Proc. Natl. Acad. Sci. USA
Vol. 77, No. 7, pp. 4270–4274, July 1980
Immunology

κ chain joining segments and structural diversity of antibody combining sites

(amino acid sequence/binding affinity/nucleotide recombination)

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Communicated by Elvin A. Kabat, April 23, 1980

ABSTRACT **Immunoglobulin κ light chains are coded for by at least three distinct gene segments designated variable, joining, and constant. The joining gene codes for the 13 amino acid segment linking the variable and constant regions. This peptide includes the last amino acid (96) in the third complementarity-determining region and thus could introduce structural diversity. We have determined the light chain variable region sequences from three myeloma proteins with β(1,6)galactan-binding specificity, bringing to six the number of light chains sequenced from proteins demonstrating this specificity. Five of these have isoleucine at position 96 and the sixth tryptophan. This substitution appears to be accommodated with no significant change in association constant for a β(1,6)galactan hapten. Additionally, as many as nine substitutions are found in both light and heavy chain complementarity-determining regions between members of this group although only minimal variations in hapten binding affinity are observed. The isoleucine found at position 96 in five of the κ chains could not be coded for by any of the joining gene nucleotide sequences previously observed and would require a novel nucleotide sequence at the recombination site between variable and joining genes to produce the observed protein structure. Alternatively, there may exist joining gene segments not yet detected.**

The study of antibody structure and diversity has long been of great interest in the field of immunology. The immunoglobulin molecule is composed of two polypeptide chains designated light (L) and heavy (H), each of which is encoded on a separate chromosome (1). Each of these two chains in turn is coded for by at least three distinct genetic regions termed variable (V), constant (C), and joining (J). The V regions of both H and L chains exhibit primary amino acid sequence differences that are responsible for the great variety of antigen binding specificities exhibited by vertebrate organisms. All L chain C regions may be divided into two classes, κ and λ; the corresponding region in heavy chains may represent several classes—i.e., μ, γ, α, δ, and ε.

Recent studies at both the protein (2–4) and nucleic acid levels (5, 6) have identified a third segment of the immunoglobulin chain, the J segment which bridges the V and C regions. The size and boundaries of this segment have been defined for mouse κ chains and include the last residue in the third complementarity-determining region. A corresponding J segment has been identified in H chains by protein sequence determination (4, 7) and by nucleic acid analysis (8).

For a number of years this laboratory has attempted to explore various aspects of antibody structure and diversity by analyzing groups of antigen-binding myeloma proteins. One such group is composed of proteins with binding specificity for β(1,6)-linked galactan moieties. Six of these proteins have been studied in terms of specificity (9, 10), structure (3, 4, 11), and idiotypy (12). We have reported the L chain V region sequences from three of these molecules. This analysis suggested the association of multiple J segments with a single V region. We have now determined the L chain V region sequences of three additional proteins in this group, and in the present communication we describe the expression of J segments in these chains and their potential role in generating binding site diversity.

MATERIALS AND METHODS

Proteins. IgA, κ myeloma proteins J-539, S-10, and T-191 were purified by affinity chromatography as described (9). The J-539 plasmacytoma was kindly supplied by Melvin Cohn (Salk Institute). After partial reduction and alkylation, H and L chains were separated on Sephadex G-100 columns in 6 M urea/1 M acetic acid.

Cyanogen Bromide Fragments and Sequence Determination. L chains were cleaved with CNBr at a CNBr/protein weight ratio of 4:1, and the fragments were isolated by gel filtration. Appropriate peptides were subjected to automated Edman degradation on a Beckman 890C sequencer using either a dimethylallylamine or 0.1 M Quadrol buffer system. Phenylthiohydantoin amino acids from each sequencer cycle were identified by a combination of high-pressure liquid chromatography (13) and hydrolysis to the free amino acid followed by amino acid analysis (14).

Binding Constants. Values were determined by fluorescence titration as described by Jolley *et al.* (9).

Enzymatic Digestions. Appropriate peptides were succinylated as described (3), dissolved in 0.1 M NH_4HCO_3, and digested with trypsin (1:50) for 2 hr at 37°C. V8 protease from *Staphylococcus aureus* was used to cleave at glutamic acid residues (15) under similar conditions at a 1:25 ratio for 6 hr.

