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Molecular Cloning of Seven Mouse Immunoglobulin κ Chain Messenger Ribonucleic Acids†

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ABSTRACT: To provide access to multiple immunoglobulin κ chain variable region sequences, we have constructed plasmid clones bearing duplex DNA sequences complementary to κ mRNAs isolated from seven different mouse plasmacytomas (McPC 603, MOPC 41A, MOPC 173, MOPC 21, BFPC 61, HPC 76, and S107). Duplex complementary DNA (cDNA) was synthesized by using reverse transcriptase and DNA polymerase I, and the longest cDNA molecules, selected by gel filtration, were inserted into the *Pst*I site of the vector pBR322 by the dG·dC tailing procedure, which permits subsequent excision of the cDNA insert [Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., & Boyer, H. W. (1977) *Gene 2*, 95]. The oligo(dG·dC) tails, sized by depurination of insert sequences labeled at 3′ termini, were between 11 and 23 nucleotides long. Clones bearing κ sequences were identified by in situ colony hybridization using [^{32}P]cDNA made on a substantially purified κ mRNA as a probe. Identity was confirmed in several ways, including the ability of a cloned cDNA sequence to specifically arrest translation of κ mRNA and the precise correlation of restriction endonuclease cleavage maps with known amino acid sequences. Clones derived from each of the seven mRNAs have insert sequences 915–1000 base pairs long, which represent nearly the full length of the κ mRNA. Detailed restriction maps confirm that the clones bear seven distinct V_κ sequences, as well as C_κ and 3′ noncoding regions. The maps of the C region halves of all clones are identical, providing evidence for a unique C_κ sequence. The maps of two clones confirm that there is a 5′ noncoding region in κ mRNAs and indicate that it is between 68 and 210 nucleotides in length. Restriction mapping has also revealed an apparent cloning aberration, the presence of an *Mbo*II site toward the 3′ terminus of one of the cDNA sequences, at a position where there is no *Mbo*II site in the mRNA sequence.

Immunoglobulins are composed of light and heavy chains, each having an N-terminal variable (V)[1] region and a C-terminal constant (C) region (Hilschmann & Craig, 1965; for a review, see Gally, 1973). A key molecular problem presented by the immunoglobulin (Ig) gene system is how V region diversity is acquired—through evolutionary or somatic processes, or both (Weigert et al., 1970; Hood et al., 1975). κ chains, the predominant class of light chains in the mouse, exhibit extensive variability, although V_κ regions can be divided into "subgroups" or "isotypes" which have very similar N-terminal amino acid sequences (Potter, 1977). Each subgroup presumably is encoded in the germ line, but it remains uncertain whether each member of such a group is inherited. Evidence that the germ line contains multiple related V_κ genes has come recently from molecular cloning (Seidman et al., 1978a), but somatic processes have also been implicated by evidence from saturation hybridization that there are not enough related V_κ genes to account for the diversity within an isotype (Valbuena et al., 1978).

A deeper analysis of κ chain diversity requires access to a variety of V_κ nucleotide sequences representing different isotypes. The difficulty of isolating κ mRNAs in sufficient purity and quantity can be surmounted by recombinant DNA techniques. In particular, chimeric plasmids containing DNA sequences complementary to individual mRNAs allow production of large quantities of pure structural gene sequences, thus greatly facilitating hybridization and sequence studies (Rougeon et al., 1975; Higuchi et al., 1976; Maniatis et al., 1976; Rabbitts, 1976; Rougeon & Mach, 1976). Plasmids carrying sequences complementary to five κ mRNAs have recently been reported (Mach et al., 1977; Lenhard-Schuller et al., 1978; Schibler et al., 1978; Seidman et al., 1978b; Strathearn et al., 1978; Wall et al., 1978).

