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Mouse κ light-chain recombination signal sequences mediate recombination more frequently than do those of λ light chain

(immunoglobulin gene rearrangement/isotype exclusion)

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ABSTRACT **Immunoglobulin and T-cell receptor genes are somatically rearranged by site-specific recombination. Recombination signal sequences (RSS) have been identified as the major targeting element of this process. Recent reports demonstrate that differences in RSS affect the frequency of recombination, suggesting a role for RSS in the development of the B-cell repertoire. Examination of mouse light-chain RSS indicates that κ light-chain RSS consistently show a greater degree of similarity to a consensus sequence than do those of λ light chain. To determine whether this difference in natural RSS could affect the patterns of light-chain gene rearrangement and expression, we have constructed recombination substrates containing both a typical mouse κ RSS pair and a typical mouse λ RSS pair. Experiments using these substrates demonstrate that the κ RSS pair mediates recombination at a vastly higher frequency than does the λ RSS pair. This result argues that RSS differences may contribute significantly to the patterns of mouse immunoglobulin light-chain rearrangement, ultimately resulting in a high proportion of κ light chain relative to λ.**

Recombination signal sequences (RSS), the family of sequences that target immunoglobulin gene rearrangement (1), have been described by consensus comparison (2–4) as well as more recent functional studies (3, 4). One study indicates that no member of the RSS family mediates recombination at a frequency higher than that of the consensus. Substitutions in the most strongly conserved positions appear to have the most critical effects, and the effects of multiple nonconsensus substitutions appear to be cumulative (4). It seems likely that the differences in natural RSS influence rearrangement to their respective gene segments. RSS may thus have an as yet unanticipated role in the establishment of patterns of rearrangement during lymphoid development. In this report we present evidence that the patterns of light-chain rearrangement in mouse B-cell development, which establish both isotype exclusion and the ratio of expression of κ light chain to λ light chain, can be ascribed to differences in their RSS.

The immunoglobulin κ light-chain isotype and λ light-chain isotype are encoded by separate loci. Mouse κ gene-segment RSS and λ gene-segment RSS differ significantly in the degree of their similarity to the consensus RSS (Fig. 1). The majority of reported mouse κ variable (V)-segment and joining (J)-segment RSS have zero or one nonconsensus nucleotides, while all reported mouse λ V and J RSS have three nonconsensus nucleotides each.

Only one light chain is expressed on the surface of a mature B cell (5). This "isotype exclusion" occurs despite the existence of κ and λ isotypes as well as three λ subtypes. B cells expressing the κ light chain usually do not have λ rearrangements, whereas B cells expressing the λ light chain have usually exhaustively rearranged their κ loci (aberrantly rearranged, or deleted, the constant-region gene; refs. 6 and 7). There are also ≈20-fold more κ-expressing B cells than λ-expressing B cells in the mouse, and this ratio appears to remain constant through the life of the mouse (8, 9). These observations suggest that λ rearrangement may obligatorily follow κ rearrangement (7, 10). An alternative possibility is that the rearrangements are simultaneous and independent; however, rearrangements occur much less frequently at the λ locus than at the κ locus (6, 11–13).

Differences in the frequency at which the mouse light-chain loci rearrange could be established by sensitivity of the recombination machinery to the differences in light-chain RSS. In this paper we compare the frequency at which a typical mouse κ light-chain RSS pair mediates recombination with the frequency at which a typical mouse λ light-chain RSS pair mediates recombination. We chose a V_κ segment with a perfect consensus (V_κL8 modified as in ref. 4) and J_κ1 because they are typical of other κ RSS and allow for more direct comparison of our results with those from ref. 4. The λ RSS pair used was V_λ1 and J_λ1, both of which have three nonconsensus substitutions. This pair was chosen because the V_λ1/J_λ1 light chain is the most commonly found mouse λ light chain and thus should have the strongest λ RSS. We report here that a typical κ RSS pair mediates recombination at a much higher frequency than a typical λ RSS pair.