RESULTS

J-539 L CNBr Fragments. Intact L chains were subjected to 35 cycles of degradation, and the sequence obtained is presented in Fig. 1. CNBr-cleaved L chains were dissolved in 5 M guanidine/0.05 M Tris, pH 8.2, and completely reduced and ^{14}C-alkylated. The resulting peptides were applied to a Sephadex G-100 column equilibrated in 5 M guanidine/0.2 M NH_4HCO_3 and eluted as shown in Fig. 2. Fractions were pooled as indicated, dialyzed against 0.2 M NH_4HCO_3, and lyophilized. The two peaks eluting prior to F1 appeared to contain aggregated or uncleaved material and were not analyzed further. Preliminary analysis, consisting of five or six steps of degradation, indicated that each pool contained a single peptide

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Abbreviations: L1, L2, and L3 refer to the three complementarity-determining regions of the light chain which form the light chain portion of the antigen contacting surface. The corresponding areas of the heavy chain are designated H1, H2, and H3. V, variable; C, constant; J, joining.

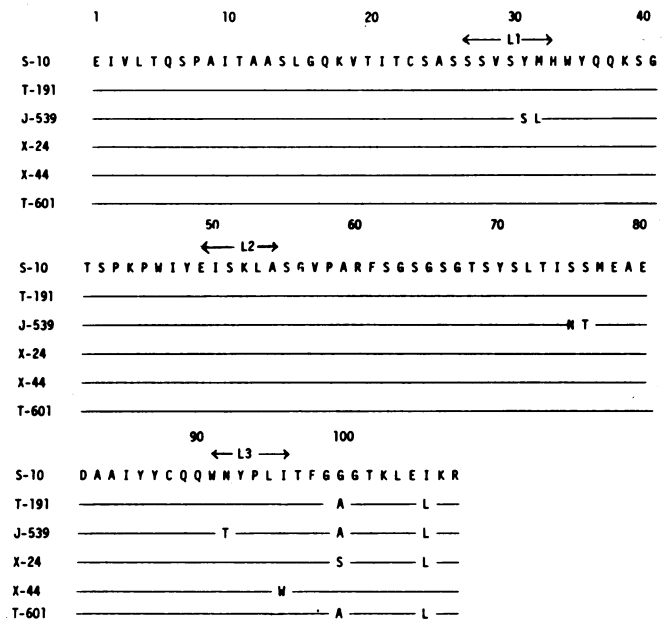


FIG. 1. L chain V region sequences from galactan-binding myeloma proteins. Numbering is according to Kabat *et al.* (16).

(homogeneity >90%). The F1 peptide begins at position 79 in the V region and, from its size and composition, extends to the COOH terminus of the L chain. F2 also begins at residue 79 and ends at 175 in the C region. The difference in length of these two peptides results from the expected partial cleavage of Met-Ser at position 175. F3 is the NH_2-terminal peptide comprising residues 1–78 and F4 is the small C-region peptide beginning at position 176 and extending to the COOH terminus.

Based on the preliminary analysis, 300–400 nmol of F1 (residues 79–214) was applied to the sequencer and subjected to 31 cycles of degradation (repetitive yield, 93%). The sequence obtained is shown in Fig. 1. The F3 peptide (residues 1–78) was degraded in two experiments for 52 and 55 cycles. The sequence of the COOH-terminal portion of this peptide was determined from two enzymatically derived peptides. By composition, the F3 peptide was known to contain two glutamic

