LACK OF K1b9 LIGHT CHAINS IN BASILEA RABBITS IS PROBABLY DUE TO A MUTATION IN AN ACCEPTOR SITE FOR mRNA SPlicing

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Four forms of k immunoglobulin chains, known as the b4, b5, b6, and b9 allotypes, are products of allelic genes at the k1 locus in domestic rabbit populations (1). A gene (k2) for a second k chain isotype (K2) is apparently present in all rabbits (2-5); one allelic form of this gene is expressed at low levels in wild rabbits and some rabbits of b9 allotype (6-10). The Basilea strain was developed by Kelus and Weiss (11) from such a b9 rabbit in which an apparent mutation resulted in failure to produce the major type of Ig k light chains (K1). In these rabbits, the majority of Ig bear k light chains, and a small proportion bear k chains of the second isotype (K2) (8, 11-13). To understand the unusual k chain expression in Basilea rabbits, we have undertaken an analysis of the k genes in these animals. This report describes our investigations of the molecular basis for the loss of expression of K1 chains in the Basilea mutant.

Rabbits are unusual in that they have two constant region k chain (Ck)1 genes (4, 5, 14-16). Although they have not yet been physically linked (4), the k1 and k2 genes are transmitted as linked traits in breeding colonies, and thus far no recombinants have been observed (6, 7, 9-11). The k1 and k2 genes each have their own J (joining region) clusters (3, 5, 15). However, it is not known whether both Ck genes associate with the same variable region k chain (Vk) gene pool. Ck1 genes show extensive polymorphism. Although the allelic b allotypes were first defined by serological and chemical analyses, the polymorphisms have now been studied by DNA sequencing (reviewed in 1, 16-18). The DNA sequence homologies of the portions encoding K1 constant regions (Ck1) range from 79% (b5 to b9) to 86% (b4 to b5) (16, 17). The known Ck2 sequences are most similar to Ck1 b9 (~89% homology) (17, 18). We used probes from the previously characterized cDNA (5, 18) and genomic clones to show, by Southern hybridization and restriction mapping, that both k1 and k2 genes are present in DNA of Basilea rabbits, and that there are no detectable gross insertions, deletions, or other rearrangements in the k1 gene. For a finer analysis, we isolated and sequenced the mutant k1b9 gene. We found a G -> A change in the highly conserved AG dinucleotide of the 3' acceptor splice site of the J-C intron. This substitution at

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1 Abbreviations used in this paper: C, constant region; IVS, intervening sequence; J, joining region; SSC, saline sodium citrate; SDS, sodium dodecyl sulfate; UT, untranslated region; V, variable region; Y, pyrimidine.
a position required for normal intron splicing (19–24) provides a likely molecular explanation for the loss of K1 expression in these animals.

Materials and Methods

Rabbits and Breeding. All of our b9 rabbits derive from a single male (R3) obtained from Dr. S. Dubiski (University of Toronto, Canada) in 1968. All other rabbits were from closed pedigreed breeding colonies. The discovery of the Basilea mutant and development of the Basilea strain have been described (10, 11). Donors of kidneys for DNA preparation were: AV251-7 and 507VT-3 (homozygous Basilea), 498FE-3 (homozygous b5), and Z211-4 (homozygous b9).

Enzymes. Restriction enzymes were from New England Biolabs (Beverly, MA) or Boehringer Mannheim Diagnostics, Inc. (Indianapolis, IN).

DNA Preparation. High molecular weight DNA was prepared from fresh kidneys according to a modification (25) of the procedure of Gross-Bellard et al. (26).

Probes. The cDNA clone pbas-3C8 that encodes the expressed K2 light chain from a Basilea rabbit (18) was the source of C, and V, probes. Digestion of pbas-3C8 with Alu I results in a 248 basepair (bp) fragment encoding C region sequence from amino acids 125–208 (C2 probe) and a 335 bp fragment containing 248 bp of V sequence, as well as J region and 48 nucleotides of C region (V, probe). The C2 probe is 90.3% and 82.8% homologous to the C1 genes from b9 and b5 rabbits, respectively. The X2 probe is a previously described 490 bp Pst I fragment from the portion of the J-C intron of xlb4 genes that is deleted from x2 genes (5). All probes were labelled with α-[32P]dCTP (Amersham Corp., Arlington Heights, IL) by nick-translation (27).

