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Immunology

Diversity at the variable–joining region boundary of λ light chains has a pronounced effect on immunoglobulin ligand-binding activity

(antibody structure/myeloma tumor/hybridoma/monoclonal antibody)

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ABSTRACT **By recombining λ light (L) chains having known variable (V) region amino acid or nucleotide sequences with a heavy (H) chain from a myeloma protein or a monoclonal antibody, we obtained reconstituted Igs that differed from each other in sequence by only one or a few amino acid substitutions at known L chain positions. Differences in affinity of the reconstituted Igs for 2,4-dinitrophenyl (DNP) ligands revealed a pronounced effect on Ig binding activity of amino acids at the V–J boundary of the λ chains. In one instance, two reconstituted Igs that differed about 1000-fold in affinity for ε-DNP-aminocaproate differed in primary structure by only a single tyrosine-phenylalanine substitution at the V–J junction (position 98) of their λ2 chains—i.e., by only one out of approximately 660 amino acid residues (L + H chains). By focusing on affinity changes, chains with unusual Vλ–Jλ junctional residues were identified. It is possible that because of a critical effect on tertiary structure junctional amino acid variations arising from gene segment assembly (*V/J* and perhaps *V/D/J*) constitute an important source of ligand-binding diversity of antibodies.**

The genetic basis for the enormous sequence diversity of variable (V) regions of immunoglobulins (Igs) has been greatly illuminated by recent studies of the gene segments that encode these regions (1). However, the relationship between particular variations in sequence and binding activity has been difficult to discern. To study these relationships, we took advantage of the well-known procedures by which the heavy (H) and light (L) chains isolated from different Ig molecules can reassociate noncovalently to establish new H–L combinations—i.e., new Igs (2, 3). Thus, we reassociated the H chain from one Ig with L chains from other Igs, choosing L chains that differ from each other at only one or few V region positions. Functional (ligand–binding) differences among the reconstituted Igs could then be related to particular residues in the V regions of the L chains. We also measured the binding activity of the isolated L chains themselves.

Mouse L chains of the λ type were used in this study, because the germ line DNA sequences of all the *V*λ and *J*λ (J, joining) gene segments of inbred BALB/c mice have been established (4, 5); therefore, somatic mutations and junctional variations (6, 7), due to imprecision in recombination of *V* and *J* gene segments, can be readily identified in sequenced λ chains of these mice. We report here that substitutions at the V–J junction of λ chains have a surprisingly great effect on Ig ligand-binding activity, much greater than that of amino acid substitutions elsewhere in the V regions of these L chains.‡

MATERIALS AND METHODS

Myelomas. Myeloma tumors (HOPC-1, H-2020, MOPC-315, TEPC-952, and CBPC-49) were originally obtained from M. Potter, National Institutes of Health. The myeloma proteins they produced are designated H1, H2020, M315, T952, and C49, respectively (see Table 1).

Myeloma Proteins and Monoclonal Antibodies. Hybridomas MRG-8-13 and MRG-8-47 (Table 1) were donated by A. Marshak-Rothstein and M. Gefter (10). Hybridomas of the RZ series (5-7, 6-2, 5-8, and 5-5) were made by R. Zaugg by fusing SP2/0 cells with spleen cells from BALB/c mice immunized against 2,4-dinitrophenyl (DNP)-*N*-(2-aminoethyl)carbamoylmethyl-Ficoll. Protein M315 and the monoclonal antibodies (mAbs) made by these hybridomas (nos. 3 and 6–11 in Table 1) bind DNP ligands and were purified by affinity chromatography (8). The other proteins in Table 1 were purified by gel filtration and QAE-Sephadex ion exchange chromatography (9).

