

EVIDENCE INDICATING INDEPENDENT ASSORTMENT OF  
FRAMEWORK AND COMPLEMENTARITY-DETERMINING  
SEGMENTS OF THE VARIABLE REGIONS OF  
RABBIT LIGHT CHAINS

Delineation of a Possible J Minigene\*

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The variable (V)<sup>1</sup> regions of immunoglobulin light chains when aligned for maximum homology can be divided into four framework regions (FR) separated by three complementarity-determining (CDR) (hypervariable [1]) regions or segments (2, 3). The latter as predicted (1), together with the corresponding three CDR of the heavy chain (4), form the antibody-combining sites (3-11). Light chains FR1, FR2, FR3, and FR4 comprise residues 1-23, 35-49, 57-88, and 98-107 and CDR1, CDR2, and CDR3 residues 24-34, 50-56, and 89-97. If the FR segments were grouped into sets of identical sequence and the members of each set were traced, it was shown (12) that members of a given FR1 set could be associated with different FR2, FR3, and FR4 sets. This independent assortment suggested that the FR sets, and by implication the CDR sets, were under different genetic control, and the hypothesis was put forward that the individual FR and CDR sets were controlled by minigenes assembled somatically by recombination at the DNA level (12). A minigene is defined as a segment of DNA coding for a portion of a domain and which shows evidence of segregation as a functional unit independent of the rest of the DNA coding for the V region (13). Because we only assorted FR segments, the findings would be independent of whether one or two residues of a given CDR assorted with any FR segment. Studies by Tonegawa et al. with cloned mouse V<sub>λ</sub> (14, 15) and V<sub>κ</sub> (16) genes and by Seidman et al. (17, 18) with mouse V<sub>κ</sub> genes showed that in 12-d-old embryo DNA, genes

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<sup>1</sup> Abbreviations used in this paper: CDR, complementarity-determining regions; FR, framework region(s); V, variable.

coding for residues 1-95 of the V region were followed by an intervening sequence. Other genes for the mouse  $\lambda$ -region coded for residues 96-108, termed the J segment and were followed by an intervening sequence of about 1.2 Kb and then by the  $C_\lambda$  coding segment (15). In mouse  $V_\kappa$  chains (19, 20), a clone coding for  $C_\kappa$  contained five J segments, each separated by an intervening sequence that varied in length from 246 to 310 base pairs and with an intervening sequence of ~2.5 Kb between the J closest to C and the C region. Thus, as defined, the J segments are clearly minigenes (12, 13).

The amino acid (21) and nucleic acid (22, 23) sequence data on the hinge region also show that these nucleotides code only for a portion of a domain and therefore constitute a minigene. It has been suggested (22, 23) that it evolved from a complete domain by a shift of a splice site followed by mutational divergence of the left end of the domain to become a portion of an intervening sequence with preservation of considerable homology in nucleotide sequence with the 5' flanking end of the  $C_{H1}$  domain.

The assortment principle was also used by Weigert et al. (24) to define the J segment from amino acid sequences with 96 as the site of the recombination, e.g., including two residues of CDR3 in J. They proposed that some diversity of antibody-combining sites could be generated by V-J joining because residue 96 is highly hypervariable (1, 2, 25). Because adult myeloma  $V_L$  DNA clones code for a contiguous stretch of amino acids 1-107 (or 108) (26), the joining of the J minigene to the DNA of the region coding for amino acids 1-95 has taken place during embryogenesis.

Because it was subsequently shown (27) that the five clones coding for  $V_\kappa$  and the three coding for  $V_\lambda$  all had segments matching at from 6/7 to 9/10 nucleotides plus the Trp codon at position 35, which could serve as recognition sites for recombination or insertion of CDR nucleotides between the nucleotides coding for the FR segments and for the CDR3-J junction, it was considered of interest to examine the amino acid sequences of rabbit  $V_\kappa$  chains for evidence of independent assortment of FR and CDR segments and to see if a J segment in the rabbit could be defined by assorting residues 96 and 97 of CDR3 with FR4 as has been found for the mouse.