In order to generate an expanded set of pure κ nucleotide sequences, we have cloned κ mRNAs from seven different mouse plasmacytomas, representative of different V_κ isotypes. To ensure that V region sequences were represented, we prepared duplex cDNA under conditions that favor longer transcripts (Efstratiadis et al., 1976; Kacian & Myers, 1976)

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[1] Ig, immunoglobulin; C, constant region; V, variable region; cDNA, complementary DNA; poly(A), poly(adenylic acid); EDTA, ethylenediaminetetraacetic acid; bp, base pair(s); $NaDodSO_4$, sodium dodecyl sulfate; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

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and enriched for the longest duplex molecules by gel filtration. To facilitate subsequent structural analysis, we inserted the cDNA molecules into the *Pst*I site of the plasmid pBR322 by the dG·dC tailing procedure, which re-forms *Pst*I sites at each end of the cDNA sequence and hence allows the inserts to be cleanly excised (Bolivar et al., 1977; Villa-Komaroff et al., 1978). We present evidence for the identity of the clones and describe the characterization of the DNA sequences, including derivation of detailed restriction endonuclease cleavage maps. We show that each of the clones bears a distinctive V_κ sequence, as well as the C_κ and 3′ noncoding regions. Hence, they represent a set of well-characterized nucleotide sequences for analysis of V region diversity and gene rearrangement.

Materials and Methods

Ig mRNA Preparations. Messenger RNA was isolated, essentially as described by Cory et al. (1976), from microsomes of the following κ-producing plasmacytomas: MOPC 41A, S107, BFPC 61, McPC 603, MOPC 173, MOPC 21 (for a review, see Potter, 1972), and HPC 76 (obtained from Dr. N. Warner). The 13S microsomal mRNA fractions programmed synthesis of κ chains in a cell-free system (S. Cory, unpublished experiments).

Synthesis of Duplex cDNA. The protocol was derived from one developed by D. Kemp (personal communication). Synthesis of the first strand was carried out for 40 min at 39–42 °C in a 50-μL reaction containing 4 μg of a partially purified Ig mRNA, 0.5 μg of dT_{10} (Miles), 50 mM Tris-Cl, pH 8.3, 4 mM dithiothreitol, 6.5 mM magnesium acetate, 1.0 mM dATP, dGTP, and dTTP, 400 μM [^{3}H]- or [^{32}P]dCTP, and 50 units of avian myeloblastosis virus reverse transcriptase, the generous gift of Dr. J. W. Beard. The mixture was then boiled for 60 s, and the pH and salt concentration were adjusted by adding 6.25 μL of 0.4 M K Hepes, pH 7.2, and 0.2 M KCl to 45 μL of first-strand mixture. Extra [^{3}H]dCTP was added to monitor second-strand synthesis, which was carried out at 15 °C for 3 h in the presence of 10 units of *Escherichia coli* DNA polymerase I. The reaction was stopped by adding 50 μL of 10 mM EDTA, and the cDNA precipitated with ethanol in the presence of 4 μg of *E. coli* tRNA.

For S1 nuclease treatment, the duplex cDNA was dissolved with vigorous vortexing in 200 μL of water, and then 200 μL of 0.6 M NaCl, 0.1 M sodium acetate, pH 4.5, and 2 mM $ZnSO_4$ was added. Digestion was carried out for 30 min at 37 °C with 100 units of S1 nuclease. The digest was made 0.1 M in Tris-Cl, pH 9.0, and 10 mM in EDTA and extracted with buffer-saturated phenol and then twice with ether.

S1-treated duplex cDNA was fractionated on an 8 mm × 360 mm column of 100–200 mesh Bio-Gel A150m agarose (Bio-Rad) in 0.15 M NaCl and 2 mM EDTA, pH 8.0. (The column had been calibrated by chromatographing a mixture of restriction fragments of known size and subjecting DNA from various column fractions to gel electrophoresis.) Ten-drop fractions were collected, and selected fractions were pooled and made 10 mM in magnesium acetate. The cDNA was precipitated with ethanol for several hours at −70 °C and recovered by centrifugation in polyallomer tubes for 2 h at 40 000 rpm (Beckman SW40 rotor).

