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Genetics

Immunoglobulin heavy chain gene organization in mice: Analysis of a myeloma genomic clone containing variable and α constant regions

(heavy chain genomic clone/intervening DNA sequences/restriction maps/R loop mapping/V and C gene segments)

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ABSTRACT **We have isolated a myeloma genomic DNA clone containing the variable and constant regions of a mouse α chain. Restriction enzyme analyses and electron microscopic R loop mapping have demonstrated that the variable region is separated from the constant region by 6.8 kilobases of intervening DNA. In addition, two intervening DNA sequences of 100–200 bases separate the constant region into three approximately equal units. These intervening sequences may separate each of the segments coding for the three constant region domains of the α heavy chain. Southern blot analysis of embryo and myeloma DNA suggests that DNA rearrangement of heavy chain variable and constant regions occurs during the differentiation of antibody-producing cells.**

The antibody system affords a fascinating model for the study of gene organization and expression in eukaryotes. Antibodies or immunoglobulins are composed of two subunits, light and heavy chains, each of which contains an NH_2-terminal variable (V) region and a COOH-terminal constant (C) region (1). The antibody polypeptides are divided into discrete domains or homology units, each encompassing approximately 110 residues. Accordingly, the light chain has two domains (V and C) and the α heavy chain has four (V, C_H1, C_H2, and C_H3) (2). The variable and constant regions of light chains are encoded by three distinct gene segments, V (approximately residues 1–99), J or joining (approximately residues 100–112), and C (approximately residues 113–219) (3, 4). The V and J segments encode the classical V region. Each of these DNA segments is separated by intervening nucleotide sequences in embryo or undifferentiated DNA (4). Studies of myeloma DNA suggest that these gene segments are rearranged during the differentiation of antibody-producing cells. However, in myeloma DNA, intervening sequences still separate the V and C segments (4). These intervening sequences must be removed from nuclear RNA transcripts of light chain genes by RNA splicing (5). Thus, DNA rearrangements and RNA splicing appear to be important events in the differentiation of antibody-producing cells (6).

We are interested in analyzing the genes coding for heavy chains in embryo and myeloma DNA to determine whether sequence rearrangements occur during differentiation that are comparable to those seen for light chain gene segments (4). Our initial approach has been to isolate genomic clones from a library of recombinant Charon 4A bacteriophage containing long fragments of M603 myeloma DNA. In this paper we report the characterization of one clone containing both V_H and C_α regions.

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MATERIALS AND METHODS

Biological and Physical Containment. Work described in this report was conducted in a P3 physical containment facility using EK2 host-vector systems, in compliance with National Institutes of Health guidelines for recombinant DNA research as published in the *Federal Register* [(1976) **41**, 27902–27943].

Bacterial and Phage Strains. *Escherichia coli* K-12 strain χ1776 (7) was provided by R. Curtiss. *E. coli* K-12 strain DP50SupF (8) and Charon 4A phage (9) were provided by F. Blattner. *E. coli* strains NS428 and NS433 (10) used for *in vitro* packaging were obtained from T. Maniatis.

mRNA Preparation. BALB/c mouse myeloma tumors originally obtained from M. Potter or the Salk Institute were propagated subcutaneously. A postnuclear supernatant prepared from homogenized tissue was used to prepare poly(A)$^+$ RNA from membrane-bound polysomes (11). Heavy chain mRNA, identified by *in vitro* translation (12), was isolated by sucrose gradient fractionation.

cDNA Synthesis and Cloning. Double-stranded cDNA was synthesized by sequential reactions with avian myeloblastosis virus reverse transcriptase and *E. coli* polymerase I (13). After exclusion on Sephadex G-100, the major component of this cDNA migrated as a band of 1550 base pairs on a nondenaturing agarose gel.

Double-stranded cDNA was joined to *Eco*RI-cut pMB9 either by poly(dA), poly(dT) tailing (14), or by ligation to synthetic *Eco*RI linkers (15). Annealing or ligation mixtures were used directly to transform *E. coli* χ1776 (16). Positive transformants were identified by the Grunstein–Hogness technique (17) using ^{32}P-labeled M603 heavy chain mRNA.