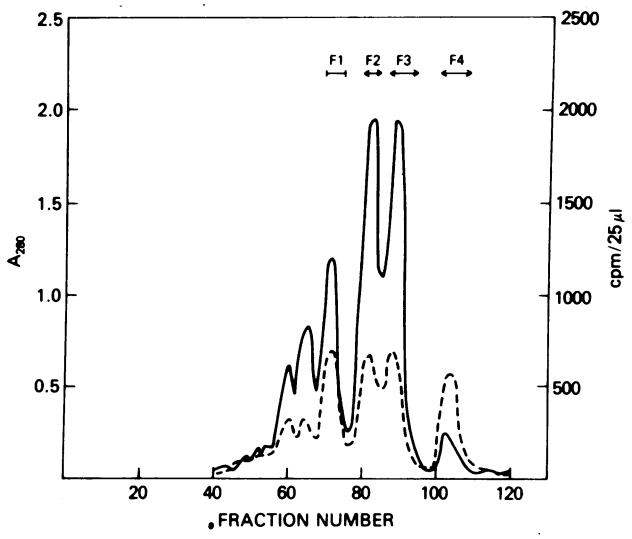


FIG. 2. Sephadex G-100 chromatography of CNBr-cleaved J-539 L chain after complete reduction and ^{14}C-alkylation. Columns were equilibrated in 5 M guanidine/0.2 M NH_4HCO_3. —, A_{280}; - - -, cpm; fraction size, ≈3.7 ml.

Table 1. Association constants of galactan-binding myeloma proteins with β(1,6) D-galactotriose hapten

Protein	K_a, M^{-1}
J-539	1.45×10^5
S-10	0.65×10^5
X-44	0.58×10^5
T-601*	0.86×10^5
X-24*	1.75×10^5

* K_a values are from Manjula *et al.* (19).

acid residues which were found at positions 1 and 50. An aliquot of F3 was subjected to two cycles of degradation to remove the NH_2-terminal glutamic acid after which the peptide was removed from the sequencer, succinylated, and digested with V8 protease. When this digest was reapplied to the sequencer in the presence of Polybrene (17), a single sequence beginning at position 51 was obtained for 15 cycles extending the determination from residues 51 to 65 (Fig. 1). A second aliquot of the peptide was then succinylated to block the NH_2 terminus and the lysine side chains and then digested with trypsin which cleaves only at the arginine at position 61. The tryptic peptide comprising residues 62–78 was isolated by gel filtration on Sephadex G-25 (superfine) in 0.1 M NH_4OH (not shown). Sequence analysis of this peptide in the presence of Polybrene (dimethylallylamine program) resulted in identification of all positions with the exception of the COOH-terminal homoserine which was placed by compositional analysis, thus completing the analysis of peptide F3. No overlap was obtained for positions 78 and 79.

S-10 and T-191 L Chains. CNBr fragments from S-10 and T-191 L chains were generated and sequenced by methods identical to those reported for three other anti-galactan L chains (3). Fragments from S-10 and T-191 differed from those obtained with J-539 as a result of an extra methionine present at position 32. The results of the sequence determination are presented in Fig. 1.

Association Constants. Association constants were determined by fluorescence titration using a galacto-triose hapten. The K_a obtained for J-539 agrees with previously published values (9) and is presented in Table 1 along with those for X-44 and S-10. T-191 gave no fluorescence change although galacto-triose has been shown to be as effective an inhibitor of precipitation as for J-539.

DISCUSSION

One of the intriguing problems related to antibody structural diversity is the question of how a small number of amino acid substitutions affect binding specificity. This premise is basic to the somatic mutation theory for generation of antibody diversity. The somatic mutation model proposes that a relatively small number of genes coding for antibodies are inherited and that the accumulation of somatic mutations throughout lymphocyte ontogeny accounts for the bulk of antibody diversity (18). One approach to this problem is the structural analysis of groups of antibodies or myeloma proteins with well-defined antigen or hapten binding specificities. One such group consists of myeloma proteins with specificity for β(1,6)galactan moieties. We have previously reported L chain V region sequences from three of these proteins (X-44, X-24, and T-601) and H chain V region sequences from four (X-44, X-24, T-601, and J-539). We have now determined L chain V region sequences from an additional three κ chains of this group (S-10, T-191, and J-539) and have begun to assess the effect of observed amino acid substitutions on binding affinity and specificity.

