cDNA clones encoding rabbit immunoglobulin λ chains

Evidence for length variation of the third hypervariable region and for a novel constant region

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Five cDNA clones designated pDH2, pDH8, pDH9, pDH31 and pDH101 encoding rabbit immunoglobulin λ light chain sequences have been characterized. Comparison of the V λ sequences suggests that, in addition to an increased divergence in all of the complementarity-determining regions (CDRs), variable-region diversity is amplified by the length heterogeneity of the CDR3, at the V λ –J λ junction. An insertion of four codons at positions 48a–d has been noted in three cDNA sequences. This insert, not found in λ nor κ light chains of other species, has a variable sequence, suggesting its possible implication in expanding variability of the CDR2. One of the cDNA clones was shown to encode a novel C λ region which differs by four amino acid substitutions from the C λ region common to all the other clones. Thus, the rabbit can use two different C λ genes, which might correlate with the expression of the two known allotypes of λ chains, C7 and C21. Southern blotting experiments indicate a small number of germ-line V λ genes and the cDNA nucleotide sequence data reported here suggest that several of these genes can be expressed. The possibility of at least two V–J–C gene clusters is discussed.

INTRODUCTION

Immunoglobulin κ and λ light chains have been shown to be encoded by three distinct genetic elements: a V gene and a joining (J) mini-gene encode the light-chain variable domain, whereas the constant domain is encoded by the C gene (for reviews, see, e.g., Tonegawa, 1983; Yancopoulos & Alt, 1986). Unprocessed mRNA also includes two introns, one of about 100 bp within the leader segment of the V gene and the other of 1–5 kb situated between the V–J and C regions (Honjo, 1983; Yancopoulos & Alt, 1986).

Both the mouse and human κ systems comprise 50–100 germ-line V κ gene segments separated by an unknown distance from a cluster of five J κ mini-gene segments. A unique C κ gene is located 2.5–4 kb downstream of the J κ mini-genes (Lenhard-Schuller *et al.*, 1978; Seidman *et al.*, 1978; Max *et al.*, 1979). Much of the diversity of the V κ -region is due to combinatorial association of the multiple V κ and J κ genes and junctional flexibility during the V κ –J κ recombination process (Tonegawa, 1983; Yancopoulos & Alt, 1986), while further amplification of the V-region diversity arises from somatic mutation mechanisms (Honjo, 1983; Tonegawa, 1983; Yancopoulos & Alt, 1986).

The rabbit κ system, by contrast, displays a much more complex organization. The C κ 1 gene in the domestic rabbit exhibits extensive polymorphism reflected in the expression of multiple allotypes, namely b4, b4 var, b5, b6 and b9 (Mage, 1981, 1985). The predominant rabbit light chain isotype is that encoded by the b4 allele. A second C κ gene, C κ 2 (Emorine & Max, 1983; Heidmann & Rougeon, 1983a), encodes the *bas* light-chain and is predominantly expressed in the *Basilea* rabbit variant strain which characteristically has an elevated level of λ -chains (Kelus & Weiss, 1977; Jaton & Kelus, 1977;

Garcia & Jaton, 1979). The C κ 1 gene is linked to a cluster of five J κ segments, of which only one appears to be functional in the case of the b4 allele (Emorine *et al.*, 1983; Heidmann & Rougeon, 1983b), although in the b9 allele, two out of the five J κ segments are expressed (Akimenko *et al.*, 1986). The V κ gene diversity is expanded by length heterogeneity at the 3' ends of V κ germ-line gene segments, four of which have been sequenced and shown to have as many as four extra codons (Heidmann & Rougeon, 1984). An analogous variation in the lengths of mouse and human V κ germ-line genes has not been observed.