Construction of M603 Library. High molecular weight genomic DNA (18) prepared from M603 subcutaneous tumors was partially digested with *Eco*RI, and fragments in the range of 12 to 20 kilobases (kb) were isolated on a sucrose gradient. Ten micrograms of M603 DNA fragments was ligated to Charon 4A arms and packaged *in vitro* to obtain a library of 3×10^6 recombinant phage (19). The library was amplified on DP50SupF as a plate lysate prior to screening.

Isolation of Clones from M603 Library. The constant region plasmid p603α1 labeled by nick translation with deoxynucleotide [^{32}P]triphosphates (20) was used to screen 400,000 clones from the M603 library plated on DP50SupF (19, 21). Duplicate nitrocellulose filters from each plate were prehybridized in 1 M NaCl/0.045 M trisodium citrate/0.2% bovine serum albumin/0.2% Ficoll/0.2% polyvinylpyrrolidone/0.1% sodium

Abbreviations: V, variable; C, constant; $NaDodSO_4$, sodium dodecyl sulfate; kb, kilobase(s); IVS, intervening sequence(s).

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dodecyl sulfate ($NaDodSO_4$) at 68°C in a rotary water bath (D. Engel and J. Dodgson, personal communication). Denatured plasmid DNA was added to 10 ng/ml and hybridization was continued for 48 hr. Filters were washed extensively in 0.15 M NaCl/0.015 M trisodium citrate/0.1% $NaDodSO_4$/10 mM $Na_4P_2O_7$ at 68°C. Duplicated spots of hybridization were identified by autoradiography, and plaques corresponding to these locations on the filters were picked and rescreened at a low plating density to obtain pure clones.

Electron Microscopic R Loop Mapping. Duplex Ch603α6 DNA was photochemically crosslinked (one crosslink per 4 kb) in the presence of 4,5',8-trimethylpsoralen (trioxsalen). Crosslinking prevents DNA strand separation, thus permitting the R loop hybridization (22) to be carried out at a temperature close to or above the DNA strand separation temperature (unpublished data). Crosslinked DNA (5 μg/ml) was hybridized to mRNA (2 μg/ml) in 70% recrystallized formamide/0.4 M NaCl/0.1 M 1,4-piperazinediethanesulfonic acid (Pipes), pH 7.2/10 mM EDTA at 56°C for 24 hr. The R-looped DNA was either spread from 70% onto 15% formamide or treated with 1 M glyoxal at 12°C to stabilize the R loops against branch migration (unpublished observations) and then spread from 50% formamide (23).

RESULTS AND DISCUSSION

Sequence Homologies of α Heavy Chain mRNAs. We isolated heavy chain mRNA from three mouse myeloma tumors secreting IgA immunoglobulins. Both the M603 and S107 immunoglobulins bind phosphorylcholine and contain nearly identical V region protein sequences (24). The M315 V region differs from M603 at 60% of its amino acid residues. To assay the extent of nucleotide homology between these mRNA species, ^{32}P-labeled single stranded M603 cDNA was hybridized to each mRNA. The hybrid was digested with nuclease S1 (25), and the resulting cDNA cleavage products were fractionated on an alkaline agarose gel (Fig. 1). M315 mRNA protects an 1100-nucleotide piece of M603 cDNA from S1 digestion; S107 and M603 mRNA both protect the full length of 1550 nucleotides. We conclude that S107 and M603 mRNAs are closely homologous over the entire sequence and that M315 mRNA shares this homology only for the C region, as expected from the protein sequences. In subsequent experiments with M603-like genomic sequences, we used S107 mRNA as a probe for the V and C regions and M315 mRNA as a probe for the C region only.

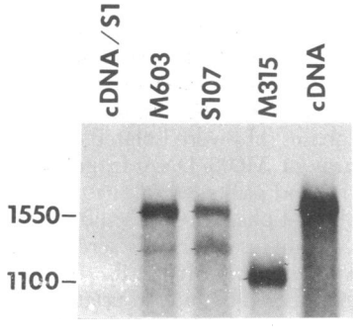

FIG. 1. Hybridization of mRNAs to M603 cDNA. Five nanograms of first-strand M603 cDNA (specific activity, 10^6 cpm/μg) was hybridized to 50 ng of the indicated heavy chain mRNA followed by digestion with nuclease S1 (25) and separation on a 2% alkaline agarose gel (26), a portion of which is shown by autoradiography. The first lane contains cDNA alone, hybridized and digested as above, and the last lane contains undigested input cDNA. The cDNA is somewhat longer than 1550 nucleotides, presumably due to an unfolded "hairpin" structure at the 5' end (27).

