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Two rearranged immunoglobulin kappa light chain genes in one mouse myeloma

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ABSTRACT

The organization of immunoglobulin gene segments coding for kappa light chains has been studied in uncloned and cloned DNA from mouse liver and a mouse myeloma. It is known that the C (constant, ref. 2) gene segment is present in the tumor DNA on two EcoRI fragments of 14 and 20 kb and in liver DNA on a 15 kb fragment. The 14 kb myeloma and the 15 kb liver fragment have been cloned previously. Here we report on the cloning of the 20 kb myeloma fragment and present detailed restriction maps covering about 22 kb of DNA surrounding the C gene segment in liver and tumor DNA. The region on the 20 kb fragment has been localized where a DNA rearrangement had occurred. The presence of two rearranged kappa light chain genes in one tumor is discussed in regard to the molecular basis of allelic exclusion.

INTRODUCTION

Among the eucaryotic genes studied so far the immunoglobulin genes are unique because a rearrangement of these genes seems to be a prerequisite for their functional expression. Structural studies on DNA fragments containing immunoglobulin light chain gene segments cloned from mouse myeloma and embryo DNA (or DNA from other non-committed tissues) have revealed that the rearrangement is due to a site-specific recombination event between a V (variable) gene segment and a J (joining) gene segment which were separated by a DNA sequence of unknown length (3,4). For kappa light chains five J gene segments have been identified which are located fairly regularly at a distance of about 300 bp from each other about 2.5-4.0 kb upstream of the C (constant) gene segment (5,6). The V and the J gene segments code for amino acids 1-95 and 96-108, respectively, of the conventionally defined variable region and the C gene segment codes for the constant region sequence (amino acids 109-214) of

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kappa light chains.

In a previous publication we have reported on a kappa chain producing mouse myeloma which reveals by Southern blot hybridization with a C region specific probe two EcoRI fragments of about 14 and 20 kb in length which are both different from the 15 kb EcoRI fragment of non-committed tissue, that is mouse liver in our case (7; for details see also Fig. 1 of the present paper). It was shown (7) by cloning and characterization of the 15 kb fragment from liver DNA and the 14 kb myeloma DNA fragment that a rearrangement 2.7 kb upstream of the C gene segment had occurred in the myeloma DNA (a more accurate value is 2.5 kb). In the present publication we report on the cloning and characterization of the 20 kb EcoRI fragment. The finding that also on this 20 kb fragment a DNA translocation had occurred upstream of the C gene segment is relevant to the discussions on the molecular basis of allelic exclusion.

The mouse myeloma we used in the previous (7) and the present paper is the one from which the kappa chain cDNA clone K38 was derived by Mach et al. (8) and which was thought to be identical to MOPC173 (7,8). We now know, however, that the nucleotide sequences of the V genes on K38 and on the two rearranged EcoRI fragments of the myeloma investigated do not correspond to the known (9) amino acid sequence of the MOPC173 kappa light chain (10,11). Apparently not MOPC173 but another myeloma had been used for the cloning of the cDNA (8). In the present publications we call the tumor myeloma T and do not introduce a specific designation for it since its light chain sequence (10,11) could not yet be correlated with one of the known amino acid sequences of mouse myelomas. For the time being the myeloma is characterized (10,11) by the sequence of its kappa light chain gene on the 14 kb EcoRI fragment which corresponds to the sequence of the cDNA clone K38.