Preparation of Reconstituted Igs. H and L chains were isolated from mildy reduced and carboxamidomethylated Igs as described (10); H chains were rerun under the same conditions to eliminate traces of L chains. The H chains (from M315 and mAb 8-47) were each mixed at 100–500 μg/ml with a 2- to 4-fold molar excess of various λ chains and dialyzed under conditions in which H and L chains reassociate (2, 3). The reconstituted Igs were concentrated by collodion bag dialysis; some precipitated H chains were removed, and gel filtration of the soluble protein [on Sephadex G-200 in phosphate-buffered saline (pH 7.4)] yielded three fractions. The first, at the void volume, usually consisted mostly of aggregated H chain. The second, at 1.2–1.3 times the void volume, emerged at the position of native IgA or IgG. The third fraction consisted of excess λ chains. $NaDodSO_4$/polyacrylamide gel electrophoresis of the second fraction yielded H and L chains in the same proportion as in native Ig. The second fraction was passed through a column of ε-DNP-lysine-Sepharose 4B to isolate by affinity chromatography those reconstituted Igs with anti-DNP specificity. Adsorbed protein was eluted with 50 mM DNP-glycine. After passage through Dowex 1 X8, followed by dialysis against phosphate-buffered saline (pH 7.4) to remove traces of hapten, the eluted protein was tested for ligand binding. Reconstituted Igs that were not retained by the DNP-lysine-Sepharose column had no measurable affinity for DNP.

Measurement of Affinity. Fluorescence-quenching titrations were carried out, plotted, and analyzed as described (11, 12). The tryptophan fluorescence of mAb 8-47 and the reconstituted Igs made with its H chain was not quenched by DNP ligands. Therefore, the binding of ^{3}H-ε-DNP-L-lysine by these proteins was measured by ultracentrifugation (13).

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Abbreviations: H, L, heavy and light (chains); V, D, J, respectively, variable, diversity, and joining [gene segment (italic type) or protein chain segment (Roman type)]; CDR, complementarity-determining region; mAb, monoclonal antibody; DNP, 2,4-dinitrophenyl.

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For other anti-DNP Abs, the equilibrium constants measured by fluorescence quenching and ultracentrifugation are in excellent agreement (12).

Binding of ε-DNP-Lysine by Free L Chains. One volume of ε-DNP-L-lysine-Sepharose 4B beads was suspended in 3 vol of phosphate-buffered saline (pH 7.4) and 0.5 ml of the suspension was mixed with 0.5 ml of phosphate-buffered saline containing free λ chains at 0.2–2.0 mg/ml. After gentle shaking at room temperature for 1–2 hr, followed by centrifugation (15 min at 2000 rpm), the amount of unbound λ chains (in the supernatant) was determined by absorbance at 280 nm. No correction for nonspecific binding was made because some myeloma proteins (H1, T952, C49) showed no decrease in protein concentration after incubation with the beads.

Partial Amino Acid Sequence of L^{5-7}. The peptide corresponding to residues 88–174 of this chain was isolated as described (10) and subjected (with help from Kathleen Hehir and Robert T. Sauer) to automated Edman degradation in a Beckman 890C sequencer (for references to procedures, see ref. 14).

RESULTS AND DISCUSSION

In inbred mice there are three λ chain subtypes (λ1, λ2, λ3; refs. 10, 15, and 16) and several chains of each subtype were reassociated with each of two H chains, one (H^{315}) from M315, a BALB/c mouse myeloma protein with high affinity for DNP- and 2,4,6-trinitrophenyl-labeled ligands and the other (H^{8-47}) from a monoclonal anti-TNP antibody, mAb 8-47, that also binds DNP ligands. Intact proteins M315 and mAb 8-47 were therefore used as standards in comparing the DNP-binding activity of each of the reconstituted Igs. The L chains and the Igs from which they were derived are given in Table 1.