Characterization of cDNA Clones. Double-stranded cDNA prepared from M603 mRNA was used to construct the cDNA restriction map in Fig. 2*a*. We used poly(dA), poly(dT) tailing to obtain one recombinant plasmid, p603α1, which was shown by electron microscopy to contain an insert of approximately 600 nucleotides. Comparison of the cDNA restriction map with that of the plasmid indicates that p603α1 contains part of the C_α sequence (Fig. 2*b*). Subsequently, synthetic *Eco*RI linkers were used to clone S107 double-stranded cDNA. Regions of the cDNA included in two of these plasmids, p107αR5 and p107αR6, are indicated in Fig. 2*b*. In order to verify that the cloned sequences are derived from S107 heavy chain mRNA, a *Hin*fI restriction fragment of p107αR6 was isolated, annealed to S107 mRNA, and used to prime cDNA synthesis in the dideoxynucleotide sequencing procedure (29). The partial sequence so determined matched the known protein sequence of the S107 heavy chain between amino acids 92 and 125 (24) (data not shown).

Characterization of Genomic Clones. We screened 400,000 plaques from the M603 library with the C region plasmid p603α1, labeled with ^{32}P by nick translation. Twenty-five clones hybridized to the probe. Only three of these clones also hybridized to a nick-translated V region probe (the portion of p107αR6 shown to the left of the *Hha* I site in Fig. 2*b*). These three clones showed identical *Eco*RI restriction patterns; one, Ch603α6, was selected for further characterization. Ch603α6 contained 16.4 kb of mouse DNA with two internal *Eco*RI cleavage sites yielding fragments of 7.2, 4.8, and 4.4 kb, which are ordered in the restriction map shown in Fig. 2*c*. Filter hybridizations by the Southern blot procedure (30) localized the variable region in Ch603α6 to the 7.2-kb *Eco*RI fragment (Fig. 3). C region probes (p603α1 and the portion of p107αR6 to the right of the *Hha* I site in Fig. 2*b*) hybridized only to a 2.4-kb section bounded by *Xho* I and *Sma* I sites in the 4.8- and 4.4-kb *Eco*RI fragments. These results, displayed schematically in Fig. 2*c*, demonstrate that the V_H and C_α regions in Ch603α6 are separated by at least 4 kb of intervening DNA.

Identification of Genomic DNA Fragments Hybridizing to cDNA Clones. Hybridization of the nearly full-length cDNA probe p107αR5 to Southern blots of *Eco*RI-digested chromosomal DNA showed that the 7.2-, 4.8-, and 4.4-kb fragments of Ch603α6 all correspond to bands present in the M603 genome (Fig. 4). In embryonic DNA, the 4.8-kb middle piece of Ch603α6 was absent, although bands at 7.2 and 4.4 kb were present. These observations are consistent with a reduction in the distance between the Ch603α6 V and C regions having occurred in the derivation of the M603 genome from the embryo genome. Genetic evidence suggests that a closely related V_H region, S107, may be several hundred thousand base pairs from the C_α region in embryonic DNA (31, 32; but see ref. 33). The other changes evident from the Southern blots of embryonic and M603 DNAs cannot yet be fully explained.

Electron Microscopy Indicates That There Are Three Intervening Sequences in the Genomic Clone Ch603α6. S107 or M315 mRNA was hybridized to trioxsalen-crosslinked Ch603α6 DNA under conditions favoring R-loop formation. At least 80% of the DNA molecules examined in the electron microscope were full length and >90% of these contained R loops. Typical micrographs are shown and interpreted in Fig. 5. Data from 52 molecules were combined to generate the map shown in Fig. 2*d*. These studies indicate that Ch603α6 contains three intervening sequences (IVS). IVS 1 has a length of 6.8 kb. The R loop to the left of IVS 1 in Fig. 2*d* is due to the V_H region because it is seen in hybridizations of Ch603α6 DNA with S107 mRNA but not with M315 mRNA. The C region structures discussed below are seen in hybridizations with either S107 or