MATERIALS AND METHODS

Cell Lines. The cell lines used were pre-B cells generated by Abelson murine leukemia virus (Ab-MuLV) transformation. Ab-MuLV cell lines have been frequently used as a source of lymphoid "recombinase" activity (3, 4) and are known to rearrange their immunoglobulin loci in culture (14–16). Three BALB/c mouse-derived Ab-MuLV lines were used as recombination-competent hosts. PD31 (gift of D. Baltimore, Rockefeller University) is an Ab-MuLV line known to undergo κ rearrangement in culture, but there is no evidence of λ rearrangement (15). ABC1.1 and ABC1.2 are two subclones of ABC1 (gift of E. Selsing, Brandeis University), an Ab-MuLV line known to be capable of both κ and λ rearrangement in culture (16). All Ab-MuLV lines were cultured as described (14). The hybridoma SP2/0 (gift of M. Shulman, University of Toronto) was used as a non-pre-B-cell control.

Extrachromosomal V(D)J Recombination Substrates. Transiently transfected recombination substrates (similar to those described in this paper) have previously been shown to reliably assay many aspects of lymphoid V(D)J rearrangement (4, 17–19). The substrates are introduced into Ab-MuLV cell lines by electroporation (Bio-Rad) (20) and incu-

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Abbreviations: RSS, recombination signal sequence(s); Ab-MuLV, Abelson murine leukemia virus; CAT, chloramphenicol acetyltransferase.

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                        Mouse Lambda V RSS
                                                          #of consensus
Consensus CACAGTG xxxxxxxxxxxxxxxxxxxxxx ACAAAAACC  substitutions
       V1 ----a-- acatgtgtagatggggaagtaga ----g---a         3
       V2 ----a-- acatgtgtagatggggaagtaga ----g---a         3

                        Mouse Lambda J RSS

Consensus GGTTTTTGT xxxxxxxxxxxx CACTGTG
       J3 -----ag-g ttgggtttcagt -------                   3
       J1 -t------c atgagtctatat ---a---                   3
       J2 ------g-g ttgggttttagt --t----                   3

                        Mouse Kappa V RSS

Consensus CACAGTG xxxxxxxxxxxx ACAAAAACC
          ------- attcaagccatg ---t-----                   1
          ------- atacaaatcata ---t-----                   1
          ------- atacaaatcaca ---t-----                   1
          ------- agacaagtcata ---t-----                   1
          ------- atgcagaccata g------t-                   2
          ------- atagagccctga ---------                   0
          ------- ctacagactgga --------a                   1
          ------- cttcagcctcct ---c-----                   1
          ------- cttcagcctcct ---c-----                   1
          ----t-- atactgcactga ---------                   1
          ----t-- atactgcactgg ---------                   1
          ------- atacaaacctga ---------                   0
          ---g--- atacagccctga ---------                   1
          ------- atacagccctga --------a                   1
          ------- atacagacccta -------ta                   2
          ------- atgcagacccta --------a                   1
          ------- ctacatactgaa --------a                   1
          ------- ctccagggctga ---------                   0
          ------- ctccagggctga ---------                   0
          ------- ctccagggctga ---------                   0
          ------- atagagccctga ---------                   0
          ------- atgcagccctga ---------                   0
          ------- atacagccctga --------a                   1


                        Mouse Kappa J RSS

Consensus GGTTTTTGT xxxxxxxxxxxxxxxxxxxxxx CACTGTG
       J1 --------- acagccagacagtggagtactac -------         0
       J2 a-------- atgggggttgagtgaagggacac -------         1
       J4 --------- aaaggggggcgcagtgatatgaat -------        0
       J5 --------- agagaggggcatgtcatagtcct -------         0
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FIG. 1. Alignment of reported mouse κ and λ RSS. Sequences are from GenBank version 60.0.

bated for 48 hr. Low molecular weight DNA is harvested by an alkaline "miniprep" procedure and introduced into bacteria (SURE, Stratagene) by electroporation (21). Recombinants are selected on plates containing ampicillin (100 μg/ml) and chloramphenicol (50 μg/ml), as substrate recombination at RSS sites allows the substrate to confer chloramphenicol resistance in bacteria.