As illustrated in Fig. 1 and summarized in Fig. 2, the binding assays showed that reconstituted Igs made with λ1 chains were inactive whereas those made with λ2 or λ3 chains (aside from a few notable exceptions) bound DNP ligands with about the same affinity as the standard Ig (intact M315) and the "homologous" reconstituted Ig (H^{315}–L^{315}). Similarly, a reconstituted Ig made with H^{8-47} and a λ1 chain (L^{H1}) was inactive whereas those made with H^{8-47} and λ2 and λ3 chains (L^{5-8}, L^{C49}, L^{8-13}, and L^{952}) had about the same affinity (4–9 × 10^6 L/M) for ^{3}H-ε-DNP-lysine as intact mAb 8-47 and the homologous reconstituted Ig, H^{8-47}–L^{8-47} (data not shown). From the amino acid residues at which the λ chains differ from each other and from the germ line gene segments that encode them (Fig. 2), it is clear that the functional differences among the three subtypes are not accounted for by amino acid residues at positions 1–96. At these positions, most λ1 and λ3 chains are encoded by the same *V* gene segment (22) and many are expected to have an identical sequence. Two examples are L^{H1}, a λ1 chain, and L^{5-8}, a λ3 chain: despite their identical sequences at positions 1–96, the reconstituted Ig made with the λ1 chain (L^{H1}) was inactive, whereas the one made with the λ3 chain (L^{5-8}) was highly active (Figs. 1 and 2). Sequence differences at positions 99–110, encoded by *J*λ gene segments, also could not be correlated with differences in activity (Fig. 2).

Table 1. Myeloma proteins and monoclonal mAbs from which H and L chains were isolated

No.	Protein	H class, L type	Ligand-binding specificity
1	H1	γ_{2a},λ1	?
2	H2020	γ_{2a},λ1	?
3	M315	α,λ2	DNP, TNP
4	T952	α,λ2	?
5	C49	α,λ3	?
6	MRG 8-13	γ1,λ2	DNP, TNP
7	RZ 5-7	μ,λ2	DNP
8	RZ 6-2	μ,λ2/λ3	DNP
9	RZ 5-8	μ,λ3	DNP
10	MRG 8-47	γ1,λ3	DNP, TNP
11	RZ 5-5	μ,λ3	DNP

TNP, 2,4,6-trinitrophenyl.

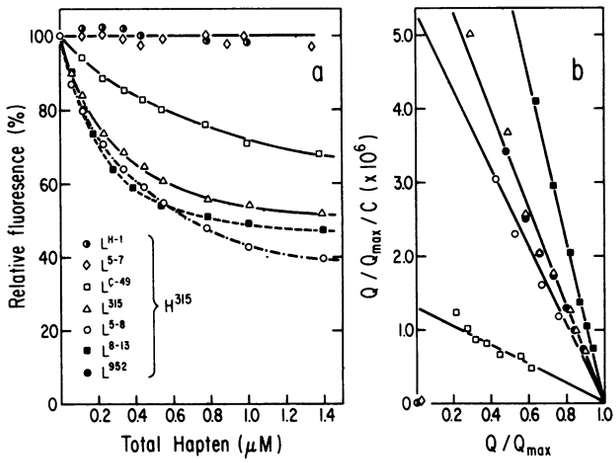

FIG. 1. Fluorescence quenching of reconstituted Igs by ε-DNP-aminocaproic acid. Q is observed quenching; Q_{max} is quenching at saturation (11, 12); C is free ligand concentrations. The calculated equilibrium constants are shown in Fig. 2. The titration curves obtained with reconstituted H^{315}–L^{315} (△) and with the intact M315 myeloma protein were indistinguishable. Titrations in *a* are plotted in *b* according to the Scatchard equation: linearity in *b* indicates that binding site uniformity was retained in the reconstituted Igs.

The crucial difference appears to be at the Vλ–Jλ boundary (positions 97 and 98, see below). At position 98, the inactive λ1 chains have tryptophan and the active λ2 and λ3 chains have phenylalanine. The significance of this difference is emphasized by a pair of λ2 chains, L^{8-13} and L^{5-7}. These chains have an identical sequence throughout the entire V region (positions 1–110), except at position 98, where the active chain (L^{8-13}) has phenylalanine and the inactive one has tyrosine (Fig. 2) (38).

