/25 cell lines). To determine whether these 4.3-kbp BamHI fragments contain independent rearrangements of the same or related germline Vk gene segments, the 4.3-kbp fragment was cloned from cell line K3-2 (Fig. 1). The 5’ region (490 bp) of this fragment was further subcloned and sequenced. A search against the Beckman Microgenie Sequence Analysis Program (Beckman Instruments, Inc., Palo Alto, CA) revealed that this region contained a member of the Vk4 family joined in frame to Jk5. The partial sequence of this Vk4 gene (codons 42–95) is identical to the published germline H4 Vk4 gene (23).

Southern Blot Analysis Reveals a Preferential Rearrangement of the Vk4 Family. Using the cloned Vk4 gene from cell line K3-2 as a hybridization probe for Southern blots of BglII-digested DNA (Fig. 2), the panel of cell lines was examined to determine if identical Vk4 rearrangements had occurred in other cell lines, and to determine the frequency of rearrangements involving Vk4 family members. This analysis revealed that 72% (18/25) of the lines had rearranged a member of the Vk4 family (Fig. 2, Table 1). In addition, a large number of the cell lines share a Vk4-hybridizing nongermline BglII fragment of 1.5 kbp (Fig. 2, Table 1). These 1.5-kbp fragments may reflect identical Vk4-Jk joins, since they contain Jk sequence as well (data not shown), or they may represent reciprocal products resulting from inversional VwJk rearrangements (24–26). Inversion of some Vk4 gene segments has been described previously (27), and inversional joins can result in rp containing adjacent, nonjoined Vx and Jk sequences in nongermline contexts. Therefore, to ascertain whether these fragments contain a reciprocal product or a VxJk join, the 1.5-kbp BglII fragment from cell line K6-22 (Fig. 2) was cloned and partially sequenced. An Alul fragment was found to contain codons 26–95 of a Vx gene identical in sequence to the germline R9 Vx4 gene (23), joined in frame to Jk5. This cloned Vx4 gene also shows a 91% nucleotide sequence identity with the rearranged Vx4 gene cloned from cell line K3-2 (discussed above). Thus, the rearranged Vx4 genes in cell lines K3-2 and K6-22 derived from distinct germline genes, indicating that more than one Vx4 gene was involved in the rearrangements observed.

As demonstrated in Fig. 2, one cell line (M46) has four detectable BglII Vk4 rearranged fragments. This feature may reflect the fact that this cell line was actively rearranging its kappa loci when the DNA was prepared. Another formal possibility is that some of these Vk4-containing fragments represent rp of Igk rearrangement. However, the excellent correlation between detectable rearranged Vk4 fragments and Vk4 RNA expression (Table 1 and discussed below), and the limited cloning and sequencing data presented here, suggest

Results and Discussion

A 4.3-kbp Igk Rearrangement Is Frequently Observed in A-MuLV-transformed Pre-B Cell Lines. 25 A-MuLV transformed

1 Abbreviations used in this paper: rp, reciprocal product.
that all or most of the V\(_{\kappa}\) rearrangements observed are V\(_{\kappa}\) to J\(_{\kappa}\) joins and not rp of rearrangement.

Blackwell et al. (28) have also recently suggested, and provided evidence for, the early and preferential rearrangement of the V\(_{\kappa}4\) family. Their recent investigation of I\(_{\kappa}\) locus rearrangements in A-MuLV transformed pre-B cell lines from C.B.17 scid mice showed that in the five signal joins (reciprocal products of inversionsal rearrangements) examined, the sequences immediately 3' of the V\(_{\kappa}\) nonamers had \(>80\%\) nucleotide sequence identity with the 3' flank of V\(_{\kappa}L8\), a member of the V\(_{\kappa}4\) family. (Blackwell et al. refer to V\(_{\kappa}L8\) as a V\(_{\kappa}5\) family member. Due to a high degree of nucleotide sequence identity the V\(_{\kappa}4\) and V\(_{\kappa}5\) families have previously been reclassified as one family [12]). In addition, a recent investigation (16) using hybridomas from fetal liver and day 1 neonatal liver, and subclones from an A-MuLV-transformed pre-B cell line, 18–81, indicated preferential usage of V\(_{\kappa}4\) as well, although with a lower frequency (32%) than was observed in our study. Although the basis for the lower frequency of V\(_{\kappa}4\) rearrangement by Lawler et al. is not known, the cell lines examined by them included hybridomas generated from fetal and neonatal tissues, sources of B cells not examined in our study, and which may represent B cells at a more mature stage of development. It is possible, therefore, that they may have missed some of the earliest recombination events. Also, the remaining cell lines all derived from one parental cell line and might not accurately reflect the repertoire of early \(\kappa\) locus rearrangements.