Plasmids. The recombination substrates were constructed with both a κ and a λ RSS pair in the same vector, in order to directly compare RSS site preference (described in Fig. 2). RSS-mediated recombination of the vectors in the eukaryotic host causes a deletion that confers chloramphenicol resistance in bacteria. The deletion removes tandemly inserted prokaryotic transcriptional terminators, including λ oop (gift of J. Greenblatt, University of Toronto) and the bacterial *rrnb* T1 and T2 terminators (excised from pKK233, Pharmacia). This deletion results in the juxtaposition of the *tac* promoter, P_{tac} (from pDR540, Pharmacia), promoter adjacent to a chloramphenicol acetyltransferase (CAT) gene (from pSV2cat, Pharmacia), allowing subsequent prokaryotic expression of the CAT gene. We constructed the vectors such that only recombination by deletion between a cognate RSS pair (e.g., V_κ to J_κ) will confer chloramphenicol resistance, and recombination of one pair excludes the possibility of recombination of the other pair. RSS were constructed by renaturation of synthetically produced oligonucleotides of both senses (sequences are shown 5′ to 3′): $J_\kappa 1$ (CACAGTGGTAGTACTCCACTGTCTGGCTGTACAAAAACC); $J_\lambda 1$ (CACTGTGATATAGACTCATGCAAAAAAC); V_κ (GGTTTTTGTTCCAGTCTGTAGCACTGTG); and $V_\lambda 1$ (TGTTCTTGTTCTACTTCCCCATCTACACATGTCATTGTG). The oligonucleotides also contained restriction endonuclease site-compatible ends to aid in cloning. Only the previously described elements of the RSS (the heptamer, spacer, and nonamer, but no flanking sequences) were included.

The vectors were based on a pUC19 (Pharmacia) variant with a deletion spanning the P_{lac} promoter. The polyoma early region was derived from pJH201, a gift of M. Gellert (National Institutes of Health).

To test whether vector context of the RSS might contain unanticipated elements that could influence recombination frequency, two vectors, with the RSS contextual locations reversed, were constructed (Fig. 2). pDR$\kappa\lambda$ was assembled by ligation of the previously described segments using compatible linking restrictions sites, resulting in the configuration

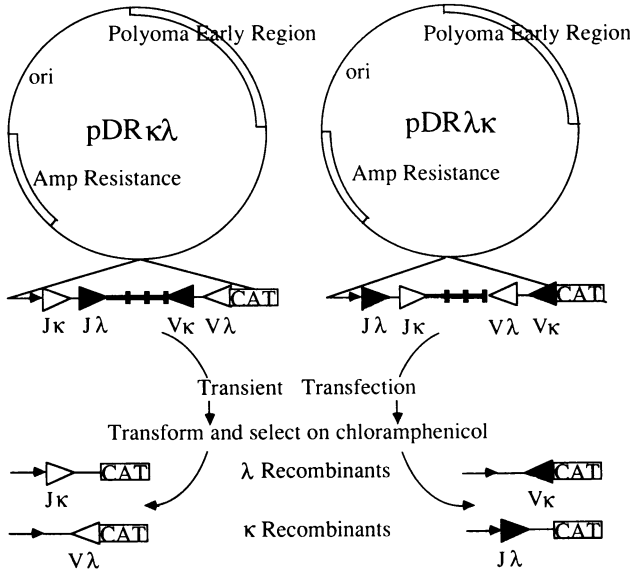

FIG. 2. Recombination substrates. Amp, ampicillin; ori, origin; arrow, P_{tac} promoter; line with vertical bars, oop T1T2 terminators; CAT, CAT gene without promoter; filled triangle, RSS with 12-base-pair spacer; open triangle, RSS with 23-bp spacer.

*P_{tac}–Bam*HI–J_κ1–*Bgl* II–*Mlu* I–J_λ1–*Bgl* II–*Mlu* I–oop–T1T2–*Xho* I–V_κ–*Sal* I–*Eco*RV–V_λ1–*Sal* I–*Eco*RV–CAT. pDRλκ was assembled by excising the RSS and religating them in the reverse order, also through the use of compatible restriction endonuclease sites. Changes introduced by this manipulation were then reversed through site-directed mutagenesis, resulting in the configuration *P_{tac}–Bam*HI–J_λ1–*Bgl* II–*Mlu* I–J_κ1–*Bgl* II–*Mlu* I–oop–T1T2–*Xho* I–V_λ1–*Sal* I–*Eco*RV–V_κ–*Sal* I–*Eco*RV–CAT. The two substrates were then sequenced, confirming that they were indeed identical in all aspects except RSS order.