MATERIALS AND METHODS

E. coli LE392 (supE supF r_K^-) was from P. Leder (12), E. coli 490A (r_K^- m_K^- met^- thr^- leu^- $recA^-$) from G. Hobom, the λ vector phage Charon 4A from F. Blattner (13), plasmid pBR322 from H. Boyer (14), plasmid K38 from B. Mach (8), and the

lysogenic E. coli strains BHB2688 and BHB2690 used for the preparation of the packaging extracts from B. Hohn (15). Nitrocellulose filters BA85 were purchased from Schleicher and Schüll, α-^{32}P-dATP and α-^{32}P-dCTP (specific activity >350 Ci/mmol) used for nick-translation from Amersham, T4 DNA ligase from Miles, calf intestinal phosphatase from Boehringer Mannheim, and SstI from Bethesda Research Laboratories. EcoRI was prepared as described (16), BamHI was a gift of U. Hänggi and T. Igo-Kemenes, XbaI of W. Hörz, and HpaI of H. Feldmann. All other restriction nucleases used were gifts of R.E. Streeck. The mouse myeloma tumor, presently characterized only by its kappa light chain sequence (10,11), was obtained from B. Mach (Department of Microbiology, University of Geneva, Switzerland) and maintained by subcutaneous transplantation in inbred Balb/c mice.

All restriction enzyme digestions were in 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 8 mM $MgCl_2$, 1 mM dithiothreitol, except HpaI digestions which were in 10 mM Tris-HCl (pH 7.4), 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol. Agarose gel electrophoresis (17), Southern transfer (18), nick-translation (19,20), DNA filter hybridization (18,21), and preparation of packaging extract (15) were carried out according to published procedures with slight variations (see legends to figures and also refs. 7,22).

Experiments involving isolation and propagation of recombinant phages and plasmids were carried out under L3/B1 and L2/B1 conditions, respectively, in accordance with the German guidelines.

Molecular cloning of the 20 kb EcoRI fragment from myeloma DNA. Fractions of EcoRI digested mouse myeloma DNA from a previously described (7) RPC-5 chromatography (25 mg of DNA, 80 fractions of 12 ml each) were used as starting material. 0.2 ml aliquots of each fraction were supplemented with 6 µg of yeast tRNA and 6 µl of glacial acetic acid, ethanol precipitated and analyzed by Southern blot hybridization for the presence of the 20 kb EcoRI fragment containing the C gene segment. The nick-translated J region subclone (Fig. 2a) was used as a probe. 4 ml each of the 5 fractions containing the 20 kb fragment and

eluting late from the column were pooled and concentrated by ethanol precipitation. The 20 kb fragment was then further enriched by fractionation on a 0.6 % preparative agarose gel with a discontinuous automatic elution device (37) according to Southern as described in ref. 7. Identification of the fragment by hybridization was as above using 400 µl aliquots of each fraction of 26 ml. DNA from the two peak fractions was precipitated by ethanol and purified by BD cellulose chromatography as in ref. 7. 6 µg of DNA were obtained.

Ligation of 1 µg of the purified DNA with 1.5 µg of Charon 4A vector "arms" (13), isolated by sucrose gradient fractionation according to ref. 23, was in 5 µl for 1 h at 12° C plus 18 h at 0° C, otherwise as in ref. 7. Phages were produced by "in vitro packaging" according to ref. 15 and plated on E. coli LE392. A titer of 10^6 plaques per µg of mouse DNA was obtained. Screening was by the plaque hybridization assay (24) using the J region subclone as a probe. Positive clones were plaque-purified. Bulk production of the recombinant phages was carried out by a plate lysate method (25) on 20x20 cm plates (NUNC, Kamstrup, 4000 Roskilde, Denmark) using E. coli LE392 as a host. The recombinant clone was designated Ch4A-T2.

Subcloning of DNA fragments. Recombinant phage DNAs [Ch4A-T1, formerly called Ch4A-173/1 (ref. 7), Ch4A-T2 (this paper) and Ch4A-L1 (ref. 7)] were digested with the appropriate restriction nucleases. After inactivation of the nucleases the digests were mixed in a molar ratio with digested DNA of the plasmid vector pBR322 and ligated at a concentration of 25 µg of DNA per ml similarly as described above. Aliquots of 50 ng of the ligated DNAs were then used to transform 0.2 ml of competent cells of E. coli 490A following the protocol given in ref. 14 with 1 h of incubation at 37° C to induce antibiotic resistance. The cells were then distributed in soft agar on 5 plates containing 50 µg/ml of ampicillin. Usually 10^4 colonies per µg of ligated DNA were obtained. Single colonies were then picked and screened by the colony hybridization assay (26).

