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Multiplicity of constant kappa light chain genes in the rabbit genome: a b4b4 homozygous rabbit contains a $\varkappa$-*bas* gene

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We have constructed a genomic library of homozygous b_4b_4 rabbit DNA in the pJB8 cosmid vector. Clones containing C$\varkappa$-like sequences were screened with a b_4 cDNA probe and were characterized by restriction mapping. One of the clones contained a C$\varkappa$ sequence different from the b_4 allotype normally expressed by the animal. We report here the nucleotide sequence of this gene and show that it probably corresponds to a $\varkappa$-*bas* form of the Basilea allotype. It appears to be a structurally complete gene without any stop codons within the coding region and containing the dinucleotide AG as a splice site acceptor for the J-C junction, just 5' of the coding block. Comparison with the b_4 cDNA nucleotide sequence shows a separate evolution of the C$\varkappa$-coding and 3'-untranslated sequences, since the 3'-untranslated regions are more conserved than the coding regions. Genomic blot analysis would suggest that the $\varkappa$-*bas* gene is isotypic in the domestic rabbit population, since it lies within a genomic *Eco*RI or *Pst*I restriction fragment, which was shown to be common to all homozygous b_4, b_5, b_6 and b_9 rabbit DNAs.
Key words: *b* allotypes/cosmid library/DNA sequence/sequence divergence

Introduction

Rabbit immunoglobulins show an extensive genetic polymorphism called allotypy. The allotypic specificities of the *b* series are located on the constant region of the $\varkappa$ light chain and segregate in a Mendelian fashion like alleles at a single genetic locus. In the domestic rabbit, serological studies have revealed four polymorphic forms of the C$\varkappa$ chain, named b_4, b_5, b_6 and b_9 (Oudin, 1960; Dubiski and Muller, 1967). Furthermore, Kelus and Weiss have developed a variant strain, called Basilea, in which the major light chain component has become lambda type (Kelus and Weiss, 1977; Jaton and Kelus, 1977). This variant strain has been shown to express a kappa-like minor component, named $\varkappa$-*bas*, lacking the *b* allotypic markers (Garcia *et al.*, 1982).

Amino acid sequence analysis of these C$\varkappa$ chains has shown a very high level of divergence, up to 33%, which is unexpected for simple alleles. Furthermore, Strosberg *et al.* (1974) and other authors have reported that individual rabbits could express a wrong or latent allotype, in addition to the nominal allotypic specificities normally found in the serum of the animals (reviewed in McCartney-Francis and Mandy, 1979). These observations have led to the hypothesis that the C$\varkappa$ genes in the rabbit were encoded by duplicated genes controlled by an allelelic regulatory mechanism (Farnsworth *et al.*, 1976).

With a b_4 cDNA probe synthesized in our laboratory (Heidmann *et al.*, 1981), we have shown, by genomic blot experiments, that homozygous b_4b_4, b_5b_5, b_6b_6 and b_9b_9 rabbit DNAs contain multiple nucleotide sequences homologous to the b_4 probe (Heidmann and Rougeon, 1982). To analyze the structure and organization of these C$\varkappa$-like sequences, we have constructed a cosmid genomic library, starting from a homozygous b_4b_4 rabbit DNA and have isolated a recombinant cosmid, C4R-47, carrying a C$\varkappa$-like gene, different from the b_4 allotype normally expressed by the animal. We report here the nucleotide sequence of this C$\varkappa$-like gene and show, by comparison with the amino acid data available, that it probably corresponds to a Basilea form of the rabbit kappa light chain.