The λ locus is much less complex in inbred strains of the mouse. Two germ-line V λ gene segments have been identified (Bernard *et al.*, 1978; Tonegawa *et al.*, 1978), each associated with its own cluster of two J λ –C λ gene pairs (Selsing *et al.*, 1982). This very restricted V λ gene repertoire is compensated for by a high level of somatic mutation events affecting the complementarity-determining regions (CDRs) which expand V-region diversity. Similarly, because the chicken λ locus has one unique functional V λ gene associated with a single J λ gene segment (Reynaud *et al.*, 1985), somatic mechanism(s) must have a major role in the establishment of a functional immune system with an adequate antigen-recognition capacity.

In the rabbit, two serologically distinct allotypic forms of λ light chains, C7 and C21, controlled by the *c* locus, have been described (for a review, see Mage, 1981, 1985). They segregate as alleles in some rabbit colonies, whereas they do not in others (Gilman-Sachs *et al.*, 1969). It has been suggested that C7 and C21 allotypes may reflect two or more different pseudo-allelic C λ subtypes or even V λ subgroups (Mage, 1985), thus making the structural correlate of these specificities uncertain. Attention has been directed to the rabbit *c*

Abbreviations used: V, J and C regions, variable, joining and constant regions; FR, framework region; CDR, complementarity-determining region; SSC, 0.15 M-NaCl/0.015 M-sodium citrate.

Fig. 1. Strategies for the determinations of the nucleotide sequences of cDNA clones encoding rabbit λ light chains

The heavy bars represent the cDNA inserts within the plasmid vector. The gaps indicated by () were added to account for sequences present only in clone pDH2. The extent of a Vλ-specific probe derived from clone pDH7 is indicated. Only those restriction endonuclease sites used to subclone fragments for sequencing are indicated: *Hind*III, ▽; *Eco*RI, ▽; *Sal*I, ▽; *Hae*III, ▽; *Pvu*II, ▽; *Pst*I, ▽; *Alu*I, ▽. Sequence determinations were either by the dideoxynucleotide chain termination procedure of Sanger *et al.* (1977), (●→, ○→) or by the chemical degradation method of Maxam & Gilbert (1977) (■→, □→). The closed symbols represent the first base of sequences read from the sense strand and open symbols indicate the readings from the mis-sense strand. The extent of each determination is given by the length of each arrow. All determinations were made at least twice.

locus due to the availability of the *Basilea* strain (Kelus & Weiss, 1977), the splenic cells of which have an elevated level of λ-chain mRNA, as reflected in the increased proportion of λ-chains among the total light chain population (Jaton & Kelus, 1977; Garcia & Jaton, 1979; Garcia *et al.*, 1982). A cDNA probe encoding a Cλ region (Duvoisin *et al.*, 1984) was used to identify four distinct germ-line Cλ genes, none of which appear to be expressed in rabbits (Duvoisin *et al.*, 1986). We have recently determined the nucleotide sequence of a cDNA encoding the entire leader and variable regions and most of the constant region of one λ-chain; the V-region exhibited remarkable features, e.g., the insertion of four codons in the vicinity of the FR2-CDR2 boundary (Kabat *et al.*, 1983) which distinguishes rabbit λ-chains

from λ and κ chains of other species, and a significant homology between the Vλ CDR2 sequence and a human D2 mini-gene sequence (Hayzer & Jaton, 1987).

As an approach to the structural characterization of rabbit λ chains, we have determined the nucleotide sequences of five cDNA clones encoding complete or partial Vλ regions, together with the Cλ regions. A comparative analysis of these data is reported below.

EXPERIMENTAL

cDNA cloning and nucleotide sequence determination

Procedures for the synthesis and the screening of a recombinant cDNA library and nucleotide sequence

determinations have been described elsewhere (Hayzer & Jaton, 1987).