C region subclone: the 2.7 kb BglII fragment containing the C gene segment (Fig. 2a) was cloned into the BamHI site of pBR322; the ligation reaction was carried out in the presence

of BamHI in order to avoid religation of vector DNA. This clone was identified using the HpaI-HhaI fragment containing the C region of the cDNA plasmid K38 (7,8) as a probe. J region subclone: the 2.8 kb EcoRI-BglII fragment comprising the J gene segments (Fig. 2a) was ligated with EcoRI + BamHI digested pBR322 DNA also in the presence of BamHI. Screening was with the HhaI-A fragment from K38 (7,8) which comprises the J5 segment (11). V-T1 region subclone: the 2.6 kb EcoRI-HindIII fragment (Fig. 2b) containing the V region gene present on the 14 kb myeloma fragment was subcloned in the appropriately digested pBR322 DNA and also identified with the HhaI-A fragment from K38 because of identical V region segments (11). V-T2 region subclone: the 3.6 kb HindIII fragment (Fig. 2c) comprising the V region gene present on the 20 kb myeloma fragment was ligated with pBR322 DNA which had been digested with HindIII and treated with calf intestinal phosphatase (27). Colony hybridization for identification of this subclone was with the J region subclone in the presence of an excess of cold pBR322 DNA as competitor.

RESULTS

Mapping of immunoglobulin gene segments in uncloned mouse liver and myeloma DNA. In order to investigate if also the 20 kb myeloma fragment had been generated by a recombination event upstream of the C gene segment we analyzed the myeloma DNA by restriction mapping of uncloned and cloned (see below) DNA. For mapping studies of chromosomal DNA from mouse liver and myeloma according to Southern's hybridization technique (18) three subclones were prepared from the previously cloned (7) 15 kb liver and 14 kb myeloma fragment and used as probes: the EcoRI-BglII and the BglII fragment from liver DNA containing the J gene segments and the C gene segment, respectively, and the EcoRI-HindIII fragment from the 14 kb myeloma fragment containing the V gene segment (Fig. 2a,b). The C region of the kappa chain cDNA plasmid K38 had also been used as a probe. The fragments identified with these four probes in liver and myeloma DNA after digestion with various restriction nucleases are summarized in Table 1. Fig. 1 shows some representative auto-