Southern Blots. Genomic DNA blots were prepared as described (28, 29) and were hybridized for 18 h at 55°–60°C in 6X saline sodium citrate (SSC) containing 5X Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS), 10 mM EDTA, and 100 μg/ml sonicated salmon sperm DNA (29). Filters were washed in 2X SSC, 0.1% SDS at 30°C for 10 min and in 0.1X SSC, 0.1% SDS for 30 min at the temperatures indicated in the Results section.

DNA Cloning. Genomic DNA (500 μg) from bas/bas rabbit 507VT-3 was digested to completion with Eco RI (3 U/μg). The digest was size-fractionated by centrifugation in 5–20% NaCl gradients for 16 h at 18,000 rpm in a Beckman SW27 rotor. Based on Southern data, fragments 10–20 kilobases (kb) long were ligated to Charon 4A Eco RI arms (Amersham Corp., Arlington Heights, IL) and packaged (30) using Packagene extracts (Promega Biotec, Madison, WI). The library was not amplified. 280,000 plaque plaques were screened with nick-translated C2 probe as described (31). The four positive plaques were isolated and rescreened with the X2 probe. The filters were hybridized as for Southern blots, and washed at 50°C in 0.1X SSC, 0.1% SDS. DNA from the recombinant phage clones 796.3λ (x1) and 552.3λ (x2) was analyzed by restriction mapping and Southern blotting. Restriction fragments carrying C, sequences, as evidenced by hybridization to the C2 probe, were isolated by preparative agarose gel electrophoresis, and used for subcloning.

Plasmid pBG35 was constructed by inserting a 3.5 kb Bgl II fragment from clone 796.3λ into the Bam H1 site of pUC-13. This subclone contains ~1,700 bp of 5' flanking region, in addition to the complete C1 exon.

Sequencing. All sequencing was done by the dideoxynucleotide termination method (32) on single-stranded M13 phage clones (33). Plasmid pBG35 was digested with Pvu II, and with Bam HI plus Sst I. A 1.25 kb Bam H1–Sst I fragment, which contains the entire C1 exon, was isolated and subsequently digested with Sau 3AI. Individual Pvu II and Sau 3AI fragments were cloned into the M13 mp8 Sma I and M13 mp9 Bam HI vectors, respectively. Bam H1–Sst I and Sau 3AI–Sst I fragments were directionally cloned into Bam H1–Sst I–digested M13 mp18. The sequence reported here was obtained on both strands. The region spanning the 3' acceptor splice site of the J-C intron was sequenced in five independent clones. Sequences were analyzed using the National Biomedical Research Foundation's ALIGN and COMPARE programs.
Results

Identification of \( C_1 \) and \( V_\kappa \) Genes in Southern Blots. One explanation for the loss of expression of the \( \kappa 1 \) gene in Basilea rabbits could be deletion of \( C_1 \) or \( V_\kappa \) structural genes. To determine whether the Basilea rabbit had suffered a deletion of the \( C_1 \) gene, we carried out a series of Southern blot hybridization analyses using a 248 bp fragment from the C region of \( \kappa 2 \) cDNA clone pxbas-3C8 (18). Because the probe is \( \sim 90\% \) homologous to the \( C_1 \) from b9 rabbits, we used conditions known (5, 14) to permit detection of crosshybridizing genes. Basilea kidney DNA was digested with Eco RI, Bgl II, Pst I, Bam HI, and Sst I, electrophoresed, and hybridized to the \( C_\kappa \) probe. As can be seen in Fig. 1, two bands of similar intensity were detected in each digest. The sizes of the bands in the Sst I digests (5.4 and 3.3 kb) correspond to those previously reported (5, 14) to contain \( C_1 \) and \( C_2 \) genes in DNA from b4, b5, b6, and b9 rabbits. These results suggested that lack of expression of \( \kappa 1 \) light chains in Basilea rabbits is not due to a deletion of the \( C_1 \) gene.