The results with this pair of λ2 chains were reinforced by the findings with a pair of λ3 chains (L^{5-8} and L^{C49}). The V regions of these chains, determined by the cDNA sequence of their respective mRNAs, are also identical except for a T–G difference in the codon at position 97; this codon corresponds to glutamine in L^{C49} and to histidine in L^{5-8} (14). The functional difference between the reconstituted molecules made with each of these chains and H^{315} was modest (about 4-fold in affinity for DNP, Figs. 1 and 2), but the two chains differed conspicuously when tested, in absence of H chains, for their ability to bind to ε-DNP-lysine Sepharose beads (Fig. 3): L^{5-8}, with histidine-97, bound well but L^{C49}, with glutamine-97, was the only λ2 or λ3 chain that did not bind at all to the DNP-beads. The isolated λ1 chains also did not bind to these beads: though these chains have His-97, they also have tryptophan, not phenylalanine, at position 98 (e.g., compare L^{H1} and L^{5-8} in Figs. 2 and 3). The binding activity of isolated L chains is discussed further below.

It is likely that both the phenylalanine vs. tyrosine difference at position 98 and the histidine vs. glutamine difference at position 97 arose from alternative recombination sites for *V*λ → *J*λ rearrangement, generating codons for phenylalanine or tyrosine at position 98 of λ2 chains and for histidine or glutamine at position 97 of λ3 chains (Fig. 4). Point mutation as an alternative mechanism cannot be excluded. How-

L chain	Variable region gene segments Vλ	Jλ	Sequence differences between λ chains and germ line forms of Vλ and Jλ gene segments	Affinity*, $M^{-1} \times 10^{-6}$	Ref(s).
			Vλ: 1 16 19 54 62 85 87 96 — Jλ: 97 98 100 92 6 110		4, 5, 17, 18
			Vλ1: E T N A E I N — Jλ1: V G L		
			Vλ2: G I S V D M T — Jλ2: V G V; Jλ3: I S V		
λ1 HOPC-1	1	1	H W	<0.005	19
H2020	1	1	① ⑥ (26 30) H W	<0.005	19
λ2 8-13	2	2	H F	11.0	a
T952	2	2	H F ⑨⑨	6.3	a
M315	2	2	① (38) ⑨④⑤⑥ (94 95 96) H F	6.7	16
5-7	2	2	H Y	0.05-0.1	14, a, b
λ3 5-8	1	3	H F	5.0	14
5-5	1	3		5.3	
C49	1	3	Q F	1.3	14
8-47	1	3		1.5	
6-2	2	3	H F	3.5	20, a

*for DNP-aminocaproate; 20°C.

FIG. 2. Correlation between sequences of λ chains and affinity for ε-DNP-aminocaproate of the reconstituted Igs made with these chains and H^{315}. The sequenced λ chains differ from the germ line sequences of their encoding *V* and *J* gene segments, listed in columns 3 and 4, by the circled amino acid substitutions shown (position no. is below each circled residue). The germ line sequences of the *Vλ1* and *Vλ2* and the *Jλ1*, *Jλ2*, and *Jλ3* gene segments are compared at the top of the figure: except for the ten positions indicated, and the amino acids shown at each of these positions, the amino acids encoded by the two *Vλ* segments are the same (17, 18) and those encoded by the three *Jλ* segments (4, 5) are also the same. Amino acids are represented by the one-letter code (21). Sequences are from the references listed in the right hand column: ref. a is ref. 38; ref. b is this paper (positions 88–121). Affinity value for L^{5-7} was too low to be measured by fluorescence quenching; estimate was based on equilibrium dialysis.

ever, this seems unlikely, especially for the histidine vs. glutamine difference between L^{5-8} and L^{C49}: the *V* region nucleotide sequences of cDNAs for these chains are identical, except for the single T–G difference in codon 97, and both correspond precisely to germ line sequences of the *Vλ* and *Jλ* gene segments that encode them (14). As is well known, junctional variants have previously been inferred for κ and H chains (6, 7, 23).