Southern blot analysis of rabbit genomic DNA

High- M_r rabbit liver DNA was extracted by the procedure of Jeffreys (1979). DNA (10 μ g) was digested with *Eco*RI and analysed on a 0.7% agarose gel. Southern transfer to nitrocellulose was by the procedure of Maniatis *et al.* (1982). A 419 bp *Hind*III-*Pvu*II fragment from cDNA clone pDH7 (Hayzer & Jaton, 1987) consisting of 8 bp of the multiple cloning region of plasmid vector pUC8, the poly(dG)-poly(dC) tail, 20 bp of the 5'-untranslated region, the entire leader and V λ regions, and all but the last five codons of the J-region (see Figs. 1 and 2) was used as a specific V λ probe. The latter was polymerized by self-ligation (Heidmann *et al.*, 1981) and nick-translated (Maniatis *et al.*, 1982). Hybridizations were as according to Thomas (1980) and the blot was washed twice at room temperature in $2 \times$ SSC/0.1% SDS and twice at 50 °C with $2 \times$ SSC/0.1% SDS, before exposure to Kodak XAR-5 film for 2 days at -70 °C with intensifying screens.

RESULTS

Cloning and sequence determination

The cDNA library constructed with purified poly(A)-containing mRNA isolated from the spleen of a hyperimmunized *Basilea* rabbit, when screened with a C λ -region specific probe, yielded 13 strongly hybridizing recombinant plasmids (Hayzer & Jaton, 1987). Of these clones, five, pDH2, pDH8, pDH9, pDH31 and pDH101, were selected for further characterization on the primary basis of the longest inserts and subsequently on the lengths of those portions of the inserts not hybridizing to the C λ specific probe (and, therefore, most likely encoding V λ -regions). Partial restriction maps and the strategy for the determination of the nucleotide sequence of each cDNA insert are shown in Fig. 1, and the sequences are compared in Fig. 2 with that of the first cDNA clone, pDH7, encoding a λ -light chain recently determined in our laboratory (Hayzer & Jaton, 1987).

Nucleotide sequences of cDNAs encoding rabbit λ -light chains

Of the five clones examined, pDH101 contains the longest cDNA insert and encodes an entire λ -chain together with the 3'-untranslated region, except for the leader segment and the N-terminal 18 codons of the mature protein. Three other cDNAs, pDH31, pDH2 and pDH9, encode differing lengths of V-regions, entire C-regions and portions of the 3'-untranslated regions. Clone pDH8 encodes five amino acids of the leader, the entire V and J regions and a partial C region. It ends at the same base position as does clone pDH7 (Fig. 2), 12 codons before the expected termination codon [i.e., if it is assumed that the C-region of clone pDH8 is identical to that found in clone pCL14 (Duvoisin *et al.*, 1984), a cDNA which extends across the TAG termination codon into the 3'-untranslated region]. This coincidence was unexpected; the short inverted repeat, apparent in the other sequences, which immediately follows the last base of the clones pDH7 and pDH8 (boxed in Fig. 2) may have resulted in a premature termination of the second strand due to the formation of a stem-loop structure.

Variable domain

It is evident from Fig. 2 that base substitutions occur in both the FRs and the CDRs, with a higher frequency in the latter. Only in the CDR3 do replacement substitutions predominate. Two of the sequences, pDH8 and pDH101, although not identical, exhibit a number of linked base changes (indicated by arrows in Fig. 2), when compared with the sequence of pDH7. Most of these lead to amino acid changes, at positions 31,34 (CDR1), 45 (FR2), 53 (CDR2) and 90 (CDR3) (Fig. 3).

We have identified (Hayzer & Jaton, 1987) a stretch of twelve nucleotides in the vicinity of the FR2-CDR2 boundary of clone pDH7, after the ATG (Met) codon at position 48, which are not found in homologous positions in human, mouse, chicken or pig κ and λ chains (Kabat *et al.*, 1983). The data of Figs. 2 and 3 confirm and extend this observation to two additional rabbit cDNA-encoded sequences.