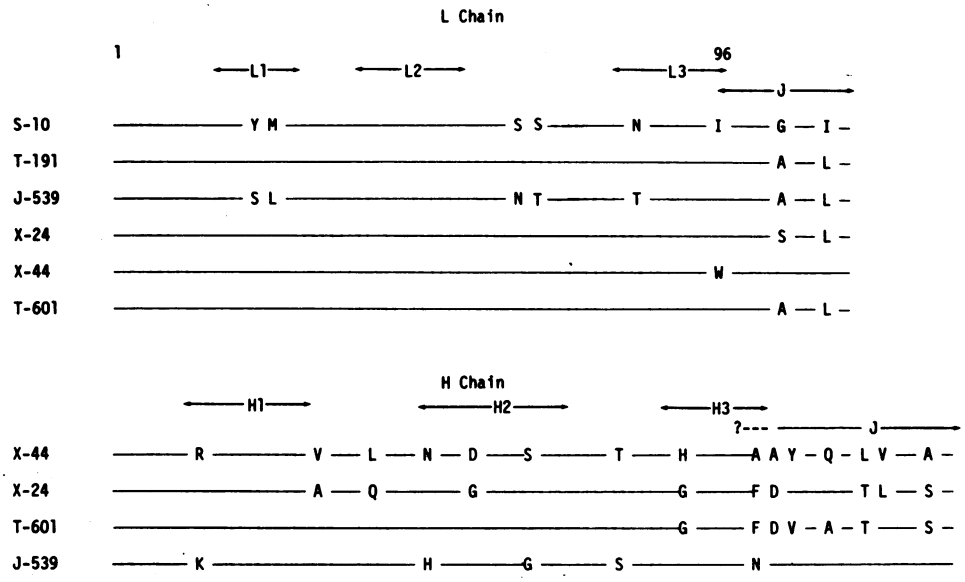


FIG. 3. Summary of sequence differences found in anti-galactan myeloma proteins. Solid lines indicate identity of sequences.

The sequence differences found in the S-10, T-191 and J-539 L chains are summarized and compared with those previously determined (3) in Fig. 3. With the exception of J-539, the V regions are identical in positions 1–95. J-539 differs at two positions in L1, two in the framework, and one in L3. All of the substitutions in J-539 L chain can be explained by single base nucleotide substitutions in the prototype sequence. It is interesting that J-539 is the only protein in this group not induced in our BALB/c colony but in fact originated in the Salk Institute colony. It is possible that differences exist in the V region gene pools in these two colonies, although the J-539 sequence could either be somatically derived or merely not yet found expressed in the NIH colony. The substitution of tryptophan for isoleucine at position 96 in the X-44 L chain is of particular interest. Although this residue is structurally located in the third complementarity-determining region, it is encoded in the J segment gene and will be discussed separately.

In the H chains from four of these proteins (Fig. 3), substitutions are found in both complementarity and framework portions of the V region as well as in the J segment. Comparisons made using both L and H chains show a number of interchanges between most members of this group. For example, J-539 and X-44 differ at eight positions in the complementarity-determining regions alone. Some of these interchanges involve quite different amino acid side chains. For example, the substitution of serine for tyrosine in the L chain first complementarity-determining region results in the exchange of the CH_2-OH side chain of serine for the bulky aromatic side chain of tyrosine. In the H chain second complementarity-determining region, asparagine and serine in X-44 are replaced by histidine and glycine in J-539. In the H chain third complementarity-determining region, an alanine/asparagine interchange is found. Among other members of this group, a number of additional examples are found in which drastically different side chains are substituted.