Assorting rabbit  $V_\kappa$  sequences may have certain potential advantages in that the rabbit populations are not inbred and in that most of the sequencing has been done on antibodies with specificities largely limited to several pneumococcal type-specific and to streptococcal group-specific polysaccharides. It thus might be possible to recognize assortments associated with or not associated with certain antibody specificities in FR and CDR segments. There are also very many complete rabbit  $V_\kappa$  sequences, and yet the degree of sequence variation even among antibodies to the same antigen has not made it possible to define individual amino acid residues as contacting amino acids. Moreover, the sequence data included one pair of rabbits, K9-335 and K9-338, littermates (28, 29) producing monoclonal antibody to streptococcal group A variant carbohydrate whose light chains were identical in sequence from amino acids 1 to 108 and a second pair of completely unrelated rabbits (Ch. Brandt and J. C. Jaton. Personal communication.), 311 and 4422, one bred in the United States and the other in Switzerland; both had produced monoclonal antibodies to type II pneumococcal polysaccharide whose light chains also were identical in sequence from amino acids 1-108 (30).

The data provide considerable evidence for independent assortment of FR and CDR segments and, as also concluded by Braun et al. (31), indicate that the J segment

in the rabbit as in the mouse may include two residues of CDR3 and may contribute to the generation of antibody diversity.

### Materials and Methods

All rabbit  $V_{\kappa}$  sequences were contained in *Sequences of Immunoglobulin Chains* (25); they represent the work of numerous groups of investigators and original references may be found in (25). Sequences that have not been determined for an entire FR segment were generally not used except when an invariant Cys 23 was lacking, and the sequences fell into an FR or CDR set with other members. All chains with the identical sequence for an FR or CDR segment were grouped together as a set. Members of a set are enclosed in a box. The prototype sequence was K4820 and was selected from the set with the most members of FR2, the most commonly occurring preserved segment found to date in 1 human  $V_{\kappa}$  IV, 20 mice (6 BALB/c and 14 NZB), and 13 rabbit sequences (12, 13). Above the prototype set are listed all amino acid positions and residues at which the other sets differed from the prototype (32). Above all other sets are listed only the positions and amino acid residues at which the set differed from the prototype. It is thus easy to see which residues in each set differed from the prototype and also by comparing any two sets to see at which positions they differ from one another (32). If the amino acid in any set differed from the prototype at a given position and if this involved a two base change, this is indicated by an asterisk (\*). If the differences involved deletions or insertions or when a three base change was involved this is indicated by a dagger (†). To define a potential J segment and because of length variations in CDR3, the last two residues of CDR3 in each sequence were renumbered as 97E and 97F and assorted with FR4 as a J segment (97E, 97F-107) comparable with what had been observed in the mouse by assortment (24) and by nucleic acid sequencing of clones (26). In rabbit 2717 residue 106A had been misaligned and was made residue 107. This homologized all subsequent residues. It should be noted that FR4 is identical for all rabbit light chains except for K16-167 in which Thr replaces Gly at position 100. Chains that have not been sequenced completely are given in parenthesis with the number of unsequenced positions. Above the sets, unsequenced positions are indicated by a question mark. In assembling Fig. 1, the individual sets as typed were cut out and arranged to produce a reasonable minimum number of crossing lines in connecting the members of the sets. If this is not done (24, 33) patterns of greater complexity are generally seen that do not give a true representation of the extent of the assortment. This procedure not only makes for clarity of the figures, but is also essential conceptually when there is no reason to arrange the sets in any specific order. Indeed, when one attempts to rearrange the sets in some defined order to test an hypothesis, the finding of increased complexity of the assortment may provide data consistent with or conflicting with the hypothesis. Thus our original simplest assortment patterns of FR segments of human, mouse, and rabbit  $V_{\kappa}$  chains (12) became extremely complex when the sets were ordered in terms of the likelihood of the amino acid differences in the FR segments being ascribable to somatic mutation (32). This increase in complexity of the assortment patterns indicated that somatic mutation was not making a significant contribution in generating the different FR sets (32).