Results

Isolation of C$\varkappa$-like gene from a homozygous b_4b_4 rabbit library

Liver DNA from a homozygous b_4b_4 rabbit was partially digested by *Sau*3A and 30-45 kb fragments, purified on a sucrose gradient, were inserted into the *Bam*HI site of the pJB8 cosmid (Ish-Horowicz and Burke, 1981). Recombinant cosmids were screened with the ^{32}P-labeled b_4 cDNA probe, as described in Materials and methods. Since the *Bam*HI site of pJB8 is localized just between two *Eco*RI sites, digestion of the recombinant cosmids with *Eco*RI allows the separation of the genomic fragments from the cosmid DNA. Restriction mapping analysis showed that one of the selected cosmids, C4R-47, contained a 12-kb *Eco*RI fragment which hybridized with the probe. By *Pst*I restriction mapping, this homologous sequence was obtained in a 3-kb fragment. The totality of the genomic fragment inserted into the cosmid vector is of 31 kb, and no other C$\varkappa$-like sequence could be detected with the b_4 probe. In order to analyze the fine structure of the C4R-47 sequence, the 3-kb *Pst*I fragment was purified on an agarose gel and subcloned into the pBR322 vector.

Identification of the C$\varkappa$-like gene

Figure 1 shows the restriction map of the 3-kb *Pst*I fragment and the strategy followed for DNA sequencing. The nucleotide sequence of a 590-bp *Kpn*I-*Pvu*II fragment is given in Figure 2. In one of the reading frames, the nucleotide sequence can be translated into a C$\varkappa$-like amino acid se-

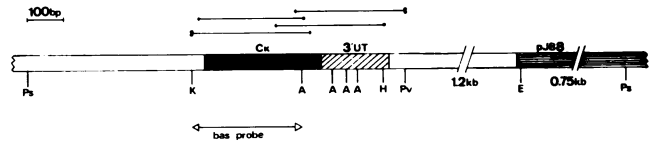

Fig. 1. Restriction map of the $\varkappa$-*bas* gene located on the 3-kb *Pst*I fragment of the C4R-47 cosmid. The coding constant region (C$\varkappa$) and 3'-untranslated region (3'UT) of the $\varkappa$-*bas* gene are located at one extremity of the genomic fragment cloned into the pJB8 cosmid vector. Ps = *Pst*I; K = *Kpn*I; A = *Ava*II; H = *Hin*fI; Pv = *Pvu*II; E = *Eco*RI. The upper part shows the strategy for DNA sequencing. The direction and extent of nucleotide reading are indicated by horizontal arrows. Filled circles specify fragments labeled at their 5' end, empty circles at their 3' end. The arrow, on the lower part, indicates the nucleotide sequence used to construct the C$\varkappa$-*bas* probe as described in Materials and methods.