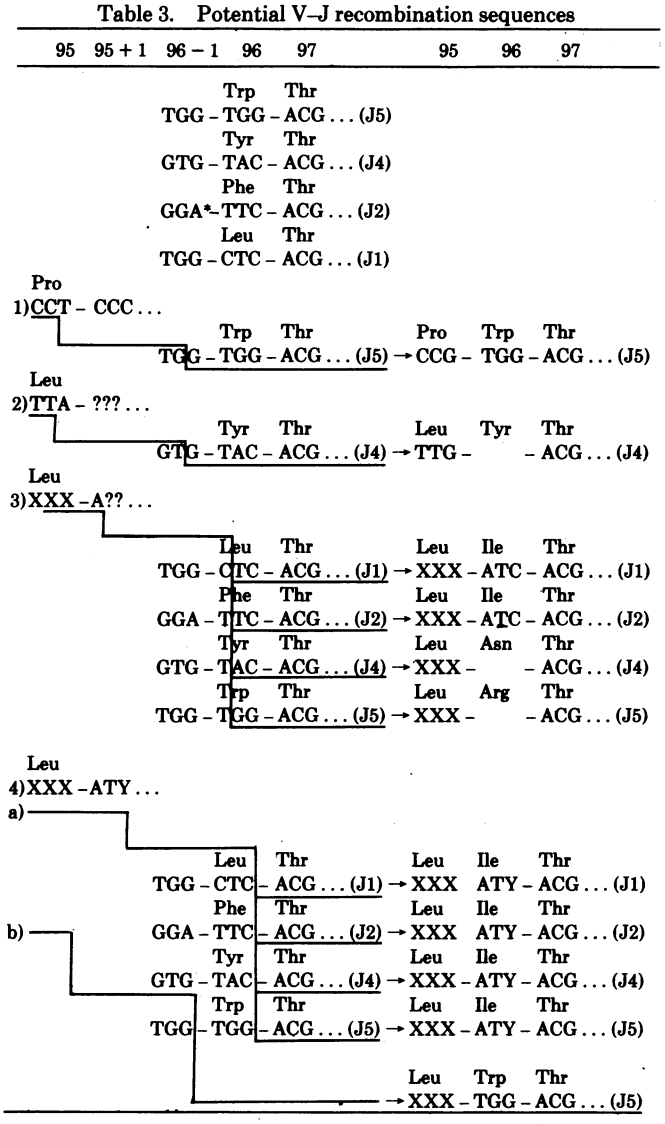
In view of the number and nature of the replacements in the complementarity-determining regions it is surprising that the association constants for these proteins are so similar (Table 1). The K_as of J-539 and X-44 for a galacto-triose hapten are 1.45 and 0.58×10^5 M^{-1}, respectively, despite eight complementarity-determining region differences. J-539 and X-24 differ at nine positions in the complementarity-determining regions and exhibit K_as of 1.45 and 1.75×10^5 M^{-1}. The K_as of the two most similar proteins, X-24 and T-601, which differ by one residue in the complementarity-determining regions, are 1.75 and 0.86×10^5. Previous studies have also shown that the K_as of this group for the disaccharide hapten or K_as of recombinant molecules made between members of this group (10, 19, 20) exhibit the same small differences shown in Table 1. We therefore conclude that for this binding specificity a relatively large number of complementarity-determining region substitutions may occur without significantly altering binding affinity. Furthermore, most of the substitutions in the complementarity-determining regions are found at positions predicted by statistical analysis (21) to be either antigen-contacting or conformationally important rather than structural in nature. It is not known whether substitutions such as those observed in the anti-galactan proteins might create new binding specificities, but clearly these interchanges do not substantially alter affinity for $\beta(1,6)$-linked galactan moieties. It also should be

Table 2. J sequences expressed in mouse κ chains*

	96												108	
	Trp	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	
TGG	TGG	ACG	TTC	GGT	GGA	GGC	ACC	AAG	CTG	GAA	ATC	AAA	CGT	J5
	Tyr	Thr	Phe	Gly	Gly	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	
GTG	TAC	ACG	TTC	GGA	GGG	GGG	ACC	AAC	CTG	GAA	ATA	AAA	CGT	J4
	Phe	Thr	Phe	Gly	Ser	Gly	Thr	Lys	Leu	Glu	Ile	Lys	Arg	
GGA	TTC	ACG	TTC	GGC	TCG	GGG	ACA	AAG	TTG	GAA	ATA	AAA	CGT	J2
	Leu	Thr	Phe	Gly	Ala	Gly	Thr	Lys	Leu	Glu	Leu	Lys	Arg	
TGG	CTC	ACG	TTC	GGT	GCT	GGG	ACC	AAG	CTG	GAG	CTG	AAA	CGT	J1

* DNA sequences are from Max *et al.* (5).