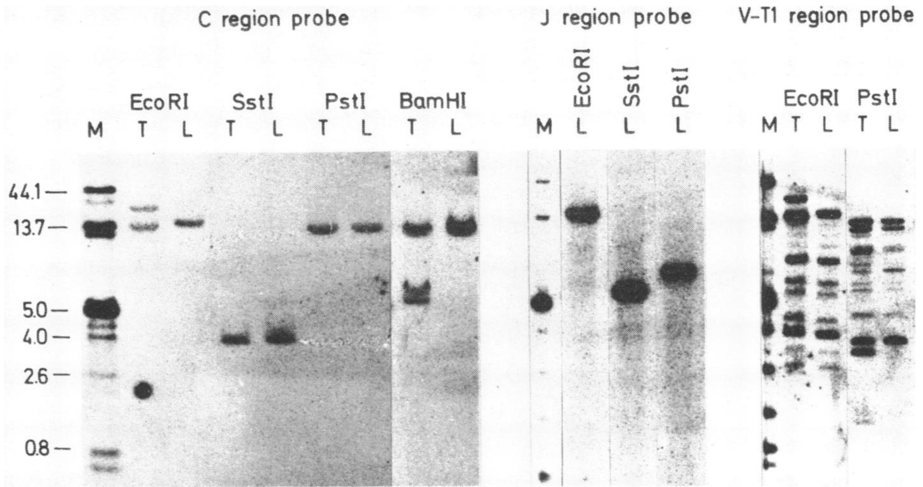

Figure 1. Autoradiograms of Southern blots of mouse liver and tumor DNA hybridized with subcloned C, J and V region probes. DNA from mouse liver (L) and tumor (T) was digested with the restriction nucleases indicated, fractionated on 0.5 % agarose gels, transferred to nitrocellulose filters and hybridized in 3xSSC, 10x Denhardt's solution at 68° C for 16 h with nick-translated plasmid probes at 50 ng/ml (specific activity 0.5-$2 \cdot 10^8$ cpm/µg) as described (7,22). After hybridization the last two washing steps were with 0.1xSSC, 0.5 % SDS for 15 min each at 65° C (7,22) and autoradiography was for 4 days at -80° C with intensifying screens. The plasmid probes are the C and J region subclones from the 15 kb liver fragment and the V-T1 region subclone from the 14 kb myeloma fragment (Fig. 2a,b and Methods). The hybridization marker (M) consisted of 100 pg each of a PstI, HindIII, BamHI, and HpaI digest of the 14 kb myeloma fragment and 100 pg of a HindIII + PstI digest of the C region subclone. Fragment sizes are given in kb. In spite of its broad appearance on the blot the 5.2 kb BamHI band corresponds to a homogeneous fragment.

radiograms and Fig. 2 gives the resulting restriction maps for liver and myeloma DNA.

The mapping data were first analyzed to investigate whether the cloned 14 and 15 kb EcoRI fragments from myeloma and liver DNA correspond to the respective regions in the chromosomal DNA and were not altered through cloning and propagation in E. coli Digestion of myeloma DNA with EcoRI plus either BamHI, HpaI, or SstI, and with BamHI + HpaI and hybridization with the C region probe from K38 led to the identification of all the expected

Table 1. Chain lengths of restriction nuclease fragments identified by hybridization in blots of liver DNA/myeloma DNA (Fig. 1).

C region probes		J region probe	
(a) EcoRI	15/20,14	EcoRI	15/n.d.
BamHI	n.d./12,5.2	BamHI	12/n.d.
HpaI	12/12	BglII	4.4/n.d.
SstI	n.d./3.6	PstI	6.6/n.d.
EcoRI + BamHI	n.d./5.5,5.2	SstI	5.4/n.d.
EcoRI + HpaI	n.d./9.7		
EcoRI + SstI	n.d./3.6	V-T1 region probe	
BamHI + HpaI	n.d./1.5	EcoRI	15/20,14
(b) EcoRI	15/20,14	BglII	n.d./4.2
BamHI	12/12,5.2	HindIII	2.7/4.1
BglII	2.7/2.7	PstI	n.d./3.0
HindIII	4.2,2.7/4.2,4.1,3.6	SstI	5.4/2.0
PstI	12/12		
SstI	3.6/3.6		

As C region probes the HpaI-HhaI fragment of the cDNA plasmid K38 (7,8) (a) and the genomic subclone (b) were used. For the V-T1 region probe only fragments are given which are different in liver and tumor DNA and which were used to construct the maps given in Fig. 2a and b. Fragment sizes are in kb; n.d. not determined. Approximate sizes are given in the table as determined from the Southern blotting experiments. For construction of the maps (Fig. 2,4) the more accurate values determined from digestion experiments with cloned fragments were used. These values were 13.7 and 20.1 for the 14 and 20 kb EcoRI fragment from myeloma DNA and 14.9 for the 15 kb EcoRI fragment from liver DNA, respectively.

fragments (Table 1 and ref. 7, Fig. 3). Furthermore digestion of liver and myeloma DNA with EcoRI, SstI, BglII, and HindIII and hybridization with either of the two C region probes yielded the expected DNA fragments (Fig. 1 and Table 1; also ref. 7, Fig. 3). We did not detect any differences in the restriction patterns of uncloned and cloned DNA and therefore felt justified to use the two maps interchangeably.