If one considers codon 96 to be the first J-codon, in accordance with Kabat *et al.* (1983), one of the most distinctive features of the rabbit V λ -domains is the heterogeneity in the length of the CDR3 at the V-J junction. The V λ -region represented in clone pDH2 contains four extra codons (designated 95a-d) when compared with clones pDH8, pDH101 and pDH31 or three extra codons relative to the λ -chains encoded by clones pDH7 and pDH9. The CDR3, which contributes to the antibody-binding site (Kabat *et al.*, 1983) is nine amino acids long in mouse λ -chains (following Cys-88; Kabat *et al.*, 1983) and is encoded by the last seven codons of the V λ 1 or V λ 2 gene segments and the first two of the J λ segments (Bernard *et al.*, 1978; Tonegawa *et al.*, 1978). The rabbit λ -chain CDR3 is either nine residues long (clones pDH8, pDH101 and pDH31), ten (clones pDH7 and pDH9) or thirteen (clone pDH2). The possible genetic significance of this finding will be discussed below.

J segment

If our assumption that codon 96 is the first J-region residue in the rabbit is correct, as has been shown for the murine (Kabat *et al.*, 1983) and chicken (Reynaud *et al.*, 1985) J λ regions, the J regions encoded by cDNA clones pDH7 and pDH101 are identical (Fig. 2); two silent substitutions, at the third base position, are evident in codons 96 and 97 of clone pDH8 and a silent substitution in codon 103 of pDH31. There is homology at the junction between the V λ and C λ regions with human and mouse J λ sequences (Kabat *et al.*, 1983), i.e. seven to nine residues among the eleven residue positions 98-107 (Fig. 3) that are not involved in the CDR3.

Constant domain

Of the seven nucleotide sequences of the rabbit C λ region available (Figs. 2 and 3), one is unique, the C region of clone pDH2. The latter differs by single-base silent substitutions in ten positions (within codons 117, 130, 132, 141, 146, 150, 152, 173, 198 and 202), one single-base replacement substitution in codon 171 (serine for asparagine) and three double-base replacement substitutions in codons 142 (glycine for arginine), 153 (threonine for asparagine) and 154 (proline for serine). The glycine-142, threonine-153 and proline-154 are common to the mouse C λ 1 sequence. Proline-154 is also present in human C λ sequences (Kabat *et al.*, 1983). Rabbits can, therefore, express two distinct constant

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Fig. 4. Southern blot analysis of rabbit liver DNA with a V λ -specific probe

DNA was extracted from the livers of rabbits 810803 (C7⁻/C21⁺), 493VB-3 (C7⁺/C21⁺) and A738-2 (C7⁺/C21⁻), digested with *Eco*RI, and hybridized with the V λ -specific probe as described in the Experimental section. A tracing from the original autoradiogram is shown to clarify the band positions. Broken lines indicate bands of weaker intensity.

were found to be heterogeneous in size at their 3' ends (Heidmann & Rougeon, 1984). Variability in the length of the CDR3 of light chains does, however, appear to be unique to rabbit κ and λ chains and to human λ chains (Kabat *et al.*, 1983). The diversity of the CDR3 in mouse and human VH regions, where VH germ-line genes are conserved at the 3' end, is augmented by size heterogeneity created by D mini-genes (Kurosawa & Tonegawa, 1982), N-region insertions (Alt & Baltimore, 1982) and junctional deletions (Tonegawa, 1983). The presence of analogous D or N-segments in the rabbit V λ sequences, although very unlikely, cannot be ruled out in the absence of germ-line V λ gene sequence data.

Our data suggest the expression of at least three distinct V λ genes, exemplified by cDNA clones pDH8, pDH31 and pDH101 representing one gene, pDH7 and pDH9 a second, and pDH2 the third. This would indicate that the expressed V λ gene repertoire of the rabbit is larger than that of the inbred mouse, with only two V λ genes (Bernard *et al.*, 1978; Tonegawa *et al.*, 1978), or the chicken, with a single expressed V λ gene (Reynaud *et al.*, 1985). The limited number of V λ genes estimated from the Southern blot data of Fig. 4 is consistent with such an interpretation. Similarly, in human, about ten genomic DNA bands hybridizing with a human V λ probe have been detected (Tsujimoto & Croce, 1984; Anderson *et al.*, 1984) and seven V λ -region subgroups are recognized (Kabat *et al.*, 1983; Anderson

et al., 1984). Because the number of V λ chain subgroups in the rabbit is unknown, it is impossible to estimate if our V λ probe detects a large proportion of the V λ gene repertoire or only a subset thereof.