All rabbit chains with the prefix K were from breeding colonies at The Rockefeller University (New York) and the Basel Institute of Immunology (Basel) and are related. Detailed geneologies may be found in Braun et al. (29, 31); the prototype rabbit K4820 was a progenitor of the Basel colony. It and K4878 and K4872 came from the State Serum Institute, Copenhagen, whereas K19, K20, K23 and K31 came from the Rockefeller University colony. Thus a portion of the data comes from families produced by matings of brother-sister and of other close

FIG. 1. Assortment of FR, CDR regions, and a proposed J segment of rabbit  $V_{\kappa}$  chains. ●, antitype III pneumococcal polysaccharide; ○, antitype VIII pneumococcal polysaccharide; ▲, antistreptococcal group A variant carbohydrate; △, antistreptococcal group C carbohydrate; ×, anti-*Micrococcus lysodeikticus*; +, anti-*p*-azophenylarsonate; ●, antitype II pneumococcal polysaccharide; □, antidi-goxin; ■, anti-*p*-azobenzoate; \*, two base changes; †, three base changes; ‡, Gln Gln Asp inserted between residues 58 and 59 and Val between 85 and 86; †, residue not known; -, deletion; ¶, residues 43 and 44 missing; #, an unidentified residue is present at position 97F. K9-335 and K9-338 were litter mates. 311 and 4422 are unrelated. One was purchased from a dealer in the U. S. and the other from a dealer in Switzerland.



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relatives, whereas the rest of the data derives from outbred and randomly selected animals. The inbred animals were immunized largely with streptococcal group C and group A variant streptococci, whereas the noninbred animals received pneumococcal polysaccharides, *Micrococcus lysodeikticus*, etc.

### Results and Discussion

Table I presents the findings indicating independent assortment of FR and CDR segments and defining a possible J segment that contains two residues of CDR3 as established earlier in the mouse (19, 20, 24) and as proposed for the rabbit (31).

Among the important findings are the following:

(a) The FR sets that contain more than a single member generally show multiple antibody specificities. This is true for all FR1 sets, and for the largest FR2 set. One FR2 set with four members and the two FR3 sets containing the pairs of identical sequences are limited to a single antibody specificity.

(b) The CDR sets tend to vary. Sets with multiple members in CDR1 have the same antibody specificity, whereas the sets in CDR2, the shortest CDR, excluding the pairs with identical V-region sequences, have members with different antibody specificities. This is perhaps not surprising because the antibody specificities are restricted to but a few antigens and because many members of these sets are related. The one CDR3 set of two related members, K16-167 and K27-306 (cousins), but that

TABLE I  
*Frequency Distribution of Members of Multiple Sets in Relation to Amount of Sequence Data*

Segment	FR1	CDR1	FR2	CDR2	FR3	CDR3	J
Amino acid residue numbers	1-23	24-34	35-49	50-56	57-88	89-97D	97E, 97F-107
Number of sequences	51	43	28 (+1*)	26	27 (+1*)	25	26
Number of sets	24	34	12 (+3*)	13 (+4*)	26	23	18
Sets/sequences, %	47	79	43	50	96	92	62
Sets with multiple members	11	4	3	4	2	3	4
Sets with single members	13	30	9	9	24	20	14
Number of sequences in sets with multiple members	38	14	21	17	4	4	12
Number of sequences in sets with multiple members/total number of sequences, %	75	32	75	65	15	16	46
Sets with single members/total number of sets, %	54	85	75	70	88	87	77
Number of sets/number of amino acid residues in segment	1.05	3.1	0.8	1.8	0.8	1.8	1.5

The J segment is made up by taking the last two residues of CDR3, called 97E, 97F, plus FR4 by analogy with the mouse J sequence.