Table 3. Potential V–J recombination sequences



Codons represented are as follows: 95, the last codon of the κ chain V-region gene; 95 + 1, the first codon of the intron on the 3′ side of the V gene; 96 − 1, the last codon of the intron preceding any given J gene; 96, the first codon of any given J gene; 97, the second codon of any J gene. Leu codons = TTA, TTG, CTT, CTC, CTA, GTG; Ile codons = ATA, ATT, ATC. XXX = any Leu codon. Y = A, T, or C.
* This codon has also been reported as TGA (6).

noted that proteins X-24 and J-539, which differ at nine positions in the complementarity-determining regions, demonstrate the same specificity for 30 different carbohydrate haptens (10).

Recent studies at both the protein (2) and nucleic acid (5, 6) levels have demonstrated that murine κ chains are composed of at least three distinct segments, designated V, J, and C. The V gene codes for amino acids 1–95 which comprise the majority of the V region. The J gene codes for the short peptide segment which consists of amino acids 96–108 and bridges the V and C regions, and the C gene codes for amino acids 109 to the COOH terminus. In embryonic DNA, V genes are followed by nontranscribed sequences termed "introns." J genes exist in a cluster consisting of five sequences which could potentially code for J protein segments. Only four of these gene sequences have been identified in corresponding protein products. The J genes are separated from each other by introns and are followed by an additional intron which separates the J cluster from the C gene. During differentiation to the mature plasma cell a rearrangement occurs such that one of the J genes is positioned adjacent to the V gene, resulting in the gene order V-J-intron-C. The intron between J and C is not translated.

In terms of antibody binding site diversity, the L chain substitution at position 96 in X-44 is of particular interest. Position 96 is the last residue in the third complementarity-determining region (Fig. 3) but is coded for by the J gene. It has been demonstrated (2) that pairing of different J segments with a single V region may introduce amino acid alternatives at position 96. Additionally, a mechanism has been suggested whereby alterations in the frame of recombination between V and J genes could produce other amino acid alternatives at position 96 which in turn might generate binding site diversity (5, 6). When X-44 is compared to T-601 it can be seen that the Trp-Ile interchange at 96 L results in the replacement of a nonpolar side chain by a large, polar, ring-structured side chain. The similarity in K_as of these two proteins suggests that the Trp-Ile interchange is accomodated without significantly altering affinity for β(1,6)galactan haptens. This observation, in conjunction with the large number of complementarity-determining region differences which appear not to change affinity or specificity, suggests that, in this system, alternative amino acids introduced at 96 L by the J segment do not generate functional binding site diversity. For other antigen-binding specificities this position may prove to be significantly involved in antigen complementation. Recent nucleic acid studies have defined two H chain J segments (8) from a germ-line DNA clone. From these data it appears that the Ala-Phe-Asn interchange in H3 found in the anti-galactan H chains (Fig. 3) is either coded for by the J gene or generated by a recombination event involving J. Again, the interchange of these structurally dissimilar amino acids produces no detectable change in β(1,6)galactan binding.

To date, complete nucleotide sequences have been reported for three mouse κ chain V regions (22, 23) and the J gene cluster (5, 6). The four germ-line J sequences that have been found expressed in protein structures and their amino acid counterparts are given in Table 2. Additional amino acids that have been observed at position 96 in κ chains can be explained by altering the frame of recombination between V and J (see ref. 6). Size differences have also been observed in related mouse κ chain protein sequences in the area of V–J joining (2), suggesting that, in some instances, insertions or deletions may result from alterations in the recombination process. The nucleotide sequence at the recombination site has been determined for two mouse κ chains, and in both instances the recombination event was found to occur between the second and third nucleotides of codon 95 (the last V region codon) and the third nucleotide of the last intron codon preceding the J sequence to be used (5, 6). An example of this recombination is presented as example 1 in Table 3. In the three κ chain embryonic DNA sequences the 95 codon was found to be CCT (proline) and the 95 + 1 codon, to be CCC.