The expression of a single J λ gene, as in the chicken (Reynaud *et al.*, 1985), would be likely if one considers the base substitutions in codons 97 and 103 (Fig. 2) as having resulted from somatic mutations. Variation in codon 96 can be accounted for by variability in the exact internucleotide site of V-J recombination, although somatic mutation cannot be totally ruled out (Tonegawa, 1983). Alternatively, if all of these differences are germ-line-encoded, the cDNA J λ sequences would be compatible with the expression of five germ-line J segments.

From the data reported in the present paper, it is clear that *Basilea* rabbits can express two different C λ genes: of seven C λ -region sequences compared, six are identical. That encoded by cDNA clone pDH2, however, differs at four amino acid positions. Comparison with the known three-dimensional structure of the human Mcg λ -chain (Schiffer *et al.*, 1973) indicates that the most probable locations of these four residues (142, 153, 154 and 171) are within hair-pin loops exposed at the surface of the C-domain. Positions 153 and 155 have been shown to correlate with rat κ chain allotypes (Sheppard & Gutman, 1981); a mouse λ chain allotype involves a substitution at position 153 (Arp *et al.*, 1982) and the human Km allotypes have been localized to positions 153 and 191 of κ chains (Hilschmann & Craig, 1965). Similarly, positions 153 and 154 could contribute to the formation of allotypes in rabbit κ chains (McCartney-Francis *et al.*, 1986); hence, the substitutions found in clone pDH2 at positions 153 and 154 might correlate with the allotypic specificities of λ chains (Mage, 1981, 1985). In this respect, the substitution of the hydrophobic residue, proline, for the hydrophilic serine residue at position 154 is expected to have a significant influence upon the folding of this region of the C-domain. It should also be mentioned that the C λ region encoded by the cDNA clone pDH2 is not identical with any of four rabbit germ-line C λ gene sequences recently reported (Duvoisin *et al.*, 1986).

The apparent association of distinct V λ -hybridizing sets of bands (Fig. 4) with either the C7 or the C21 allotypic specificity deserves some comments: the V λ -hybridizing banding pattern for C7⁺C21⁺ rabbits cannot be readily explained by the summation of the V λ -hybridizing bands found in C7⁺C21⁻ and those of C7⁻C21⁺ rabbits. DNA bands 2 (C7⁺C21⁻), 6 and 9 (C7⁻C21⁺) are not represented in the genome of the C7⁺C21⁺ animal. This observation may be compatible with the non-Mendelian segregation of the *c* locus allotypes reported to occur in some rabbit colonies (Gilman-Sachs *et al.*, 1969; Mage, 1981) and suggests that the C7 and C21 genes are present on the same chromosome. Functional inactivation of one set of V-J-C genes by mutational events (e.g., deletion, point mutation, etc.) would result in a pseudo-allelic segregation.

Two models could be envisaged to account for the data shown in Fig. 4. One model would be that groups of V λ genes are organized in a single cluster separate from a cluster of J-C gene units. Each V λ group would then preferentially rearrange with a specific J-C gene unit. A second model is that each of several J-C clusters is linked

with its own V λ gene group, an organization analogous to the mouse system where two V-(J-C)-(J-C) clusters have been described (Blomberg *et al.*, 1981; Miller *et al.*, 1981). In rabbits, however, the situation is probably more complex, as there may be more V λ gene segments expressed (the present paper) and more germ-line C λ gene segments (Duvoisin *et al.*, 1986) than in the mouse genome (Bernard *et al.*, 1978; Blomberg *et al.*, 1981; Miller *et al.*, 1981).

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