To determine if another family might also be highly represented in our panel of cell lines, the lines were screened for V\(_{\kappa}1\) rearrangement. Like V\(_{\kappa}4\), the V\(_{\kappa}1\) family is fairly complex with 12 fragments resolved on Southern blots of BamHI-digested DNA (the V\(_{\kappa}4\) probe hybridizes to 16 BamHI fragments). In contrast to the high frequency of V\(_{\kappa}4\) rearrangements, of 25 cell lines only 3 exhibited V\(_{\kappa}1\) rearrangements (K3-7, K3-9, M9). These three lines were also positive for V\(_{\kappa}4\) rearrangement.

**Analysis of RNA From A-MuLV-transformed Cell Lines also Indicates a Preferential Usage of the V\(_{\kappa}4\) Family.** Since the V\(_{\kappa}4\) probe detects 17 BglII restriction fragments on a Southern blot, it was possible that some BglII fragments were undetectable because of comigration with germline restriction fragments bearing V\(_{\kappa}4\) genes. Therefore, Northern blot analysis was performed using total RNAs from the panel of cell lines (data not shown), and it was revealed that three of the lines without a detectable V\(_{\kappa}4\) gene rearrangement express mature V\(_{\kappa}4\) mRNAs (K10, N4, E5). Furthermore, 11 of the 18 V\(_{\kappa}4\)-rearranged cell lines also express mature V\(_{\kappa}4\) mRNA transcripts (Table 1), verifying the use of a V\(_{\kappa}4\) family member by these lines. Six of the seven V\(_{\kappa}4\)-rearranged, V\(_{\kappa}4\) mRNA-negative cell lines (except one, K3-9), were negative for expression of the \(\kappa\) locus (Table 1), as determined by Northern blots hybridized with the \(\kappa\) probe (data not shown). Together, the Southern and Northern data show that 84% (21/25) of the cell lines have rearranged at least one V\(_{\kappa}4\) gene.

Although the present data clearly establish a preferential usage of the V\(_{\kappa}4\) family, the possibility of another family also being preferentially rearranged cannot be excluded. How-
Table 1. Summary of Igk Locus Rearrangements and RNA Expression in A-MuLV-transformed Cell Lines