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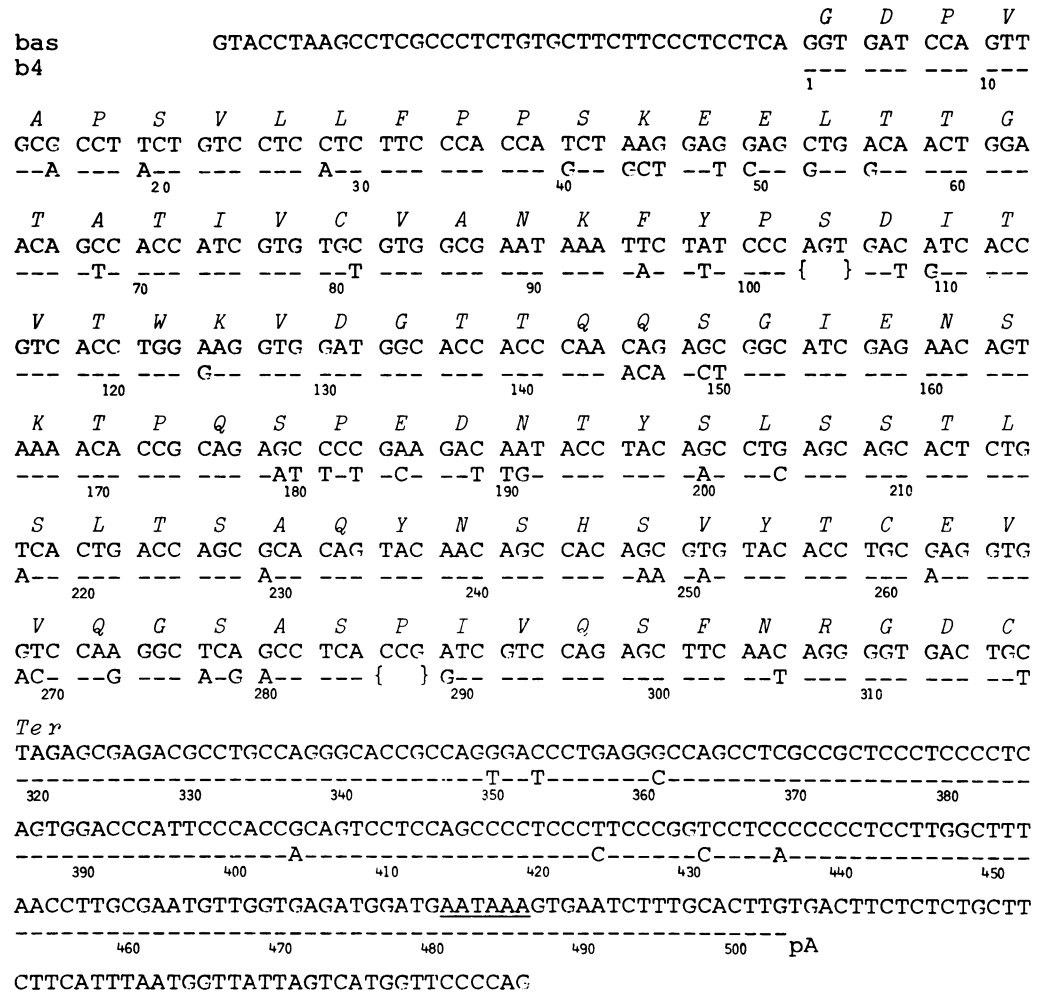

Fig. 2. Nucleotide sequence of the 590-bp *Kpn*I-*Pvu*II fragment containing the *x-bas* gene. The *x-bas* nucleotide sequence was compared with the b_4-cDNA sequence (Heidmann *et al.*, 1981). Only the nucleotides which differ from the *x-bas* chain are mentioned for the *x*-b_4 chain. The homologous positions are indicated by dashes. Parentheses in the b_4 sequence indicate the deletions required to maximize the homology. In the upper line, the amino acid sequence predicted from the *x-bas* nucleotide sequence is indicated by the one-letter-code (Dayhoff, 1976). Ter = chain termination codon. The sequence AATAAA in the 3'-untranslated region is underlined.

quence of 106 amino acids, without any internal stop codons. This C*x*-like sequence starts with a glycine at position 108 and ends with a cysteine at position 214, before the TAG termination codon (Figure 3). The two cysteines, which are involved in the secondary structure of the molecule, are present at position 135 and 195.

This C*x*-like sequence was compared with the known amino acid sequence of b_4, b_5, b_6 and b_9 allotypes (Figure 3) and shows, respectively, only 71, 57, 72 and 76% homology. However, comparison with the partial amino acid sequence of the C*x-bas* allotype (Garcia *et al.*, 1982) shows a very strong homology (Figure 3). Only one amino acid, out of 43 reported, is different: Garcia *et al.* described a hydroxyproline at position 123, whereas the nucleotide sequence of C4R-47 codes for a lysine at that position. We do not know if this difference could correspond to an artefact in the amino acid sequence determination, or if it could represent an allelic variant of the *x-bas* sequence. One feature of the *x-bas* protein sequence is the presence of an asparagine at position 172, replacing the cysteine which is common to all other *b* allotypes. Since the C4R-47 also codes for an asparagine at this position, it seems very likely that the C4R-47 C*x* gene corresponds to the *x-bas* gene.