To extend these findings, Eco RI digests of DNA from Basilea, b5, and b9 homozygous rabbits were compared (Fig. 2). The bands of \( \sim 11 \) and \( \sim 14 \) kb seen in Basilea DNA were indistinguishable from those found in normal b9 DNA, strengthening the conclusion that the \( C_1 \) gene in Basilea is not deleted. Three fragments that hybridized to the \( C_\kappa \) probe were detected in DNA from the b5 rabbit; an \( \sim 14 \) kb band shared in common with the other DNA, and two smaller bands corresponding to the \( C_1 \) gene, which is known to have an internal Eco RI site (14, 34).

When the same blot was hybridized with a \( V_\kappa \) probe, \( \sim 30 \) bands of differing intensity were detected. The patterns obtained with the b5, b9 and Basilea DNA were strikingly similar, suggesting that the defect in Basilea rabbits is probably not due to a loss of \( V \)-region genes.

Identification of \( C_1 \) and \( C_2 \) Genes in Southern Blots. We attempted to find differences between normal \( \kappa 1 \)b9 and Basilea \( \kappa 1 \)b9 genes by Southern analysis. As described above, two bands that hybridize strongly to a \( C_2 \) probe were detected in digests of b9 and Basilea DNA. Probing with \( C_2 \) under high stringency conditions allowed us to distinguish \( C_1 \) and \( C_2 \) in the various digests. The identification of \( \kappa 1 \) was confirmed by hybridization to the X2 probe derived from a region in the J-C intron of \( \kappa 1 \) genes that is absent from \( \kappa 2 \) genes (5). Fig. 3 shows representative results with the Sst I digests of Basilea and control DNA. Similar identifications were made on blots of Bam HI, Pst I, Eco RI, and Bgl II digests (not shown). In Eco RI digests of Basilea and b9 DNA, the \( \sim 11 \) and \( \sim 14 \) kb bands represent the \( C_1 \) and \( C_2 \) genes, respectively. \( C_1 \) genes were always found in fragments of the same size in Basilea and normal b9 DNA, although there was restriction fragment length polymorphism of \( \kappa 2 \) fragments in some digests. These experiments did not reveal gross rearrangements or deletions in the vicinity of the \( C_1 \) exon in Basilea DNA. Furthermore, at least a portion of the intron detected by the X2 probe and present in \( \kappa 1 \) genes is also found in the nonexpressed \( \kappa 1 \) gene of Basilea. Thus, we hypothesized that the loss of expression of the \( C_1 \) gene might be due to a subtle change within or in the vicinity of the \( C_1 \) gene itself. To investigate this possibility we cloned and sequenced the \( \kappa 1 \) gene from Basilea.
Cloning of κ Genes from Basilea. Genomic clones containing the κ2 gene (552.3Å) and the κ1 gene (796.3Å) were identified based on hybridization to the C2 and X2 (κ1-specific) probes. Restriction mapping of these clones with EcoR1, SstI, PstI, BglII, and BamHI and Southern hybridization revealed Cκ-bearing fragments of sizes entirely consistent with the results of genomic blots.
FIGURE 2. Southern analyses of Eco RI digests of DNA from homozygous b5, Basilea, and b9 rabbits. The electrophoresis, C,2 probe, and washing conditions were as described in Fig. 1. The V probe is a 335 bp fragment from pbas-3C8, containing 248 bp of V region sequence, as well as J region, and 48 nucleotides of C region. The positions of molecular weight markers are indicated.

Sequencing of the C,1 Gene from Basilea. A C,1-bearing 3.5 kb Bgl II fragment from the insert of phage clone 796.3A was subcloned, and the sequence of 849 bp that includes the complete C,1 exon, ~300 bp of 5' intron and ~250 bp of 3' flanking region was obtained (Fig. 4). There is complete identity of the C,1 coding block and 3' untranslated (UT) nucleotide sequences with that of a cDNA derived from a normal b9 rabbit (17).