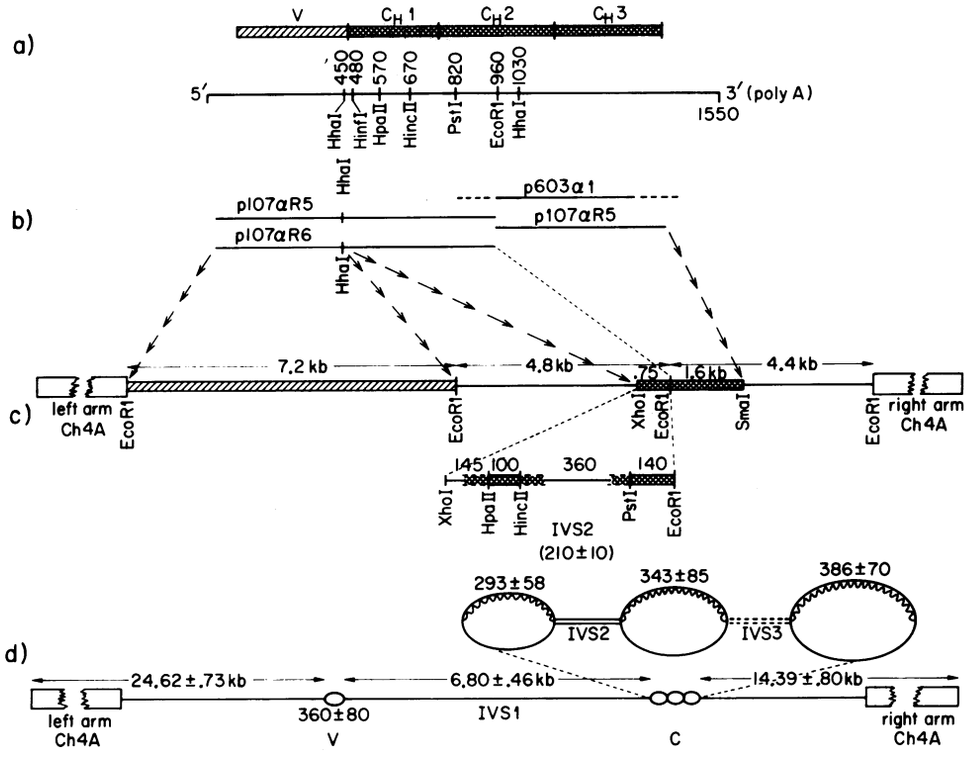

FIG. 2. Comparison of genomic and cDNA segments. (*a*) Restriction map of M603 double-stranded cDNA. The presence of a "hairpin" structure (determined by alkaline agarose gels) served as a marker for the 5′ end (27). Only the *Hin*fI site identified by mRNA sequencing is shown. The structural domains depicted above the cDNA are based on a partial protein sequence of M315 and M511 heavy chains and analogy with other IgA proteins (28). The protein sequence has been aligned so that the *Hha* I site sequenced in S107 mRNA coincides with the *Hha* I site mapped in M603 cDNA. This alignment gives good correspondence between the *Hpa* II and *Hinc*II sites mapped in M603 cDNA and possible cleavage sites of these enzymes at codons for amino acids 158 and 191. (*b*) Sequences included in cDNA plasmid clones. The ends of p603α1 are uncertain, as indicated by dotted lines. The larger of the two *Eco*RI fragments in p107αR5 is present in inverted orientation relative to its position in the cDNA. This is presumably due to the independent ligation of these two fragments during the cDNA cloning procedure. The restriction fragments of Ch603α6 within which the various parts of the plasmid clones hybridize are indicated by arrows connecting the plasmid and genomic clones. The dotted line connects the internal *Eco*RI site of the cDNA plasmids with the corresponding site in Ch603α6. (*c*) Restriction map and sequence organization of Ch603α6. Restriction fragments to which V and C region probes (see arrows from plasmids) hybridize are shaded in correspondence to the protein domains. The enlarged portion of the figure shows one of the short intervening sequences (IVS 2). Compare distances between restriction sites here and those shown in *a*. The *Xho* I site does not lie within the coding sequence. (*d*) R-Loop map of Ch603α6. The right and left arms of Ch4A were taken to be 10.7 and 20.1 kb long, respectively. The enlargement of the C region R loops is drawn with the total length of the RNA·DNA duplex equal to the length of the cDNA from the 3′ end to the junction with the V region shown in *a*. IVS 2 and IVS 3 are both assumed to be the same length. The *Eco*RI site shown in the enlargement of the Ch603α6 restriction map above is aligned with a point 590 nucleotides (measured on the RNA·DNA duplex regions) from the 3′ end of the C region R-loop complex. This corresponds to the distance of the *Eco*RI site from the 3′ end of the cDNA. The wavy line in the R loops represents RNA. The dashed line for IVS 3 indicates that its length has not been accurately measured. Errors given are SD.