Comparison of Cx-bas and Cx-b$_4$ nucleotide sequences

To define completely the C*x-bas* gene, the nucleotide sequence of C4R-47 was compared with the cDNA b_4 nucleotide sequence, as shown in Figure 2. Gaps were introduced in the b_4-C*x* sequence to maximize the homology. In the C*x*-coding sequence, the overall homology is 83% at the nucleotide level. Over the 318 bp compared, there are 48 differences and two deletions of three nucleotides at nucleotide position 103 – 105 and 286 – 289. There are only eight silent mutations involving a single change of the third base of the codon, whereas there are 29 amino acid substitutions: 20 resulting from one base substitution, seven from two base substitutions and two from three base substitutions. It can be noticed that the nucleotide changes are essentially concentrated in four segments: four differences between nucleotides 40 and 55, seven between bp 95 and 109, eight between bp 179 and 191 and six between bp 268 and 289.

The same stop codon TAG is used in the *x-bas* and *x*-b_4 genes. At 3' of the stop codon, the nucleotide sequence of C4R-47 can be perfectly aligned with the 3'-untranslated nucleotide sequence of the C*x*-b_4 gene, without introducing any gaps. Thus, out of 185 bp compared, there are only seven

```
          110                 120                 130                 140
C4R-47  G D P V{ }A P S V L L F P P S K E E L T T G T A T I V C V A N K F Y P S
b4      - - - -{ }- - T - - I - - - A A D Q V A - - - V - - - - - - - - Y F -{ }
b5        A T L{ }- A T - - I - - - A S A Q - A - - - V - - - - - - - - Y F -{ }
b6        A T L{ }- - T - - I - - - - S A - - A - - - - - - - - - - - - Y F -{ }
b9        - - P I - - T - - - - - - - A D Q - - - Z - V - - - - - - - - - R - D
bas       - - Z{ }- - - - - - - - - - hP- - - - - - - - - Z- - - - (
            M                                               F

                  150                 160                 170
C4R-47  D I T V T W K V D G T T Q Q S G I E N S K T P Q S P E D N T Y S L S S T
b4      - V - - - - E - - - - - - T T - - - - - - - - N S A - C - - N - - - -
b5      - G - - - - E - - - K P L T T - - - T - - - - - N S D - C - - N - - - -
b6      - G - - - - - - - - - I - - S - - - - - - - - - N G A - C - - N - - - -
b9      - - - - - - - - - D E I - - - - - - - - T - - - - - - - C - -( )- - - -
bas                                             )- - - - - - - - - - - - - - -

        180                 190                 200                 210
C4R-47  L S L T S A Q Y N S H S V Y T C E V V Q G S A S P I V Q S F N R G D C
b4      - T - - - T - - - - - K E - - - K - T - - T T -{ }V - - - - - - - - -
b5      - T - Q K S N - - - - N Q - - - Q L P - - A G -{ }V - - - - S - K N -
b6      - T - -(                                                    )K S -
b9      - - - - K - - - - - - -{ }- - - Q - H N S A G -{ }- - Z - - - - - - -
bas     (                                                             )
```

Fig. 3. Amino acid comparison of domestic rabbit light chain constant regions. The amino acid sequence predicted from the C4R-47 nucleotide sequence has been compared with b_4, b_5, b_6, b_9 and *bas* allotypes. Brackets indicate gaps introduced to maximize the homology. Parentheses represent uncharacterized amino acid residues. Dashes indicate the amino acid residues which are identical to the C4R-47 sequence. Data for the protein sequences are from Chen *et al.* (1975) and Heidmann *et al.* (1981) for b_4; Emorine *et al.* (1979) and Chersi *et al.* (1980) for b_5; Emorine *et al.* (1979) for b_6; Farnsworth *et al.* (1976) for b_9; Garcia *et al.* (1982) for *bas*

nucleotide changes, which gives an overall homology of 96.2%. This 3′-untranslated region contains the hexanucleotide AATAAA and by homology with the Cκ-b_4 sequence, the putative polyadenylation site can be located at nucleotide position 503.