We next used the blotting and hybridization technique to map restriction sites on the DNA outside of the cloned liver and myeloma fragments. Restriction fragments overlapping the EcoRI

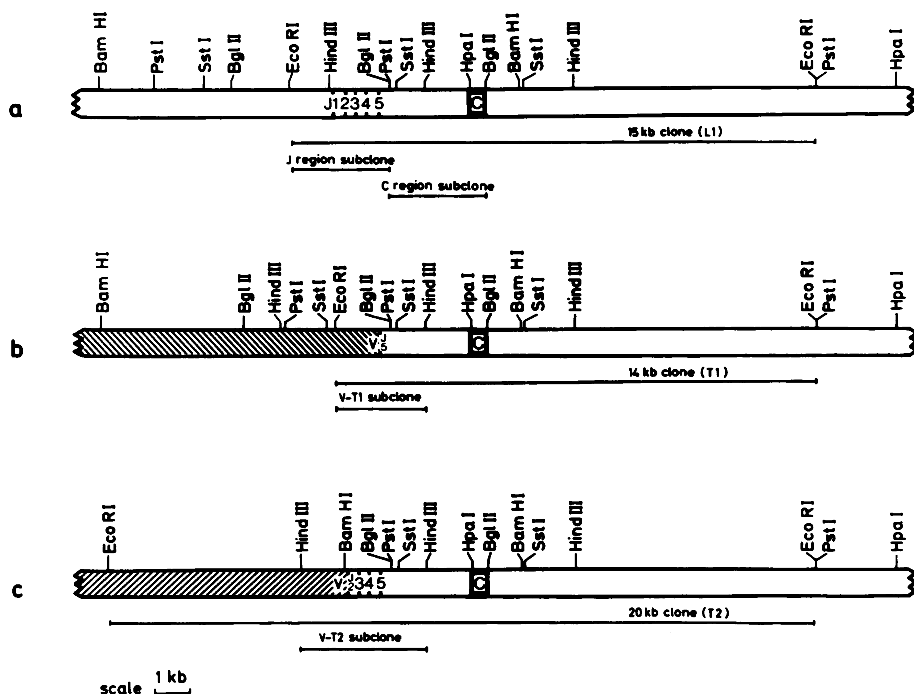

Figure 2. Comparison of restriction maps of chromosomal DNA from mouse liver and myeloma. a, liver DNA; b and c, myeloma DNA. The restriction maps were constructed from the information provided by the Southern blotting experiments (Fig.1 and Table 1) and the restriction maps of the previously cloned (7) 14 kb myeloma and 15 kb liver EcoRI fragments. (Compared to the map in Fig. 3 of ref. 7 the order of the BamHI and SstI cleavage sites to the right of the C gene segment has been reversed in further mapping studies.) The localization of immunoglobulin gene segments (V, J1-J5, C) given in the figure has been obtained from sequence information [embryo DNA (5,6), liver DNA (11), 14 kb myeloma fragment (10,11), and 20 kb myeloma fragment (11)] and from mapping studies on the 20 kb myeloma clone (Fig. 4). Translocated DNA sequences which include the two V gene segments are indicated by hatching.

site upstream of the C gene segment in liver DNA were identified by hybridization of BamHI, BglII, PstI, and SstI digests with the J region probe (Fig. 1, Table 1, Fig. 2a). The 12 kb BamHI fragment thus identified was also found by hybridization with the C region probe as expected from the map. Likewise fragments overlapping the EcoRI site downstream of the C gene segment in

liver DNA were revealed by hybridization of HpaI and PstI digests with the C region probes. The same fragments were found in myeloma DNA indicating that the DNA has not been rearranged in the region of about 14 kb between the PstI site near the J segments and the HpaI site at the downstream end of the map (Fig. 1 and 2, Table 1). This was confirmed in a number of other hybridization experiments of uncloned DNA (Table 1) and by restriction mapping of cloned DNA fragments (see below and ref. 7).