A Defect in the 3' Acceptor Site for Intron Splicing. In Fig. 5 the region 5' of the C, exon is compared to the published sequences of normal K1b9 (16), K2(b4) (5), K1b4 (3), and K1b5 (14) genomic clones. As noted previously (14, 16), these
**FIGURE 3.** Identification of $\kappa_1$ and $\kappa_2$ genes in Sst I digests of DNA from b5, Basilea and normal b9 rabbits. The blots were washed in 0.1X SSC, 0.1% SDS, for 30 min at the temperatures indicated (°C). The X2 probe is a 490 bp Pst I fragment from the J-C intron of a $\kappa$4 gene (5). The sizes of restriction fragments indicated were calculated from standard curves plotted using Hind III-digested bacteriophage $\lambda$ molecular weight markers.

**FIGURE 4.** Nucleotide sequence of the C1,1 exon and flanking regions of the $\kappa$b9 gene from Basilea DNA. The deduced amino acid sequence of the $\kappa$1b9 light chains is shown in italics. The amino acids are numbered in italics according to the standardized system of Kabat et al. (35). The polyadenylation signal is underlined. Poly(A) is found after position 802 in cDNA (17).
regions are highly conserved. The 5′ region from the Basilea κ1 gene differs at only two positions from a normal K1b9 gene, and is slightly more homologous to κ2(b4) (97.9%) than to the other genes (96.5% and 96.8%). Starting at position 171, there is a length difference in the 5′ intron of κ2(b4), κ1b4, and κ1b5. This appears to be due to a deletion, because the human and mouse J-C introns (36–38) have homology with κ1b9 in this region. The presence or absence of this 11 bp stretch does not seem to correlate with expression of the associated Cκ gene. However, in the consensus 3′ acceptor site for intron splicing TcG the AG preceded by a pyrimidine appears to be required for normal function (19–24). We find a G → A transition at position 295, which changes the normal CAG/G acceptor site to CAA/G. This G → A point mutation provides a likely explanation for the absence of K1b9 light chains in the Basilea strain.

Discussion

The Basilea strain was developed from a mutant rabbit found in a laboratory colony about 12 years ago (11). The spontaneous mutant lost the capacity to produce K1b9 light chains; the other isotype (K2) was unaffected (10). Homozygous Basilea rabbits compensate for this loss by producing elevated levels of λ chains (8, 11–13).
To elucidate the molecular basis for the mutant phenotype, we conducted a series of experiments to determine first whether the $\kappa$1 structural gene had been deleted. Not only did we find that the $\kappa$1b9 gene was not deleted, but we found by Southern blotting that all Basilea DNA restriction fragments that we detected containing $C_{1}$ were indistinguishable in size from those in normal b9 rabbits.

We characterized the $\kappa$1b9 gene from Basilea by sequence analysis. The $C_{1}$ coding block and 3'UT sequences were completely identical to those of cDNA clones derived from normal b9 rabbits. However, comparison with several genomic $\kappa$ chain clones shows a single G→A substitution in the acceptor splice signal of the J-C intron of the Basilea $\kappa$1b9 gene (Fig. 5). The consensus sequence at the 3' acceptor splice site has been found to be a pyrimidine (Y)-rich stretch of $\geq 11$ nucleotides, a nonconserved position, another pyrimidine, and an AG dinucleotide that appears (19–24) to be absolutely required for function: (Y)$_{n}$N$_{3}$AG/. We postulate that the change from (Y)$_{13}$CAG/G to (Y)$_{13}$CAA/G in $\kappa$1b9 of Basilea (Fig. 5) either eliminates normal RNA splicing or makes splicing at this acceptor site less efficient. A large number of mutations that result in $\beta$-thalassemia in man involve changes that alter or destroy the function of RNA splice sites. Point mutations that alter the invariant GT or AG dinucleotides of donor or acceptor sites completely abolish the synthesis of normal globin mRNA. Frequently, mutations lead to activation of otherwise cryptic splice sites in coding or intervening sequences. In addition, changes in consensus nucleotides have been found to markedly affect the efficiency of normal mRNA processing (20, 21).