Screening of Recombinants. Recombination between a cognate RSS pair deletes one member of the other cognate pair; thus recombinations are mutually exclusive and can be identified by the presence of a single remaining RSS (see Fig. 2). κ recombinants have a λ RSS remaining. In the case of pDRκλ, it is V_λ1, while in the case of pDRλκ, it is J_λ1. Conversely, λ recombinants retain a κ RSS. In the case of pDRκλ, it is J_κ1, while in the case of pDRλκ, it is V_κ.

Chloramphenicol-resistant colonies were first transferred by toothpick to a fresh plate and regrown, after which *in situ* lysis and nucleic acid fixing to nitrocellulose circles was carried out by standard techniques. Oligonucleotides specific to different RSS consisted of 15-mers derived from the RSS spacers. They were labeled by terminal deoxyribonucleotidyltransferase tailing with digoxigenin-labeled dUTP (Boehringer Mannheim). The nitrocellulose circles were then hybridized with these nonradioactive oligonucleotide probes, washed, and developed using a colorimetric method (see Fig. 3) according to the manufacturer's instructions. The four probes were used sequentially without intervening stripping of the filters. The oligonucleotide probes and the order in which they were used were as follows: (*i*) V_κ, CTACAGACTGGAACA; (*ii*) J_κ1, TGTAGATGGGGAAGT; (*iii*) V_λ1, TGGTAGTACTCCACT; (*iv*) J_λ1, GATATAGACTCATGC.

RESULTS

Characterization of the Substrates. We first determined that the recombination substrates used in our experiments function in a manner similar to those reported (17, 18). The parent, unrecombined substrates failed to form chloramphenicol-resistant colonies, while examples of chloramphenicol-resistant recombinant plasmids demonstrated plating efficiencies of ≈95% on chloramphenicol plates. No chloramphenicol-resistant substrates were recovered after transfection into SP2/0, a mature B-cell hybridoma. Approximately 3% of the replicated (as determined by insensitivity to *Dpn* I; see ref. 18) substrate recovered after transfection into the PD31 Ab-MuLV host, however, conferred resistance to chloramphenicol. Taken together, these results confirm the suitability of these substrates for the reliable detection of lymphoid V(D)J recombination; that is, the substrates acquired the ability to confer chloramphenicol resistance at a significant frequency only after transfection into a pre-B-cell line.

Screening of randomly picked chloramphenicol-resistant colonies did indicate a source of false positives, however. Three of 48 randomly picked chloramphenicol-resistant colonies (derived from a transfection into PD31) contained some contaminating unrecombined plasmid in addition to a recombined plasmid (as detected by restriction digest and Southern blotting) and were thus positive with all four RSS probes. All chloramphenicol-resistant colonies initially positive with the first probe were therefore rescreened in parallel with the other three probes. In this manner, colonies positive with all four probes (containing contaminating unrecombined plasmid) could be eliminated as false positives. No chloramphenicol-resistant colonies screened with either of the two more exhaustive methods were positive with zero, two, or three RSS. The absence of recombinants retaining zero or two remaining RSS argues against frequent "illegitimate" recombination between noncognate RSS (e.g., J_κ to V_λ or J_λ to V_κ). An example of the raw data, including two false positives, is shown in Fig. 3.