Restriction fragments overlapping the EcoRI site upstream of the C gene segment of the 14 kb myeloma fragment (Fig. 2b) were identified by hybridization of the V-T1 region subclone to BglII, HindIII, PstI, and SstI digests. About 10 bands (Fig. 1) were always detected in Southern blot hybridization experiments of liver and myeloma DNA with the V region probe. This is obviously due to a cross-hybridization of the probe to related V gene sequences (28-30). For construction of the map only strong bands were used and only those which occur either in the liver or in the myeloma digest and not in both digests. Such fragments must be derived from the region comprising the site of recombination. An example is the 3 kb PstI fragment in myeloma DNA (Fig. 1).

Distinct fragments comprising the second C gene segment present in the myeloma DNA (Fig. 2c; 20 kb fragment, see below) were detected in digests with EcoRI, BamHI, HindIII, and EcoRI + BamHI which had been hybridized with either of the C region probes. This strongly indicates that two C gene segments are present in the myeloma DNA which are located on differently rearranged DNA fragments. From the mapping data the recombination site on the 20 kb EcoRI fragment can be confined to the 1.4 kb BamHI-BglII DNA segment 2.2 kb upstream of the C gene segment (Fig. 2c).

Cloning and restriction mapping of a 20 kb EcoRI fragment containing immunoglobulin gene segments from myeloma DNA. The 20 kb EcoRI fragment from myeloma DNA which had been detected (7) and mapped (see above) by hybridization was cloned in the λ phage Charon 4A after enrichment by RPC-5 chromatography and preparative gel electrophoresis (Methods). The RPC-5 fractions

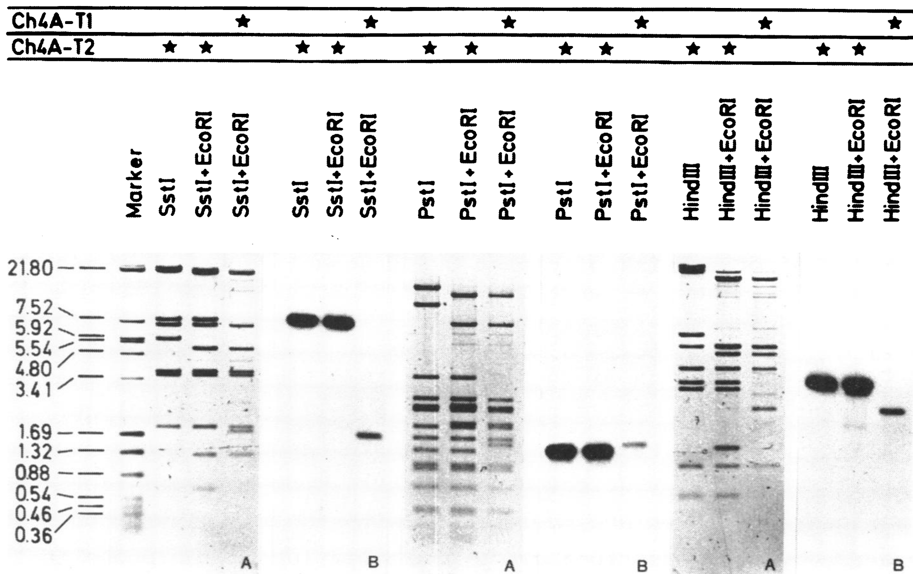

Figure 3. Restriction endonuclease analysis of the 20 kb EcoRI fragment cloned in Charon 4A. (A) Ch4A-T1 and Ch4A-T2 phage DNAs (0.5 µg per slot) were digested with various restriction nucleases and analyzed on 0.5 % agarose gels in the presence of ethidium bromide. The marker fragments consisted of a mixture of EcoRI fragments from phage λ DNA (32) and BspRI fragments from plasmid λdv1 DNA (33 , electrophoresis in the absence of ethidium). Sizes are in kb. (B) Autoradiograms of blots of the gels shown in (A) after hybridization with the J region subclone (Fig. 2a). Hybridization was as described in the legend of Fig. 1 with 30 ng/ml of plasmid probe and two incubations at 68° C for 15 min each in 1xSSC, 0.5 % SDS as the last washing steps. Autoradiography was overnight without intensifying screens. The sizes of the hybridizing fragments are listed in Table 2.