M315 mRNAs. These results and the measured lengths of the several R loops indicate that IVS 1 occurs approximately at the junction of the V_H and C_α regions (Fig. 2*d*). Two kinds of R-loop structures were seen for IVS 1, depending on whether a single mRNA molecule (Fig. 5 *a* and *b*) hybridized to both the C and V regions of the DNA or whether these regions were hybridized to two separate mRNA molecules (Fig. 5*c*).

In addition to the 6.8-kb IVS, 58% of the molecules contained two structures that we interpret as due to two short IVS within the C_α region (IVS 2 and IVS 3 in Fig. 2*d*). In one type of short IVS structure, the single- and double-stranded arms of a C region R loop are joined at a reproducible point inside the R loop. We interpret this as due to a base-paired IVS (bpIVS in Fig. 5). Alternatively, the two arms of an R loop are not joined, but there is a small knob at a reproducible point on the double-stranded arm. We interpret this as an IVS that is not base paired to its complement on the opposite strand (ssIVS in Fig. 5). The positions on the C region R loop where bpIVS and ssIVS structures are seen are coincident. Because IVS 2 and IVS 3 are observable as knobs but are too short to be measured by electron microscopy, we believe their lengths to be in the range of 100 to 200 nucleotides.

The reproducible junction points that we attribute to base-paired IVS could be due to site-specific trioxsalen crosslinking. However, the ssIVS structures cannot be explained this way. Furthermore, these structures occurred with approximately the same frequency in R loops with uncrosslinked Ch603α6 DNA.

Both electron microscopy and restriction mapping give the same orientation of the V_H and C_α regions relative to the right and left arms of Charon 4A. The position of the 3′ poly(A) end of the mRNA also has been independently determined in some R loops by an electron microscopic labeling technique (34) (Fig. 5*c*).

IVS 2 Is 210 Nucleotides in Length by Restriction Mapping. By comparing the sizes of restriction fragments produced from DNA of the genomic clone Ch603α6 with those from the cDNA clone p107αR6, we have confirmed the presence and determined the length of one of the short IVS in the C_α region (Fig. 6). Gel electrophoresis showed that the *Hinc*II and *Pst* I

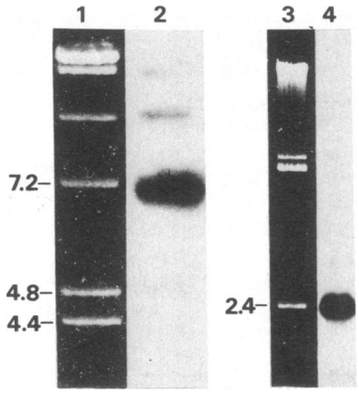

FIG. 3. Localization of the V and C regions in Ch603α6 by hybridization to Southern blots. Restriction enzyme digests of Ch603α6 DNA were electrophoresed on 1% agarose gels, transferred to nitrocellulose filters (30), and hybridized to V or C region probes prepared from gel-purified fragments of *Hha* I-digested p107αR6 (site of cleavage indicated in Fig. 2*b*). V region probe: ethidium bromide staining (lane 1) and blot (lane 2) of *Eco*RI-digested Ch603α6 DNA. Only the 7.2-kb fragment displayed strong hybridization to the V region. C region probe: ethidium bromide staining (lane 3) and blot (lane 4) of *Sma* I/*Xho* I-digested Ch603α6 DNA. Only the 2.4-kb fragment hybridized to this C region probe. The same result was obtained with probes from other parts of the C region. Unmarked bands seen by ethidium bromide fluorescence contained Ch4A vector DNA. The origins of the gels are not shown.