\* No sequence data or sequence incomplete.

differed in sequence in the other FR and CDR segments had the same antibody specificity; CDR2 of K27-306 was not sequenced.

(c) The most striking evidence for independent assortment is the finding that the monoclonal antitype II pneumococcal light chains with identical V-region sequence from unrelated outbred rabbits 311 and 4422, one from Switzerland, the other from the United States (30), had an FR1 identical with two other antistreptococcal group A variant antibodies from two other rabbits K16-167 and K27-489. Unlike K16-167 and K27-489, 311 and 4422 had the preserved FR2 set as did the prototype K4820 originating in Denmark; this set contained segments of chains of antibodies of three specificities different from type II, one chain of no known specificity, as well as two other chains with type II specificity.

(d) The pair of littermates with an identical sequence K9-335 and K9-338 assorted in the same set in CDR2 with three related rabbits of the same specificity and with three unrelated outbred rabbits 3381, BS-1, and K25—these having another specificity. It is of interest that a second population of antibodies of the same specificity from one member of the pair, rabbit K9-335I, had different FR1, CDR1, shared the preserved FR2 set, and yet was in a CDR2 set that contained antibodies of four specificities.

(e) The J segment contained 18 sets. This is a large number, especially because the FR4 portion, residues 98-107, was identical in all but one sequence, K16-167, which had Thr at position 100 instead of Gly. All members of the prototype set had the same specificity as did the set with K16-167, and, indeed, except for this substitution, it would have fallen into the prototype J set because it also has Ile-Val at positions 97E and 97F. Of the three sets with two members, one set had a chain 3547 not known to have antibody activity (34) although it had been immunized with streptococcal vaccine. The other had antistreptococcal group A variant activity like the prototype set; another set had one chain with antipneumococcal type III activity and the other with antistreptococcal group A variant activity; the third set had the two identical chains from unrelated rabbits with type II antipneumococcal activity.

(f) Of the remaining 13 sets of J segments that differed from each other and from the prototype at one or both of the two positions 97E, 97F which could contribute to the generation of diversity, 8 were from light chains of antibodies to type III pneumococcal polysaccharide, 2 to *p*-azobenzoate, and 1 each to type VIII pneumococcal polysaccharide, to group C streptococcal polysaccharide and to *M. lysodeikticus*. The variations in sequence at positions 97E and 97F for the J segments from chains with type III antipneumococcal antibody give no indications of how they could influence site specificity, but there is no evidence that the different type III sites are necessarily the same.

(g) In the mouse, the first three nucleotides in the intervening sequence following the codon for amino acid 95 are CCC, and there appears to be considerable preservation of nucleotide sequences at the junction of J and CDR3 (26). Sakano et al. (19), Max et al. (20), and Weigert et al. (24) have proposed that some diversity in J could have been generated by intracodon recombination when the J nucleotides are joined to the rest of the V region. If one attempts such intracodon recombinations assuming CCC to be preserved at the beginning of the intervening sequence in the rabbit as in the mouse, the prototype set Ile Val would generate Leu or Pro at position 97E, neither of which has been found. Indeed, with CCC by the proposed intracodon

recombination it would be possible to produce, in addition to Leu and Pro, only His, Gln, and Arg, and only Arg has been found at position 97E in one J segment. Thus the amount of diversity that may be introduced by VJ joining in the rabbit is not very great, and 11 different amino acids have been found at position 97E in the 18 J segments. The outbred rabbit may prove, therefore, to have more  $J_k$  minigenes than the inbred mouse<sup>2</sup> or a more complex mechanism to generate diversity (35) as also proposed for the variable portion of Ig heavy chain (33). The precise location of the *N*-terminal end of J segments cannot be identified from amino acid sequences alone (24), and nucleotide sequence data will be needed to determine whether the two last amino acid residues in CDR3 should indeed be included with FR4 to form the J segments of rabbit kappa light chains, as has been found for mouse light chains. Indeed much of the diversity generated by intracodon recombination in the mouse derives from an extra Pro between residues 95 and 96 in a single NZB light chain PC7132 (24) which was assigned to J, thus permitting intracodon recombination involving six nucleotides of the intervening sequence following residues 95. It is possible that such recombination between the other FR and CDR minigene segments could also contribute somatically to diversity.