Characterization of the genomic restriction fragment carrying the Cκ-bas gene

We have previously shown by genomic blot analysis that individual rabbits of different allotypes contain several restriction fragments hybridizing with a b_4 cDNA probe (Heidmann and Rougeon, 1982). Whatever the enzyme used, one band was common to all animals (16.5 kb in *Eco*RI digests or 7 kb in *Pst*I digests).

By restriction mapping analysis of the C4R-47 recombinant cosmid, only a 12-kb *Eco*RI fragment and a 3-kb *Pst*I fragment hybridize with the b_4 probe. Since in the C4R-47 cosmid, the Cκ-*bas* gene lies 1.2 kb before the *Eco*RI site of pJB8 (Figure 1), the 12-kb *Eco*RI fragment could correspond to the common 16.5-kb *Eco*RI genomic fragment and thus, the 3-kb *Pst*I fragment to the 7-kb *Pst*I genomic fragment. To test this hypothesis, genomic blot experiments were carried out using a specific Cκ-*bas* probe lacking the conserved 3′-untranslated region (300 bp *Kpn*I-*Ava*II fragment in Figure 1). Liver DNA from a homozygous b_4b_4 rabbit was digested either by *Eco*RI or *Pst*I, blotted on to a nitrocellulose filter and hybridized with the ^{32}P-labeled Cκ-*bas* probe. The filter was washed under high stringency conditions to reveal only highly homologous sequences. The autoradiogram is shown in Figure 4. In an *Eco*RI digest, the Cκ-*bas* probe hybridizes to the 16.5-kb common fragment and to a 8.5-kb fragment, while in a *Pst*I digest the probe hybridizes mainly to the 7-kb common fragment. Further experiments on other recombinant cosmid clones indicate that the 8.5-kb *Eco*RI

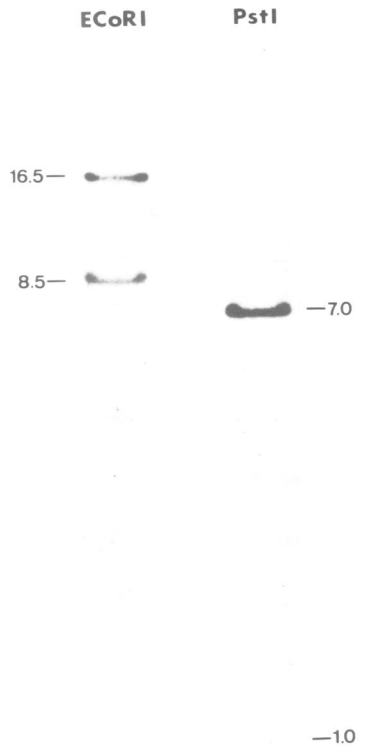

Fig. 4. Genomic blot analysis of b_4b_4 homozygous rabbit DNA with a κ-*bas* specific probe. 25 μg of a homozygous b_4b_4 rabbit DNA, digested with *Eco*RI or *Pst*I, were electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose filter and hybridized to a ^{32}P-labeled Cκ-*bas* probe, constructed as described in Materials and methods. The filter was washed under high stringency conditions (0.1 x SSC, 0.1% SDS, 65°C, 30 min as final wash). Fragment sizes were estimated from an *Eco*RI digest of λ bacteriophage DNA run in a parallel lane.

fragment contains the 5′ part of a Cκ-b_4 gene (data not shown); this Cκ-b_4 sequence is also included in a 1-kb *Pst*I fragment which is faintly revealed by the Cκ-*bas* probe and can be better seen on a longer exposure (data not shown). So, the 12-kb *Eco*RI fragment and the 3-kb *Pst*I fragment, which contain a κ-*bas* gene in the C4R-47 cosmid, correspond to the common bands present in the restriction patterns of all homozygous rabbit DNAs.

Discussion

We have previously reported that the DNA of individual rabbits, whatever their allotype, contains several nucleotide sequences that are more or less homologous to a b_4 cDNA Cκ probe (Heidmann and Rougeon, 1982). The results reported here show that a homozygous b_4b_4 rabbit contains in its genome a Cκ-like gene, different from the normally expressed b_4 allotype and which is probably present in all domestic rabbits.