used in the cloning experiment were from a column chromatography of one and a half years ago and had been kept in the cold room. (Also fractions of an RPC-5 chromatography run of a liver DNA digest have been successfully used for cloning after a similar period of time (31)). In both purification steps only the peak fractions were taken in order to obtain high enrichment because we have had difficulties in earlier experiments to clone the 20 kb fragment (7). By screening about 30 000 recombinants with the J region subclone as a probe 16

Table 2. Chain lengths of some restriction nuclease fragments from the 20 kb myeloma fragment.

EcoRI + BamHI	2.5, 1.45, 2.7, 5.2
EcoRI + BglII	6.3, 1.8
EcoRI + HindIII	1.6, 0.6, 3.3, 3.6
EcoRI + HpaI	2.45, 7.9
EcoRI + PstI	2.3, 0.5, 3.8, 1.5
EcoRI + SstI	0.65, 7.6
EcoRI + XbaI	3.55, 2.2, 2.4

Fragments from double digests are listed which are unique to Ch4A-T2 and are not also derived from Ch4A-T1. Sizes are given in kb and fragments are listed according to their order from left to right on the 20 kb EcoRI fragment (Fig. 4). Fragments which hybridize to the J region subclone (Fig. 2a) are underlined.

positive plaques were detected, four of which were picked and plaque-purified. Phage DNA was prepared and analyzed by EcoRI and EcoRI + HindIII digestion (not shown). EcoRI digestion of the four DNAs revealed a fragment of about 20 kb in length which comigrated with the large Charon 4A vector "arm" (11) on 0.5 % agarose gels and hybridized to the J region subclone. Also EcoRI + HindIII digestions gave identical digestion patterns. We therefore analyzed only one of the four clones (designated Ch4A-T2) in more detail.

DNA from the phage Ch4A-T2 and, for comparison, DNA from the phage Ch4A-T1 were digested with a variety of restriction nucleases (Fig. 3). The digestion patterns were analyzed in the following way: first, fragments of the 20 kb myeloma clone containing translocated DNA sequences were identified by their occurrence in double digests of Ch4A-T2 but not in those of Ch4A-T1 DNA; second, fragments containing the EcoRI site of the rearranged DNA sequence were identified by their occurrence exclusively in Ch4A-T2 double digests; and third, fragments containing the site of recombination were detected by blotting and hybridization with the J region subclone (Fig. 3, Table 2). From these and other nuclease digestion and hybridization data the map in Fig. 4 was constructed which is in agreement with the chromosomal mapping data.