Is the V–J boundary effect limited to λ chains? Other studies suggest that substitutions at homologous positions in κ and H chains may also have a pronounced effect on ligand-binding activity. Thus, the κ chains for a mAb to oxazalone and for several mAbs to benzenearsenate have identical V region sequences except at the Vκ–Jκ boundary [designated position 96 (24) but corresponding to position 98 in the sequential numbering system used here for λ chains]. At this position the anti-oxazalone mAb has Tyr (25) and the anti-benzenearsenate mAbs have arginine (26); however the functional significance of this difference is obscured because there are also multiple V region differences in the H chains with which each of these κ chains is associated. It is also notable that the phosphorylcholine-binding myeloma protein S107 and a variant of this protein that has lost the ability to bind phosphorylcholine-protein conjugates (but not the ability to bind phosphorylcholine itself) differ in V_H amino acid sequence at only a single position (no. 105, ref. 27). This position, though encoded by a J_H gene segment, may be structurally homologous to λ-98 (and κ-96) in that it also lies at the amino terminus of a highly conserved J region hexa- or heptapeptide sequence that is found in many Ig chains (24): X-Y-Z-(Z)-G-U-G, where X is the position of interest (λ-98, κ-96, H105), Y is usually an aliphatic amino acid, Z is an aromatic amino acid (or two contiguous ones), G is glycine, and U is usually glycine, serine, or glutamine.

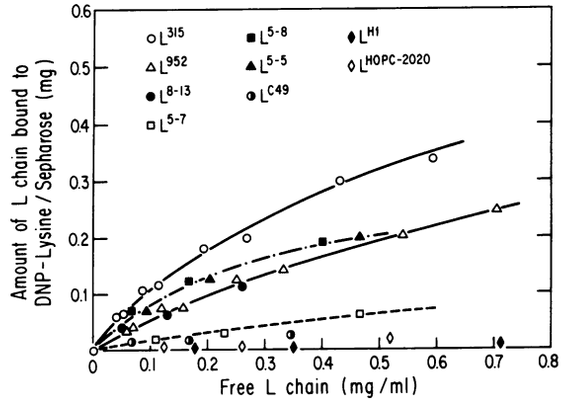

FIG. 3. Binding of free λ chains to ε-DNP-L-lysine-Sepharose 4B beads.

It is possible that the amino acid residue at the V–J junction makes contact with bound ligand and provides a major energetic contribution to the binding. An x-ray crystallographic study of myeloma protein M603 (phosphorylcholine binding; ref. 28) and some models (29, 30) of the combining site of M315 (DNP binding) do indeed suggest that in these proteins a V–J junction residue (κ-96 in L^{603} and λ-98 in L^{315}) makes contact with bound ligand. However, another interpretation of the x-ray crystallographic data (31) and a different model (32) of the M315 combining site indicate that these L chain V–J boundary residues are virtually entirely in con-