This Cκ-like gene appears to be a functional gene. The nucleotide sequence codes for a constant region of 106 amino acids without any stop codons within the gene. Comparison with the b_4 cDNA sequence allows the determination of a 3′-untranslated region of 185 bp, beginning with TAG as a termination codon of the coding sequence, and containing, as a polyadenylation signal, the hexanucleotide AATAAA (Proudfoot and Brownlee, 1976), 17 nucleotides before the

putative polyadenylation site. Just 5′ to the coding block, it also contains the dinucleotide AG as a splice site acceptor for the J-C junction (Breathnach *et al.*, 1978), at the same position as in the mouse and human Cκ genes (Hieter *et al.*, 1980). Whether this Cκ gene is associated with its own Jκ segment or not, and whether it is located near the normally expressed b_4-Cκ gene is under investigation. It can be noted, however, that no other Cκ gene is detectable with the b_4-Cκ probe, on a 29-kb genomic fragment, 5′ to this Cκ-like gene.

Comparison with the b_4 nucleotide sequence also shows a separate evolution of the Cκ-coding sequence and the 3′-untranslated region between the two duplicated genes, since the nucleotide coding sequences are 83% homologous, whereas the 3′-untranslated regions exhibit up to 96.2% homology. Such patterns of evolution between segments of duplicated genes have already been observed, particularly in the globin (Slightom *et al.*, 1980) and in the immunoglobulin gene families (for review, see Baltimore, 1981). It was shown that segmental homology between duplicated genes could result from non-reciprocal intergenic conversion (Ollo and Rougeon, 1983). In the rabbit Cκ system, however, until the nucleotide sequence of every *b* allotype is known, it is difficult to assess which part of the gene has been converted. If the b_4 and Cκ-like genes have duplicated a long time ago, the strong homology in the 3′-untranslated region could have resulted from a recent intergenic conversion between the two genes. On the other hand, if duplication occurred more recently, the four segments of high divergence could be looked on as converted elements from other Cκ genes (as for example, Cκ-b_9 for amino acid position 168–170). Since the rabbit Cκ genes represent a very polymorphic but definite system, nucleotide comparisons will probably help in the understanding of the evolutionary mechanisms of DNA sequences.

Comparison of the translated amino acid sequence with the amino acid data available shows that this Cκ-like gene is different from the b_4, b_5, b_6 and b_9 forms, normally expressed in the domestic rabbit, and suggests that this gene codes for a Basilea form of the Cκ light chain. Indeed, the cysteine at position 172 is replaced by an asparagine in the *κ-bas* chain. The single difference with the published partial amino acid sequence is a lysine instead of a hydroxyproline at position 123.

Our results show that, in a b_4b_4 homozygous rabbit, the Cκ-*bas* gene is located on a restriction fragment which is common to all rabbits tested, whatever their allotype. Thus, it seems likely that the Cκ-*bas* gene could be an isotypic gene in rabbit populations. It is interesting to observe that this gene, which appears to be potentially functional, is contained in a DNA region which does not appear polymorphic, as deduced from identity of restriction fragments. However, it is not known whether in allotype-suppressed rabbits, the Cκ-*bas* gene expression becomes enhanced or not. It has been shown that, in contrast to domestic rabbits, wild rabbits express the *κ-bas* phenotype and it has been suggested that *κ-bas*$^+$ was an allele of a *κ-bas* gene which could be expressed in domestic rabbits (Benammar and Cazenave, 1982). The Cκ-like gene described here could be an allelic form of the *κ-bas* gene at the *bas* locus. Hybridization experiments are in progress to assess that hypothesis. If it is so, the *bas* mutation could be simply regarded as a single mutational event that affected the b_9 locus, leading for example to an aberrant J-C junction or to a premature stop codon in the Cκ-coding sequence. Thus, the *bas* mutation could have occurred in any other allotype of the *b* series and analysis of the Cκ genes present in a homozygous *bas/bas* rabbit DNA will provide further light on that mutational event.