| Cell line* | Detectable Vk4 rearrangements$ | Ck mRNA$ | Vk4 mRNA$ | 4.3-kbp BamHI Ck fragment$ | 1.5-kbp BglII Vk4 fragment$ | 8-kbp BamHI rp$ |
|------------|-----------------------------|----------|-----------|----------------------------|-----------------------------|----------------|
| K10        | -                           | +        | +         | -                          | -                           | -              |
| K10-13     | + (1)                       | +        | +         | -                          | -                           | -              |
| K3-2       | + (2)                       | +        | +         | -                          | +                           | -              |
| K3-5       | + (1)                       | +        | +         | -                          | +                           | -              |
| K3-7       | + (1)                       | +        | +         | -                          | -                           | +              |
| K3-9       | + (1)                       | +        | +         | -                          | -                           | +              |
| K3-10      | + (1)                       | +        | +         | -                          | +                           | +              |
| K6-22      | + (1)                       | +        | +         | -                          | +                           | +              |
| K23-4      | + (1)                       | +        | +         | -                          | +                           | +              |
| M2         | + (1)                       | -        | -         | -                          | +                           | -              |
| M9         | + (1)                       | -        | -         | +                          | -                           | -              |
| M41        | + (2)                       | -        | -         | +                          | -                           | +              |
| M46        | + (4)                       | +        | +         | +                          | +                           | +              |
| F1         | -                           | -        | -         | -                          | +                           | -              |
| F2         | + (1)                       | -        | -         | -                          | -                           | -              |
| F12        | + (1)                       | -        | -         | -                          | -                           | -              |
| F32        | + (2)                       | -        | -         | -                          | -                           | -              |
| P5         | + (1)                       | +        | +         | +                          | +                           | -              |
| P40        | + (1)                       | +        | +         | +                          | +                           | -              |
| N4         | -                           | +        | +         | +                          | -                           | +              |
| N14        | -                           | -        | -         | -                          | +                           | -              |
| N15        | -                           | +        | -         | +                          | -                           | +              |
| N20        | + (1)                       | +        | +         | +                          | +                           | -              |
| P55.D6     | -                           | -        | -         | -                          | +                           | -              |
| E5         | -                           | +        | +         | +                          | -                           | -              |
| Totals$    | 18 (24)                     | 16       | 14        | 14                         | 10                          | 12             |

* Groups of cell lines prefixed by different capital letters were derived from independent transformations of adult bone marrow cells.

$ The number of rearranged Vk4 gene segments was determined by Southern blot analysis of BglII-digested DNA using the Vk4 probe.

$ The expression of full-length Ck transcripts was determined by Northern blot analysis using the Ck probe.

$ The expression of full-length Vk transcripts of the Vk4 family was determined by Northern blot analysis using the Vk4 probe.

$ The presence of a 4.3-kbp BamHI fragment containing Ck sequence was determined by Southern blot analysis using the Ck probe.

$ The presence of a 1.5-kbp BglII fragment was determined by Southern blot analysis using the Vk4 probe.

$ Reciprocal products, resulting from k locus rearrangements, were identified on Southern blots of BamHI-digested DNA hybridized with a probe containing sequences 5' of germline Jk1 (pRL).

$ Number of positive cell lines.

Despite the remarkably high frequency of Vk4 gene rearrangements in our cell lines, we asked whether this preferential utilization of the Vk4 family influences the primary adult antibody repertoire. To address this, a Vk cDNA phage library was constructed using poly(A)$ mRNA from adult BALB/c spleen cells cultured for 3 d with LPS. The primary library contained 340,000 recombinant phage, and it was determined by plaque lift hybridization that 70% of the cDNAs were Ck+ and >6,000 Ck+ phage were screened using the Vk4 probe, and it was calculated that 17% of the cDNAs contain a Vk4 gene. Data from a second independent cDNA library confirmed these results. A recent study...
Figure 2. Vk4 rearrangement in A-MuLV-transformed pre-B cell lines. Southern blots of BgIII-digested DNA from A-MuLV-transformed cell lines were hybridized with the Vk4 probe. Arrows indicate Vk4 rearrangements.

by Teale and Morris (15) obtained similar results using in situ hybridization for both adult and neonatal Vk4 family usage, while Kaushik et al. (14) reported an even lower frequency for Vk4 usage using a B cell colony blot assay. Therefore, in contrast to our finding that 84% of the pre-B cell lines examined have a rearranged Vk4 gene on at least one chromosome, the frequency of Vk4 expression in normal splenic B cells appears more consistent with the relative proportion of Vk4 germline genes (12, 13).