Of the available anti-galactan L chain amino acid sequences, all proteins have leucine at position 95, five have isoleucine at 96, and one has tryptophan at 96. Based on the results described above for V–J recombination, we can begin to assess this event in the anti-galactan L chains. First, from the amino acid sequences (Fig. 1), potentially all of the expressed J regions (J1, J2, J4, and J5) may be used in these L chains. Weigert *et al.* (2) also reported that closely related L chains from the V_{k21} subgroup express various J segments. However, in the anti-galactan L chains at position 96 only the tryptophan in X-44 could be encoded by a germ-line sequence (Table 2). Because the isoleucine found in five of the chains is not coded for by any of the germ-line 96 codons, and assuming there are no addi-

tional J genes, it must be generated by the recombination event between V and J. If the recombination in the anti-galactan κ chains occurred between the second and third nucleotides of codon 95, an incorrect protein sequence would be generated as shown in example 2 of Table 3. None of the six leucine codons in combination with any of the J sequences can produce the Leu-Ile dipeptide in this frame of recombination. Similarly, if the recombination event occurred between any other nucleotides of codon 95 it would still be impossible to generate the appropriate protein sequence.

A second possibility for deriving the proper protein sequence would be to alter the frame of recombination such that this event occurred in the first codon (designated 95 + 1) of the intron following V in undifferentiated DNA. As pointed out above, the same codon, CCC, has been found at this psoition in four κ chains with different V region sequences. If CCC is used as the 95 + 1 codon, again no frame of recombination can produce isoleucine at 96 because all recombined sequences would produce codons with C at either the first or first and second nucleotide positions of codon 96. All isoleucine codons must have A in the first nucleotide position (Table 3). It therefore appears that, if the recombination event involves the 95 + 1 codon, the sequence CCC is not universal. If, in fact, the first nucleotide of the 95 + 1 codon in the anti-galactan κ chain gene(s) were A, the correct sequence, Leu-Ile at 95–96, could be generated by recombination with J1 or J2 but not with J4 or J5 (example 3). However, the S-10 sequence (which has isoleucine at 96) expresses either J4 or J5, as indicated by the protein sequence and thus could not be derived from this recombination. We suggest the simplest explanation for the anti-galactan L chains is that the recombination does involve the 95 + 1 codon but that in these genes the 95 + 1 codon is actually a codon for isoleucine as in example 4a. Thus, in recombination with any J the entire 95 + 1 codon would be joined to the first nucleotide of codon 97, the second codon in the J gene, resulting in isoleucine at position 96 in all Js. The tryptophan at 96 in X-44 could then easily be explained by altering the frame of recombination so that the event occurred after codon 95, joining the V gene to the germ-line J5 sequence (example 4b) and eliminating the 95 + 1 codon.

Several points should be emphasized in regard to this proposed recombination mechanism. First, the CCC codon cannot be unique at position 95 + 1 and thus is unlikely to be the sole recognition sequence for the recombination event. Second, the recombination in this system occurs after the 95 + 1 codon in five instances and between codons 95 and 95 + 1 in one instance, in contrast to the two reported examples within codon 95. It is important to note that isoleucine has been found at position 96 in 5/6 of these L chains. Since amino acids as diverse in structure as isoleucine and tryptophan can be accomodated at this position one might expect to find several amino acid alternatives if the recombination event occurred randomly or had a high mistake frequency. The observation that alternatives are not found in high frequency suggests that for these genes the recombination process is not a random event. In the case of X-44 where tryptophan replaces isoleucine at position 96 the recombination frame appears to have altered by exactly one trinucleotide. The specificity of the recombination event is thus remarkable in that, regardless of where the actual recombination occurs, the resultant nucleotide sequence remains in a proper reading frame. Alternatively, if the mechanism lacked specificity, a large number of frame shifts likely would occur; to date these have not been observed at either the protein or nucleic acid level.

If the L chain structures described above are not generated by a mechanism such as suggested in example 4, then multiple, identical (or nearly identical) V genes which differed in their intron sequences would be required to code for the observed proteins. Clearly, the recombination sequence proposed above may not be general in nature and may be characteristic of only certain gene sets, depending on their actual structure.

We thank Ms. Janet Pumphrey for her excellent technical assistance.

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