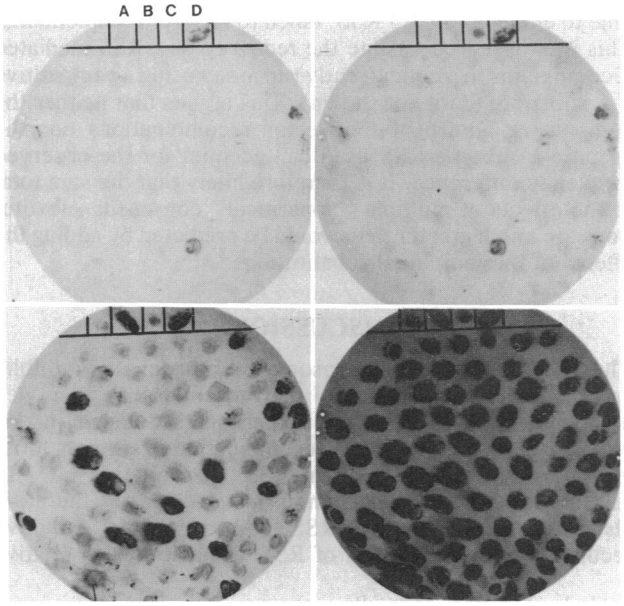

FIG. 3. Sample colony lift and hybridization. A colony lift was hybridized with digoxigenin-dUTP-tailed probes, in the following order: probe 1, V_κ spacer probe (*Upper Left*); probe 2, J_κ spacer probe (*Upper Right*); probe 3, V_λ spacer probe (*Lower Left*); and probe 4, J_λ spacer probe (*Lower Right*). The filters were not stripped between hybridizations. The top four colonies are controls: A contains a κ RSS-mediated recombinant from pDRκλ, B contains a K RSS-mediated recombinant from pDRλκ, C contains a λ RSS-mediated recombinant from pDRκλ, and D contains the parent vector. A should hybridize with probe 4 only, B with probe 3 only, and C with probe 2 only. D hybridizes with all four probes. Note that the two colonies positive in all four images of the filter were revealed as positive for all four probes in a separate screening and thus were eliminated from the analysis of data.

Comparison of Light-Chain Isotype RSS Recombination Frequencies. Results of the comparison of κ and λ RSS-mediated recombination in transfections of pDRκλ and pDRλκ into PD31, ABC1.1, and ABC1.2 are summarized in Table 1. λ RSS-mediated recombination occurred at a substantially lower frequency than κ RSS-mediated recombination: In >700 recovered recombinants, only 1 was mediated by a λ RSS pair. This RSS-mediated effect supersedes effects of both vector context and recipient cell line.

Several chloramphenicol-resistant recombinant vectors from both κ and λ RSS-mediated events were sequenced to determine their structure; the recombination junctions were similar to those demonstrated for other lymphoid recombination-mediated events. Restriction enzyme digestions of 45 recombinant structures further confirmed this conclusion (data not shown). It is unlikely that the basis for low recovery of λ RSS-mediated recombinant plasmids involved a preferential loss during recovery and selection, as the ability of a λ RSS-mediated recombinant to transform and confer chloramphenicol resistance was not significantly different from that of a κ RSS-mediated recombinant (data not shown). Moreover, an earlier assay using lower concentrations of chloramphenicol suggested a similar paucity of λ RSS-mediated recombination, despite frequent "breakthrough colonies" containing unrecombined "parent" vector.

Studies by Gellert and coworkers (4) on the effects of nonconsensus RSS substitutions on recombination frequency suggested that the positions where $V_\lambda 1$ and $J_\lambda 1$ RSS differed from the consensus were not individually "critical" for recombination activity. Nevertheless, our findings demonstrate that the cumulative effects of differences between the λ and κ RSS pairs seem to be profound. An experiment using a vector containing only λ RSS, where $V_\lambda 1$ can recombine to either $J_\lambda 1$ or $J_\lambda 3$ RSS, failed to recover recombinants. This was observed despite the recovery of κ RSS-mediated recombinants in a concurrently run assay using a competitive κ/λ substrate (data not shown). This argues that neither the competitive nature of the vector recombinations nor the specific λ subtype RSS used can account for the observed frequency difference. It is therefore likely that the sum total of the effects of multiple "noncritical" consensus substitutions are much greater than would be predicted by adding the effects of the individual substitutions.