An 8-kbp BamHI Reciprocal Product Is Frequently Found in Both A-MuLV-transformed Pre-B Cell Lines and Adult Splenic B Cells. Given the apparently nonrandom use of the Vk4 family, a family with members known to rearrange by inversion resulting in rp, we asked whether our panel of cell lines also share similar rp. All of the cell lines have undergone at least one inversional rearrangement as determined by the presence of nongermline BamHI fragments (reciprocal products) that hybridize to pRI (Fig. 3 B), a probe containing sequence 5' of the Jk region (data not shown). Furthermore, nearly half of the lines contained a reciprocal product located on BamHI fragments of the same size (8 kbp, Table 1). The presence of an rp was confirmed by cloning and sequencing a portion of this 8-kbp fragment from cell line K6-9. This rp was found to contain a back-to-back joining of the heptamer-nonamer signal sequences from Jk1 and a member of the Vk 12,13 family (Fig. 4), as determined by a 93% sequence identity with the published consensus sequence of K2 (29), a germ-line Vk 12,13 gene. This suggests that an additional rearrangement involving a Vk family other than Vk4 has occurred, and it is also, to our knowledge, the first evidence that a Vk 12,13 family member can rearrange by inversion.

If the frequent presence of 8-kbp BamHI rps in our cell lines is indicative of rearrangements that occur in vivo, then an 8-kbp BamHI rp should also be detected in normal tissue. Indeed, Southern blots of neonatal and adult spleen DNA revealed an 8-kbp BamHI fragment in adult spleen but not neonatal spleen or liver (Fig. 3 A). The absence of an 8-kbp rp in neonatal liver and spleen was most likely due to the small number of B cells present in neonatal tissues. The rearranged BamHI fragment detected in normal spleen was indistinguishable in size from the 8-kbp fragment frequently observed in our cell lines, for example K23-3 in Fig. 3 A. By densitometry, ~27% of the Igk loci from IgM-expressing spleen cells exhibited this 8-kbp rp (data not shown). Although we have no evidence regarding the nature of the 8-kbp rp in normal B cells, these data support the possibility that the in vitro rearrangements observed in pre-B cell lines reflect those occurring in vivo.

To determine if all of the 8-kbp BamHI fragments contain the identical rp, a 221-bp HaeII/XbaI fragment 3' of the Vk12,13 signal sequence (Fig. 4) was used to screen the panel of cell lines. Only one cell line (K6-22) other than K6-9, the cell line from which the rp was cloned, gave a positive signal in the 8-kbp region (data not shown). Therefore, the other 8-kbp rps are products of rearrangement events involving a Vk gene or genes different than in cell line K6-9.

Two other groups have also found 8-kbp BamHI rp-containing fragments, neither of which, however, involved
a V\(\kappa\)12,13 gene. Rather, one contained the 3' flank of a V\(\kappa\)10 gene (27), and the other contained the 3' flank of a V\(\kappa\)8 gene (Clarke, S., personal communication). Together, these findings suggest that V\(\kappa\) genes from at least four families contain conserved BamHI sites 3' of their coding regions, resulting in 8-kbp BamHI rpS upon inversional recombination. Whether these families include genes that are frequently involved in Igk locus rearrangements, secondary rearrangements in particular (discussed below), awaits further investigation.

Functionality Significance and Mechanism for the Preferential Usage of the V\(\kappa\)4 Family. The phenomenon of the preferential usage of V\(\kappa\) genes proximal to the D region, originally observed in A-MuLV-transformed cell lines, has recently been demonstrated for normal fetal tissue (4). Thus, it is possible that the preferential rearrangement of V\(\kappa\)4 genes will also be found to exist in vivo. What functional significance might a high frequency of V\(\kappa\)4 gene rearrangements have in developing cells? One possibility extends the suggestion by Shapiro and Weigert (27) that inversional rearrangements might provide a means by which more distal V\(\kappa\) gene segments can be repositioned closer to J\(\kappa\) segments, thereby increasing their probability for recombination. This speculation is consistent with the findings that multiple, sequential V\(\kappa\)4 rearrangements can occur on a single chromosome (26, 27, 30). Considering the size of the V\(\kappa\)4 family, its central position within the V\(\kappa\) region, and the ability of at least some V\(\kappa\)4 genes to rearrange by inversion, the V\(\kappa\)4 family may play a special role in bringing J\(\kappa\) segments and distal V\(\kappa\) genes together for recombination. The V\(\kappa\)4 family is not, however, the only family that could serve this function. Therefore, an additional factor, such as chromatin structure or accessibility (31) must be involved in creating this bias for V\(\kappa\)4.