Tailing the Duplex cDNA and pBR322 Vector. From 0.005 to 0.2 pmol of duplex cDNA was extended with deoxycytidylate residues in a 25-μL reaction containing 500 pmol of [^{3}H]dCTP (Amersham, ~20 Ci/mmol), 0.14 M sodium cacodylate, 0.03 M Tris-Cl, pH 7.6, 0.2 mM dithiothreitol, 1 mM $CoCl_2$, and 40 units of terminal deoxynucleotidyltransferase (the gift of Dr. R. L. Ratliff). After ~5 min at 0 °C, which yielded 10 to 40 residues per end group, 25 μL of EDTA was added; the DNA was then precipitated with ethanol together with 3 μg of *E. coli* tRNA and finally resuspended in 0.05 M Tris-Cl, pH 7.5, and 0.1 mM EDTA. (Precipitation proved necessary to remove substances inhibitory to transformation of χ1776.) The vector was tailed in a similar 50-μL reaction containing 4 mM $MgCl_2$ instead of $CoCl_2$, 190 units of terminal transferase, [^{3}H]dGTP (Amersham, 13.2 Ci/mmol), and 10 μg of pBR322 cleaved with *Pst*I; about 20 dG residues were added per end group after incubation at 30 °C for 15 min. The vector was then extracted with phenol and recovered by ethanol precipitation.

Annealing and Transformation. Tailed duplex cDNA (0.005–0.2 pmol) was annealed with 0.1 μg of vector in 100 μL of 0.2 M NaCl and 0.02 M Tris-Cl, pH 8.2. The mixture was first heated to 65 °C for 10 min, then held at 45 °C for 1 h, and finally cooled slowly to 25 °C over several hours. *E. coli* χ1776 (Curtiss et al., 1977) was then transformed essentially as described by Villa-Komaroff et al. (1978). With this procedure, which involves $MnCl_2$ and a sodium acetate buffer, we routinely obtained at least 10^5 transformants from 0.1 μg of uncut pBR322 DNA. Transformants were selected on fresh supplemented L-broth plates containing tetracycline (12.5 μg/mL) and subsequently toothpicked in ordered arrays onto a master plate containing tetracycline, a Millipore filter on a tetracycline plate, and a plate containing ampicillin (100 μg/mL) in order to identify clones bearing inserts (Bolivar et al., 1977). The Millipore filters had been boiled three times to remove detergents inhibitory to χ1776.

Colony Hybridization. For labeled probe, cDNA was prepared as described above, except that the 25-μL reaction contained 1 or 2 μg of mRNA and 50 μCi of [^{32}P]dCTP (Amersham, 2–3 Ci/μmol). After incubation at 42 °C for 1 h, the cDNA was extracted with phenol and passed through a Sephadex G100 column.

Millipore filters bearing transformant colonies were placed for a few minutes on filter paper saturated with 0.5 M NaOH and then on paper containing 1.0 M Tris-Cl, pH 7.4, and finally 0.5 M Tris-Cl, pH 7.4, and 1.5 M NaCl (J. Lis and L. Prestidge, personal communication). The filters were then sucked dry on a filter block, washed with 95% ethanol, and finally baked at 80 °C for 2 h under vacuum. Prior to hybridization, the filters were treated first at 65 °C for 3 h in 0.2% Ficoll, 0.2% polyvinylpyrrolidinone, 0.2% bovine serum albumin, 0.03 M sodium citrate, 0.3 M NaCl, and 5 mM EDTA and then treated for 1 h at 65 °C with a similar solution supplemented with denatured salmon sperm DNA (50 μg/mL), poly(A) (10 μg/mL), and 0.1% $NaDodSO_4$. Hybridization was carried out under oil in the supplemented solution containing a [^{32}P]cDNA probe (4×10^5 cpm/mL) for 15–18 h at 65 °C. Filters were then washed twice for 30 min at 65 °C in 0.3 M NaCl, 0.03 M sodium citrate, and 4 mM EDTA and then in the same solution containing 0.1% $NaDodSO_4$ for 1 h.