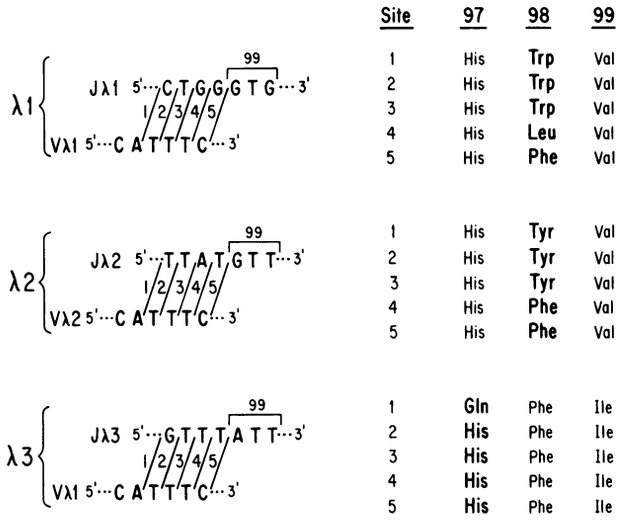

FIG. 4. Possible amino acid substitutions at the V–J boundary of each of the λ chain subtypes, due to variation in sites for joining *Vλ* and *Jλ* gene segments. The germ line sequences shown are from BALB/c mouse DNA and are at the 3′ end of the encoding sequence of the *Vλ* gene segments and at the 5′ end of the encoding sequence of the *Jλ* gene segments (4, 5). Of the junctional variants (positions 97 and 98), all but phenylalanine at λ1 position 98 have been observed. In about 5% of λ1 and λ3 chains, the joined *V–J* gene segments are *Vλ2* → *Jλ1* and *Vλ2* → *Jλ3*; these unusual rearrangements have no effect on the junctional variants shown, because the 3′ ends of *Vλ1* and *Vλ2* are the same.

tact with V_H and thus may be only marginally accessible for contact with bound ligands (see also refs. 39, 40). If the latter view is correct, a change in the side chains of such a residue could well have a dramatic effect on the shape of the combining site and on affinity. Whatever the reason, reconstituted Igs that differ in sequence by only a single amino acid at the Vλ–Jλ junction—i.e., by only 1 out of about 660 amino acids (L+H chains)—and indeed by only 1 atom out of 10,000 (the O of tyrosine-98), can differ profoundly in affinity for ligand.

Though amino acids at the λ chain V–J junction are important for ligand-binding activity, we are not suggesting that particular amino acids at the junction are required for a particular specificity or affinity. The basis for this qualification is well illustrated by $L^{5\text{-}7}$. Of the four completely sequenced λ2 chains, $L^{5\text{-}7}$ is the only one with tyrosine at the V–J boundary and the only one that did not form a good anti-DNP site with H^{315} (Figs. 1 and 2). However, Tyr at this position (λ-98) cannot, *per se*, be incompatible with an anti-DNP combining site, because the particular mAb (RZ 5-7) from which $L^{5\text{-}7}$ was derived has substantial affinity for ε-DNP-lysine (1×10^6 M^{-1}, unpublished). Moreover, a preliminary study has identified five additional anti-DNP mAbs with substantial affinity for DNP and with λ2 chains that have tyrosine at position 98 (unpublished work). Evidently, in association with the H chains from many anti-DNP mAbs, λ2 chains having Tyr-98 can form anti-DNP combining sites.

Similar considerations could account for the conclusion of Rudikoff *et al.* (33) that the V–J boundary residue of κ chains has no significant effect on hapten binding. They found that in five of six κ-containing galactan-binding myeloma proteins the κ chains had isoleucine at the V–J boundary and the sixth had tryptophan and yet all of these proteins had similar affinity for the hapten. However, the κ chains of these six Igs also differed by other substitutions (a minimum of three) and each was associated with a different H chain; thus, substitutions at other positions of V_L or V_H could well have obscured the contribution of the residue at the Vκ–Jκ boundary.

In contrast to the striking effect of substitution at the Vλ–Jλ boundary (position 98 or 97), amino acid substitutions elsewhere in the V regions, whether in framework or complementarity-determining regions (CDR), had remarkably little effect on affinity for DNP ligands. This lack of effect may appear surprising, but it is not actually in conflict with the expectation that amino acid substitutions in CDRs of H and L chains might well alter the affinity of an antibody for its antigen (or haptenic analog). This becomes clear when pairs of chains are compared. Thus, $L^{8\text{-}13}$, a λ2 chain and $L^{5\text{-}8}$, a λ3 chain, have the same amino acids at positions 97 and 98 but differ at ten other V region positions (because they are encoded by different gene segments), and yet there was no difference in affinity for DNP of the reconstituted Ig made by each of them with H315 (Fig. 2). However, of the ten differences between $L^{8\text{-}13}$ and $L^{5\text{-}8}$ only two are in CDRs (positions 54 and 96) and they are fairly conservative (asparagine vs. serine and threonine, respectively). Or, consider L^{315} and L^{952}. This pair differs at five positions: two are in framework regions, but three are contiguous in the third CDR and include substitutions that either are or could be nonconservative (phenylalanine vs. tyrosine and serine vs. arginine). Despite these differences, the effect on affinity is negligible (6.7×10^6 vs. 6.3×10^6 M^{-1}). (However, the substitutions in the third CDR of L^{315} have a pronounced effect on the reaction of M315 with antibodies to the unique idiotype of this protein; unpublished observations and ref. 34.) The lack of effect of these CDR substitutions on affinity for ε-DNP-lysine could mean that this hapten is an inappropriate ligand, perhaps because a DNP-like antigen is unlikely to have been involved *in vivo* in the development of the B cells that gave rise to the myeloma tumors (MOPC-315 and TEPC-952) from which these L chains were derived. It is also possible that, although these CDR replacements have a negligible effect on affinity for a small DNP hapten, they could have a substantial effect on the binding of large ligands (35).