Although the data presented support the idea of V\(\kappa\)4 recombination being used as a vehicle for subsequent random rearrangements of distal V\(\kappa\) families, it is not known that V\(\kappa\)4 recombination is actually occurring first in the process. However, since both V\(\kappa\)4 rearrangements that were cloned from cell lines in this report were in-frame rearrangements involving J\(\kappa\)5, and since at least some V\(\kappa\)4 genes rearrange by inversion, it is possible that an inversional recombination of a V\(\kappa\)4 gene to J\(\kappa\)5 (or J\(\kappa\)2-J\(\kappa\)4) is the primary event that occurs. Because this is an inversional event, J\(\kappa\)1-J\(\kappa\)4 are displaced on the chromosome rather than lost. Therefore, J\(\kappa\)1-J\(\kappa\)4 would remain available as substrates for recombination with proximal V\(\kappa\) genes resulting in subsequent rearrangements; however, these rearrangements would be nonfunctional given their distance from C\(\kappa\). In fact, as mentioned previously, evidence exists that indicates that more than one rearrangement can occur on a single allele, and there are documented cases of plasmacytomas that contain double recombination products, products that result from more than one rearrangement occurring on the same allele (26). These subsequent rearrangements could result in the deletion of the initial rp generated from the primary recombination event as well as subsequent rps and VJ rearrangements. This has already been proposed as an explanation for plasmacytomas that lack reciprocity between the rp identified and the expressed V\(\kappa\) gene (26, 27).
The mechanism behind the preferential usage of the Vk4 family remains unclear. Proximity to J, which has been suggested to play a role in preferential Vn gene usage in A-MuLV-transformed pre-B cells (2, 3), does not appear to be correlated with preferential Vk4 rearrangement. Current data on the organization of the Igk locus (12, 13) position several Vx families between Vk4 and Jk. However, interspersion of Vx and Vn gene families has been well documented (21, 32, 33), and it is possible that a subset of the Vx4 family may be more proximal to Jk and that these are the rearranged genes being observed. Furthermore, the relatively large size of the Vx4 family may contribute to its frequent rearrangement, but it is unlikely the sole contributing factor since Vx4 genes probably represent no more than 10–20% of the Vx segments in the Igk locus (12, 13). An intriguing possibility, recently suggested by Tutter and Riblet (34) for the Igk-V locus, is that certain Vx-gene sequences are evolutionally conserved to maintain noncoding functions; for example, recombination. Thus, Vx4 sequences may intrinsically be highly recombinogenic.

As with the preferential rearrangement of particular Vn genes in mouse (2, 3) and human (5–7), the functional significance and mechanism of preferential Vk4 rearrangements is unknown. Understanding the factors responsible for the biased expression of a particular V gene is especially important when considering certain disease states, such as chronic lymphocytic leukemia in which there is a high expression of one particular x light chain gene (35, 36). In addition, there remains the question of how the primary B cell Vx and Vn repertoires become more representative of the inherited Vx gene segments, thereby allowing the considerable germline complexity of these loci to be utilized. Specifically, it is unclear what prevents the Vk4 family from dominating the adult splenic Vx repertoire. One possibility is that Vk4-rearranged pre-B cells are unable to escape from the bone marrow into the periphery, or once in the periphery the cells may undergo gene replacement. When considering the antibody pool, it is conceivable that Vk4 protein exhibits poor Vn chain pairing, thereby thwarting an overrepresentation of Vk4-bearing antibody. Undoubtedly, the regulatory process responsible for the formation of the Vx gene repertoire will involve numerous components at various stages of development.

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