Materials and methods

Enzymes and chemicals

All restriction endonucleases, T4 DNA ligase and T4 polynucleotide kinase were obtained from New England Biolabs. Calf intestine alkaline phosphatase, *Escherichia coli* DNA polymerase I and DNase I were purchased from Boehringer Mannheim. Terminal deoxynucleotidyl transferase was from P.L.Biochemicals. α-^{32}P-labeled nucleoside triphosphates (800 Ci/mmol), [γ-^{32}P]ATP and 3′-[α-^{32}P]dATP (cordycepin triphosphate) were purchased from Amersham International.

Construction and screening of a rabbit cosmid library

500 μg of high mol. wt. rabbit liver DNA, prepared as previously described (Heidmann and Rougeon, 1982) was partially digested by *Sau*3A restriction endonuclease, according to Maniatis *et al.* (1978). DNA fragments of 30–45 kb were purified on a 5–20% sucrose gradient run for 3.5 h at 38 K. pJB8 cosmid vector (Ish-Horowicz and Burke, 1981) was digested with *Bam*HI, dephosphorylated with 0.3 U/μg of alkaline phosphatase for 1 h at 60°C, and then extracted three times with phenol and three times with ether. 5 μg of genomic DNA fragments were co-precipitated with 5 μg of cosmid DNA. Ligation was carried out at 15°C for 16 h at a final concentration of 1 μg total DNA/μl with 3 U of T4 ligase/μg. Preparation of packaging extracts, *in vitro* packaging of recombinant DNA molecules, transduction in *E. coli* HB101 cells and plating were performed according to Grosveld *et al.* (1981). The packaging efficiency was $1-5 \times 10^4$ colonies/μg of genomic DNA.

Bacterial colonies were screened at a density of $5-10 \times 10^3$/85 mm diameter plate, using the replication procedure described by Hanahan and Meselson (1980). After chloramphenicol amplification, colonies on nitrocellulose filters were lysed and treated according to Grosveld *et al.* (1981). Filters were hybridized as for Southern blots (Southern, 1975), using the nick-translated rabbit b_4-Cκ probe previously described (Heidmann and Rougeon, 1982). Positive clones were identified by rapid alkaline lysis (Birnboim and Doly, 1977) and analyzed by Southern blot hybridization (Southern, 1975).

Subcloning into pBR322

C4R-47 cosmid DNA was digested by *Pst*I and electrophoresed on a 0.7% agarose gel. The 3-kb fragment was electroeluted from the gel and co-precipitated in a molar ratio with pBR322, which was linearised by *Pst*I and dephosphorylated as described for pJB8. Ligation was carried out at 15 μg/ml with 3 U T4 ligase/μg. Transformation of *E. coli* HB101 cells and screening of *Pst*I-C4R-47 subclones were performed as previously described (Auffray *et al.*, 1980).

DNA sequencing

The nucleotide sequence was determined according to the procedure of Maxam and Gilbert (1980). The DNA fragments were labeled either at the 5′ end with T4 polynucleotide kinase and [γ-^{32}P]ATP, or at the 3′ end with terminal deoxynucleotidyl transferase and 3′-[α-^{32}P]dATP. 3′ protruding sites were labeled at their 5′ end after treatment with the Klenow fragment of *E. coli* DNA polymerase I. The products were analyzed on 20%, 8% and 6% 0.35 mm thick urea polyacrylamide gels according to Sanger and Coulson (1978).

Genomic blot experiments

Southern blot experiments were performed as previously described (Heidmann and Rougeon, 1982).

The rabbit Cκ-*bas*-specific probe was constructed as follows: a 300-bp *Kpn*I-*Ava*II fragment was purified on 5% acrylamide gel from *Pst*I-C4R-47 subclone, and ligated to itself as previously described (Heidmann and Rougeon, 1982). The ligated fragments were nick-translated to high specific activity ($5-10 \times 10^7$ c.p.m./μg) according to Rigby *et al.* (1977).

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