sites of the cDNA plasmid p107αR6 are separated by 150 nucleotides, whereas in the genomic DNA of Ch603α6 they are 360 nucleotides apart. This demonstrates the presence of an IVS of 210 nucleotides in the genomic clone which corresponds to the position assigned to IVS 2 by electron microscopy.

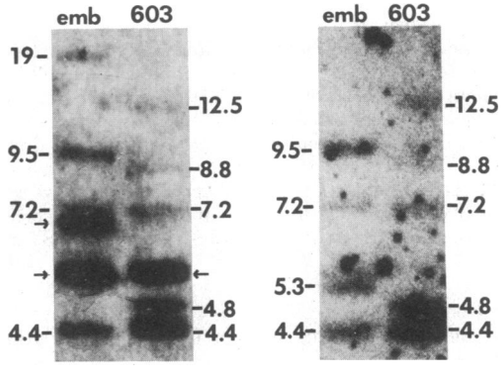

FIG. 4. Southern blots of *Eco*RI-cut genomic DNAs hybridized to p107αR5. High molecular weight genomic DNA was prepared from 12–13 day BALB/c embryos (emb) or M603 subcutaneous tumors (603) (18). Approximately 15 μg of DNA completely digested with *Eco*RI was electrophoresed on each lane of a 0.7% agarose gel. Nitrocellulose filter replicas of the gels were hybridized to ^{32}P-labeled p107αR5 (specific activity, 10^8 cpm/μg). Upper portions of the blots are not shown. In the two lanes at the left, different plasmid DNAs were added as internal standards (arrows). This obscures the 7.2- and 5.3-kb bands in the embryo DNA, which are better seen in the embryo lane at the right. The 19-kb band is not visible in the right embryo lane, probably because of poor transfer to the filter. Some clones from the M603 library contained a 4.4-kb *Eco*RI fragment hybridizing to p603α1, plus either a 12.5- or an 8.8-kb *Eco*RI fragment hybridizing to the 5′ C_α sequences in p107αR6. Thus, there may be multiple copies of the C_α gene segment, which would account for the relatively intense hybridization to the 4.4-kb band. All copies of the C_α gene in M603 DNA appeared to have undergone rearrangement or mutation from the embryo; the significance of this observation for the differentiation of antibody-producing cells is unknown.