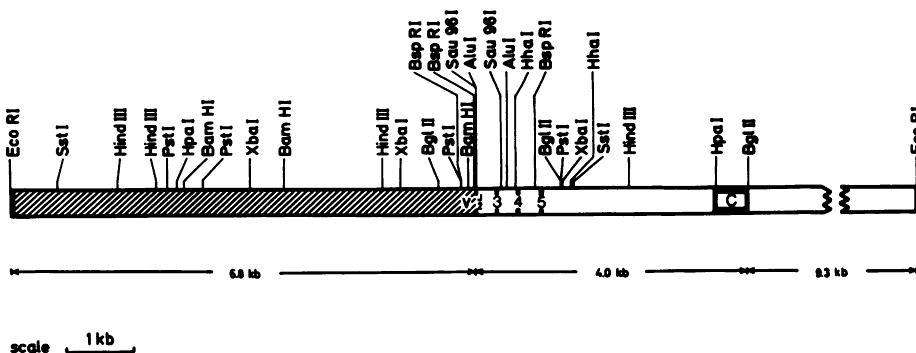

Figure 4. Physical map of the cloned 20 kb EcoRI fragment from myeloma DNA. The restriction map was constructed from the information provided by the digestion and hybridization experiments shown in Fig. 3 and Table 2 and from the results obtained with other combinations of restriction enzymes (not shown). Only some of the sites for AluI, BspRI, HhaI, and Sau96I are given. Symbols and localization of immunoglobulin gene segments are as in Fig. 2. The map region downstream of the C gene segment which is identical to the one of the 15 kb liver fragment (7) has been omitted.

The region comprising the site of recombination on the 20 kb myeloma fragment can be confined to an about 50 bp region between the BspRI and Sau96I sites next to the J2 gene segment (Fig. 4). This conclusion is based on hybridization experiments of the J region subclone to HhaI, Sau96I, AluI, and BspRI digests of the 20 kb myeloma fragment, the V-T2 subclone (Fig. 2c) derived from it, and the 15 kb liver fragment. The interpretation of the hybridization pattern was facilitated by the known sequence of the J region in embryo DNA (5,6) and the detailed restriction map of the corresponding liver DNA region (unpublished).

DISCUSSION

The results described in this paper establish that the two C gene segments present in the mouse myeloma studied are both rearranged with respect to their organization in non-committed DNA. The weak band which can be seen in some Southern blot hybridization experiments of EcoRI digested mouse myeloma DNA

(Fig. 1; also Fig. 2 of ref. 34) at the position of the 15 kb liver fragment is attributed to contamination of the myeloma by non-committed cells. We therefore conclude that a rearrangement of immunoglobulin gene segments coding for kappa light chains has occurred in this myeloma in both homologous chromosomes.

It has been suggested previously from Southern blot hybridization experiments that only one of the two homologous chromosomes is rearranged to form a complete immunoglobulin gene whereas the other remains in its germ line configuration (35). This would have explained in a simple way the phenomenon of allelic exclusion which is generally observed in immunocompetent cells, that is the expression of only one chain of a given immunoglobulin family in a single cell type. Recent experiments, however, have shown that multiple rearrangements (up to three) of light (29,30) and heavy (36) chain gene segments are found in myeloma cells which synthesize only one light and heavy chain. It is therefore interesting to ask whether a site-specific V-J recombination event leading to a complete immunoglobulin gene (3,4) is also involved in the non-productive rearrangements.

For the myeloma studied in this paper we know now from DNA sequencing data that the 14 kb EcoRI fragment is generated by a site-specific recombination event between a germ line V and the J5 gene segments and represents the expressed light chain gene of this tumor (11). This finding supersedes our previous tentative conclusion on the basis of R-loop studies that the 14 kb fragment may be derived from the non-expressed allele (7). The second gene segment in the tumor is located on a 20 kb EcoRI fragment which is generated, as we have shown in this paper, by a DNA rearrangement next to the J2 gene segment. Recent partial DNA sequencing data of this region have confirmed our map (Fig. 4) and shown unambiguously that also on the 20 kb myeloma fragment a V gene segment is located (11).

These findings indicate that allelic exclusion in immunocompetent cells is not necessarily based on only one V-J recombination event although this conclusion requires still further clarification. It is unlikely that the finding of two

rearranged kappa light chain genes is due to a heterogeneity of the tumor; the tumor has been transplanted many times during the past few years and, whenever checked, the 14 and 20 kb fragments appeared in approximately equimolar amounts in Southern blots. It may be possible, however, that for instance the second V-J rearrangement produces a non-functional immunoglobulin gene. Further work has to show whether the kappa light chain gene present on the 20 kb EcoRI fragment is transcribed.

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