Mutations that alter donor splice sites are common (20, 21, 39). Mutations in acceptor splice sites have been less frequently observed. Recently, there have been two independent reports of a single A→G change in the AG of the 3' acceptor splice site of the second intervening sequence (IVS-2) of $\beta$-globin genes from American blacks with $\beta$-thalassemia (22, 23). This change of CAG/C to CGG/C eliminates the synthesis of normal $\beta$-globin mRNA; a cryptic splice site in IVS-2 is used.

The mutation we observe in the Basilea $\kappa$1 gene does not eliminate the dinucleotide AG, because after the G→A transition, there is a G in the codon for amino acid 108 (Fig. 5). However, the AG is shifted downstream one position and preceded by an A, thus changing (Y)$_{13}$CAG/G to (Y)$_{13}$CAAG (Fig. 5). Although the invariant AG is usually preceded by a pyrimidine, it is conceivable that this altered acceptor could still function. Since the codon for amino acid 108 of rabbit appears to be formed by one base donated by the J segment and two bases donated by the $C_{1}$ exon, the splicing would result in a frameshift and early termination after only 17 additional amino acids were translated (Fig. 6). The interesting possibility arises that compensation for this frameshift could take place in those pre-B cells in which an abnormal V-J DNA joining event occurs (40, 41). The result of this 5' frameshift would be a light chain with a normal V region, a J region lacking conserved amino acids required for normal function, followed by a normal $C_{\lambda}$ sequence. Such a light chain probably could not form a normal Ig molecule. Although normal b9 light chains have not been detected in Basilea rabbits, some pre-B cells of Basilea rabbits stain with fluorescent anti-b9 (42), and small amounts of protein that have some b9-like determinants have
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FIGURE 6. The codon for amino acid 108 of rabbit C, genes appears to be formed by one base donated by the J segment and two bases donated by the C, exon. In the klb9 gene of Basilea, a frameshift would occur if splicing occurred after the AAG, as shown. The donor sites of klb9 genes are inferred from the sequence of normal klb9 cDNA (17).

been detected serologically by inhibition assays (43). The b9 inhibitory activity in sera was not found on functional antibodies in rabbits producing anti-SII antibodies (43). Possible sources of such light chains would be through some frameshift correction mechanism, through splicing to a cryptic acceptor site upstream of C, in the J-C intron or downstream within the C, sequence, or less likely, through somatic back-mutation of the A to G.

In recent analyses of RNA produced by Basilea and control parental-type b9 rabbits, sensitive S1 nuclease protection studies with uniformly labeled single-stranded C,b9 probes have shown that low levels of RNA with C,b9 sequence can be detected in spleen cells of Basilea rabbits (0.1–2% of the amounts produced by controls) (44, 45, and N. McCartney-Francis, manuscript in preparation). These RNA species could reflect messages that are unstable and aberrantly spliced, or that have frameshifts as discussed above. To further define the nature of the small amounts of RNA with detectable C,b9 sequence, we are conducting further S1 nuclease protection studies with intron probes (N. McCartney-Francis, E. Lamoyi, and R. Mage, work in progress). Characterization of the exact sequences of these postulated aberrant low-abundance messages will require cloning and sequencing of corresponding cDNA.

Summary

Rabbits of the Basilea strain do not produce normal Klb9 light chains but continue to produce immunoglobulins with light chains of the rare K2 isotype and of \( \lambda \) type. To understand the molecular basis for this unusual expression of \( \kappa \) light chains in Basilea rabbits, we undertook an analysis of their \( \kappa \) genes. We isolated and sequenced the mutant klb9 gene and found a substitution of A for G in the highly conserved AG dinucleotide of the 3' acceptor splice site. Although we cannot rule out additional alterations of portions of the gene we did not sequence, this spontaneous change of the G found in the normal gene provides a likely molecular explanation for the loss of K1 light chain expression in Basilea rabbits.

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