Some of the L chains alone, in the absence of H chain, bind to ε-DNP-lysine Sepharose beads (Fig. 3). Free λ chains are predominantly dimers in solution and the L^{315} dimer is known to bind ε-DNP-lysine (36, 37). In general, differences in the binding of various λ chains to ε-DNP-lysine beads paralleled differences in the binding activity of the reconstituted Igs formed by the same chains with H^{315} and $H^{8\text{-}47}$. In both assays, the λ1 chains were inactive and the λ2 and λ3 chains were active, except for $L^{5\text{-}7}$ and L^{49}. (There were, however, some differences between the two assays: e.g., isolated L^{315} bound better than the other λ2 or λ3 chains to the DNP-lysine beads whereas, on reassociating with H^{315}, $L^{8\text{-}13}$ formed the highest affinity Ig.) That there were some differences is not surprising because the affinity of the isolated λ chains for DNP is lower than that of the corresponding reconstituted H–L Igs by a factor of at least 1000, and there are doubtless significant differences in the way DNP ligands are bound by λ chain dimers and by Ig molecules.

Diversity at gene segment junctions is well-known for κ and H chains (6, 7, 23) but prior to the present studies (see also ref. 14) had not been reported in λ chains. This detection was a direct consequence of analysis of λ chain contributions to ligand-binding activity. The amino acid at the Vλ–Jλ boundary previously appeared to be invariant in each subtype (for refs., see Fig. 2): tryptophan at position 98 of λ1 chains, phenylalanine at position 98 of λ2 chains, and histidine at position 97 of λ3 chains. However, the nucleotide sequences of the *Vλ* and *Jλ* gene segments (4, 5) indicate that several junctional variants are possible and that their number and position vary with λ chain subtype (Fig. 4): three in λ1 (tryptophan, leucine, or phenylalanine at position 98), two in λ2 (phenylalanine or tyrosine at position 98), and two in λ3

(histidine or glutamine at position 97). By focusing on chains with unusual ligand-binding activities, we identified a λ2 chain (L^{5-7}) with tyrosine at position 98 and a λ3 chain (L^{C49}) with glutamine at position 97. A λ1 chain (from an antibody with exceptionally high affinity) with leucine instead of the usual tryptophan, at position 98 has also been identified (K. Tamoto, personal communication). It is possible that because of a critical effect of junctional residues on tertiary structure amino acid variations arising from gene segment assembly (*V–J* and perhaps *V–D–J*) constitute an important source of ligand-binding diversity of antibodies in general.§

§Numbering system: All sequenced murine λ chains have the same number of V region amino acid residues. In this paper, the positions are, accordingly, numbered sequentially, 1–110, from the amino terminus to the end of the *J* region. The numbering is not interrupted by the letter designations (for residues in the first and third CDR) introduced by Kabat and Wu (24) to maximize sequence homology of V regions of H, κ, and λ chains. As a consequence of this difference, the chain positions designated here as 96, 97, 98, and 99 correspond to positions 94, 95, 96, and 97, respectively, in the Kabat–Wu numbering system.

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