DISCUSSION

The ability of extrachromosomal substrates to faithfully assay many aspects of V(D)J rearrangement has been well documented (4, 17–19). The experiments detailed in this paper suggest that substrate recombination is strongly influenced by different light-chain RSS. The relative rates of rearrangements at the endogenous κ and λ loci may therefore also be determined by their RSS. The comparison of relative recombination frequencies of RSS in substrates to relative recombination frequencies of endogenous gene segments with the same RSS is supported by the following examples. Some pseudogene segments rarely, if ever, recombine; examination of these pseudogene segment RSS reveals that they have substitutions in specific positions that are also critical for recombination when assayed in substrates (e.g. $J_\kappa 3$, $J_\lambda 4$; GenBank and ref. 4). The sheep λ repertoire consists of two to three major rearrangements, despite the existence of ≈25 V_λ gene segments (22). The gene segments that are not rearranged often have RSS substitutions similar to those that impair recombination in extrachromosomal substrates (4).

Table 1. Summary of results

Substrate	Cell line	No. of recombinants		% λ/κ
		λ type	κ type	
pDRκλ	PD31	1	310	0–1.3
	ABC1.1	0	170	0–4
	ABC1.2	0	131	0–4
pDRλκ	PD31	0	89	0–5
	ABC1.1	0	36	0–10
	ABC1.2	0	59	0–7

Substrates are described in Fig. 2. Recombinant, chloramphenicol-resistant colonies were classified by the RSS pair that mediated the recombination. % λ/κ is expressed in 95% confidence intervals.

As described above, κ-expressing cells generally retain the λ loci in a germ-line configuration, whereas in λ-expressing cells, the κ loci are generally rearranged (6, 7). The "stochastic" model argues that rearrangement of both loci is simultaneous but that rearrangement of the κ locus is much more frequent than rearrangement of the λ locus. Light-chain rearrangement is then inhibited by expression of productive light chain (of either isotype) in association with heavy chain on the surface, and isotype exclusion is established (11–13). This model is consistent with the lack of a distinguishable order of light-chain commitment in lymphopoiesis (9, 23). Recent reports of the isolation of λ-rearranged mouse B cells without exhaustively rearranged κ loci also provide evidence that κ rearrangement is not a necessary precondition for λ rearrangement (24, 25). Lastly, an extensive study of λ rearrangement patterns in λ-expressing mouse hybridomas has prompted the argument that patterns of λ subtypic rearrangement and exclusion, at least, are stochastic (26). We have demonstrated that a typical mouse κ RSS pair recombines much more frequently than a typical mouse λ RSS pair, a result that is consistent with a stochastic model of light-chain rearrangement.

The ratio of κ expression to λ expression in humans and sheep is much different than that of the mouse: both humans and sheep have a much higher proportion of λ expression (27). Human and sheep λ RSS also exhibit much greater similarity to the consensus than mouse λ RSS (GenBank version 60.0 and ref. 28), suggesting that the frequency of their λ locus rearrangement would be correspondingly higher than that of the mouse λ locus. This is consistent with a hypothesis that the major factor influencing the ratio of κ light chain to λ light chain is the frequency at which their respective RSS mediate recombination. A corollary to this hypothesis can be found in the example of human J_λ gene segments, where only $J_\lambda 2$ and $J_\lambda 3$ have perfect consensus RSS (28); these two J_λ segments are also the most commonly found in human λ hybridomas (29).

Humans retain, however, a pattern of light-chain gene rearrangement similar to that of the mouse, in that κ-expressing cells usually have a germ-line λ locus, while λ-expressing cells usually have rearranged κ loci, a fact that is difficult to reconcile with a strictly stochastic model of light-chain rearrangement. An *in vitro* model of human B-cell differentiation does demonstrate simultaneous onset of light-chain expression, however, arguing against ordered rearrangement (23). These apparently conflicting observations can be reconciled by evidence that further light-chain rearrangement is not always arrested in culture following transformation (10, 24, 30, 31), or even in untransformed cells after expression of surface immunoglobulin (32, 33).

Recombination at immunoglobulin and T-cell receptor loci is likely to be influenced by many factors, including transcription and accessibility (34–37). The results presented in this paper introduce RSS as a previously underestimated factor affecting recombination frequency. Indeed, the magnitude of the effect that differences in light-chain RSS have on recombination suggests that RSS may be the principal factor influencing mouse light-chain gene rearrangement patterns in B-cell development.

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