(h) An important parameter in relation to any mechanism for the generation of diversity is the number of CDR and J sets in relation to FR sets. Table I is an attempt to estimate this. If there are many more CDR sets than FR sets, this would have substantial implications for the generation of diversity. Although the rabbit amino acid sequence data are the most extensive, they nevertheless represent a very highly selected group of sequences because of the limited number of antibody specificities that have been examined. The data are also skewed in that many more FR1 and CDR1 sequences have been determined as compared with the rest of the chain. The tendency to have multiple sets will also be a function of the length of each segment comprising the set. Table I attempts to obtain an estimate of the relative numbers of FR and CDR sets.

If the CDR are compared with the FR, it is clear, as seen from the last row in Table I, that the number of sets is a function of the number of amino acid residues in the FR or CDR segment, and this must be taken into account. Even without this, however, it is clear comparing FR1 and FR2 with CDR1 that there are almost twice as many sets in CDR1 as in FR1 and FR2 and that a much smaller proportion of sets in CDR1 has multiple members despite the selection for only a few antibody specificities. If these values are corrected for length of the segment, the difference in number of sets between CDR1 relative to FR1 and FR2 increases two to three times. The same results are seen if CDR3 and the proposed J segment are compared with FR1 and FR2; in each instance, the percentage of sequences in sets with multiple members is much lower for CDR3 and J. These differences in number of sets would be further increased if adjusted for length differences with CDR2, which differs strikingly in length from the other segments and which shows much less variability in rabbit  $V_k$  chains than is seen in other species (36). The proportion of sequences in sets

<sup>2</sup> Rudikoff, S., D. M. Rao, C. P. J. Glaudemans, and M. Potter (personal communication) have suggested for mouse antigalactan  $V_k$  light chains that an additional gene containing an A as the first nucleotide of the intervening sequence following the codon for amino acid 95 could also account for the additional diversity.



with multiple members is less than in FR1 and FR2. The number of sets would also almost be doubled if adjusted for length differences.

(i) The data on FR3, the segment of longest length, do not appear to fit with the other segments. The 26 sets each have but a single sequence except the 2 sets each with the completely identical  $V_{\kappa}$  chains. However, there is some indication that FR3 can be divided into two segments which show some suggestion of assortment or recombination. Such assortment can be seen if FR3 is separated into two segments 57-68 and 69-88; the data are generally similar for 57-69 and 70-88 or 57-70 and 71-88. Fig. 2 shows the data for 57-68 and 69-88; it is clear that many of the individual chains in Fig. 1 can be grouped into sets and that these sets show evidence of assortment. It may be of significance that the three-dimensional structure of immunoglobulin light chains shows residues 68 and 69 to be at a bend connecting two  $\beta$ -strands in the mouse  $V_{\kappa}$  light chain  $F_V$  dimer REI (5, 9).

The two sets of 57-68 contain 13 and 5 chains and assort with six sets of 69-88 containing two or three members each. Thus the separation of FR3 into two segments has substantially reduced the 26 distinct FR3 sets.

Although the clones from 12-d-old mouse embryo DNA indicate that nucleotides coding for amino acids 1-95 occur as a contiguous segment (14, 17, 18, 26), the evidence for assortment, by recombination with or insertion into nucleotides coding for FR segments, of nucleotides coding for CDR segments suggests that this minigene mechanism in addition to diversity generated by the joining of residues 1-95 to the J minigene is fundamental to the generation of diversity.