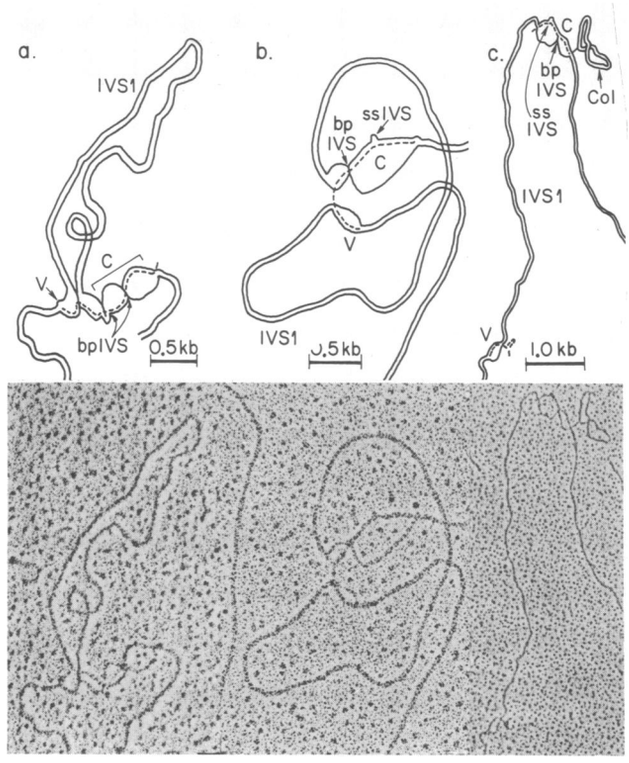

FIG. 5. Electron micrographs of R-loop structures observed with S107 mRNA hybridized to crosslinked Ch603α6 DNA. (*a*) R loop showing the large intervening sequence (IVS 1) dividing the constant (C) and variable (V) regions, and two short base-paired intervening sequences (bpIVS) in the constant region. (*b*) Similar to *a* but the C region contains a single-stranded intervening sequence (ssIVS) and a base-paired intervening sequence. (*c*) Two mRNA molecules hybridized to one Ch603α6 DNA molecule. The poly(A)$^+$ RNA at the 3′ end of the C region R loop is labeled with a poly(BrdU)-tailed circular microcolicin E1 molecule(Col). Both the V and C region R loops contain unhybridized RNA tails adjacent to IVS 1. The C region contains one bpIVS and one ssIVS. Broken lines represent RNA.

The Two Short C Region IVS May Separate the Three C_α Domains. The short IVS in the C_α gene separate the C region into three roughly equal segments. At the protein level the C_α region is divided into three roughly equal homologous structural domains: C_H1, C_H2, and C_H3 (28). Accordingly, the IVS in the DNA may separate the individual C_α domains, although this supposition will have to be verified by direct nucleic acid sequence analysis.

There are several features of immunoglobulin evolution and structure that might involve IVS separating C_H domains. First, all C regions, including C_α, contain homologous protein domains presumably derived from a common ancestral gene (2, 28, 37, 38). Second, C regions do not all contain the same number of domains, indicating that new C regions may arise by the addition or deletion of domains. Third, certain aberrant immunoglobulins (heavy chain disease proteins) often appear to involve deletions with breakpoints occurring between domains (39). Fourth, a variant myeloma cell line produced in culture shows deletion of the C_H1 domain (40). If IVS between domains facilitate unequal recombination, then the creation of new C regions with additional or deleted domains can be explained. Accordingly, the short IVS observed in the C_α region may permit the immunoglobulin domains to operate as fundamental units of evolution (41).

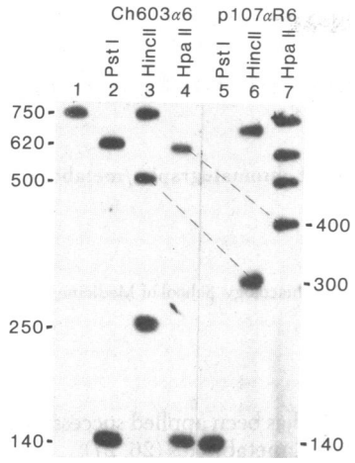

FIG. 6. Identification and measurement of IVS 2 by restriction mapping. Ch603α6 DNA digested with *Eco*RI and *Xho* I was ^{32}P end-labeled (35), and the 750-bp C region fragment (Fig. 2*c*) was eluted from a 1% agarose gel (36). An autoradiograph of part of a 5% acrylamide gel is shown comparing restriction digests of this end-labeled genomic DNA fragment and p107αR6 end-labeled after *Eco*RI digestion. Lanes: 1, Ch603α6 750-bp *Xho* I/*Eco*RI fragment; 2–4, products of digestion of this fragment with *Pst* I, *Hin*cII, and *Hpa* II, respectively (the *Hin*cII digest is incomplete); 5–7, *Pst* I, *Hin*cII, and *Hpa* II digests of p107αR6 end-labeled after *Eco*RI digestion. The *Eco*RI/*Pst* I bands of the two DNAs are the same length (140 bp), whereas the *Eco*RI/*Hin*cII and *Eco*RI/*Hpa* II bands are both 200–220 bp longer in the genomic DNA than in the cDNA (Fig. 2*c*). Comparing these results with the cDNA restriction map (Fig. 2*a*) shows that Ch603α6 contains a short intervening sequence (210 ± 10 bp) between the *Hin*cII and *Pst* I sites of the cDNA. This region includes the boundary between the C_H1 and C_H2 domains. (Other bands in lane 7 derive from *Hpa* II cleavage of the end-labeled plasmid DNA. The 790-bp fragment produced from *Hin*cII-digested p107αR6 is not shown on this part of the autoradiograph.)

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