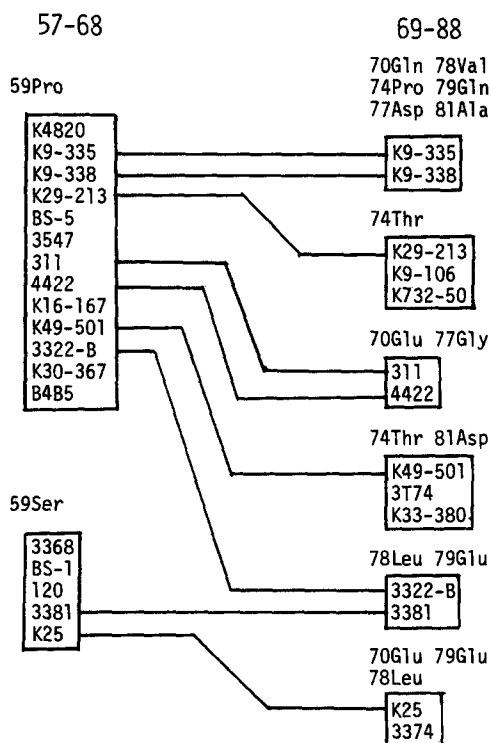


FIG. 2. Assortment of FR3 into two segments.

The recent report of Schilling et al. (33) of a segment generating diversity in CDR3 *N*-terminal to the J segment of anti- $\alpha$ 1  $\rightarrow$  3 dextran hybridomas and bearing individual idiotypic determinants provides additional evidence consistent with the minigene hypothesis.

It is important to distinguish between the generation of diversity and the generation of complementarity differences responsible for antibody specificity, e.g., for noncovalent binding of an antigenic determinant in the antibody-combining site. Although positions 96 and 97 of CDR3 of  $V_L$  are coded for by the J minigenes (19, 20) and although position 96 shows the highest variability (1, 2, 25), position 96 has been identified only once as a contacting residue in the high resolution x-ray crystallographic structures thus far reported (5-11) and in initial model building studies of antibody-combining sites without (37, 38) or with (39) nuclear magnetic resonance data in which the amino acid residues of the CDR sequences known to bind ligands were introduced on to a framework established by x-ray crystallographic studies. Padlan et al. (40) established residue 96 as a contacting residue for phosphorylcholine in the mouse myeloma protein McPC603. In mouse  $V_\kappa$  chains position 97 is essentially invariant (25). Rudikoff et al.<sup>2</sup> also consider that in the  $\beta$ 1  $\rightarrow$  6 galactans, the alternative amino acids at position 96 do not contribute to complementarity. Thus the intracodon recombination proposed (19, 20, 24) as a mechanism for V-J joining and for reducing the number of J minigenes might create sequence diversity without contributing significantly to those complementarity differences which would make for various antibody specificities. Indeed, even if antibody site complementarity were to be generated by position 96 as in McPC603 when an actual J sequence was assembled somatically by joining to the rest of the V region, it would not necessarily follow that the other amino acids if created at position 96 by intracodon recombination would necessarily function as CDR residues in CDR3 although conceivably they might influence other residues in CDR3 conformationally so that they become contacting. Indeed, although the discovery of the J minigene and of somatic assembly at the DNA level between the 12th d of embryonic life and the adult (12, 15, 17, 18, 26) is a seminal development in molecular genetics, it has tended to focus attention on position 96 and away from the other CDR residues that are crucial to the generation of antibody diversity, of idiotypic specificity, and of their interrelations.

### Summary

Amino acid sequences of rabbit light chains show considerable evidence of independent assortment of framework (FR) and complementarity-determining (CDR) segments. This suggests that they are coded for by independent genetic units (minigenes) and that individual light chains are assembled somatically by recombining these units. Identical FR sets with multiple members generally comprise chains with different specificities, whereas identical CDR sets tend to have chains of a single specificity. A J segment, which, by analogy with mouse light chains, is made up of the last two residues of CDR3 plus all of FR4, contained 18 different sets and could contribute to diversity generated by CDR3.

The longest segment, FR3, had a very large number of sets. Evidence is presented showing that the number of sets could be substantially reduced by permitting FR3 to

be formed by two independently assorting segments comprising residues 57-68 and 69-88.

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