Small-Scale Preparation of Plasmid DNA for Assessing the Length of cDNA Inserts. Plasmid-bearing clones of χ1776 were grown overnight in disposable plastic tubes. All subsequent operations were performed in 1.5-mL Eppendorf tubes, and centrifugation was carried out in the Eppendorf microcentrifuge at 12000*g*. Bacteria were killed with chloroform, harvested by centrifugation (2 min), washed once with 1 mL of 10 mM Tris-Cl, pH 8.5, and 1 mM EDTA, and then resuspended in 150 μL of 15% sucrose in 50 mM Tris-Cl, pH 8.5, and 50 mM EDTA; spheroplasts were generated by digestion with 50 μL of 4 mg/mL lysozyme for 30 min at room temperature and a further 30 min on ice. Ice-cold water (150

μL) was added and, after 5 min on ice, spheroplasts were lysed by incubation at 70–72 °C for 15 min; cell debris and chromosomal DNA were then pelleted by centrifugation (15 min). Plasmid DNA was recovered by ethanol precipitation, reprecipitated, and finally redissolved in 350 μL of water.

Large-Scale Preparation of Plasmid DNA and Excised cDNA Sequence. Plasmids were amplified overnight in χ1776 with 5 μg/mL chloramphenicol at 30 °C or in strain RR1 (Bolivar et al., 1977) with 30 μg/mL at 37 °C. Following $NaDodSO_4$ lysis (Guerry et al., 1973), plasmid DNA was precipitated from the cleared lysate with 10% poly(ethylene glycol) (Humphreys et al., 1975) and banded in a CsCl–ethidium bromide density gradient. The DNA was then extracted with phenol to remove residual nuclease. To recover inserts, chimeric plasmid DNA was digested with *Pst*I, and the excised cDNA sequence was separated from the vector by centrifugation through a gradient of 10–40% sucrose in 0.2 M sodium acetate, 10 mM Tris-Cl, pH 7.5, and 1 mM EDTA. Up to 500 μg of DNA was loaded per Beckman SW27 gradient (48 h at 27 000 rpm), or 175 μg per SW40 gradient (22 h at 40 000 rpm).

Restriction Mapping. Purified insert DNA (0.1–0.4 μg) was digested in a 5-μL reaction with about 1 unit of restriction enzyme, using the buffers recommended by New England Biolabs, but with 5 mM dithiothreitol in place of 2-mercaptoethanol and 0.01% gelatin in place of bovine serum albumin. After incubation for 2 h at 30 °C, 2 μL of 50% glycerol, 20 mM EDTA, 0.15% bromophenol blue, and 0.15% xylene cyanol FF was added, and the samples were loaded on a 0.35 mm thick 5% polyacrylamide slab gel (acrylamide:bis = 29:1) in 50 mM Tris-borate and 1 mM EDTA (Sanger & Coulson, 1978). Electrophoresis was at 5 mA until the bromophenol blue dye had migrated about 28 cm (overnight). To stain the gel, one of the glass plates (which had been siliconized) was removed, and the gel, in place on the other plate, was soaked in 1 μg/mL ethidium bromide for 1 h. After careful transfer onto clear plastic film ("Glad-Wrap"), the gel was photographed on a transilluminator.

3′ End Labeling of Restriction Fragments. Purified insert DNA (0.1 μg) was dried down together with 5 μCi of [α-^{32}P]dCTP (Amersham, 2–3 Ci/μmol) in a siliconized Eppendorf tube, redissolved in 5 μL of 50 mM Tris-Cl, pH 8.0, 5 mM $MgCl_2$, and 10 mM 2-mercaptoethanol, and incubated with 0.5 unit of *E. coli* DNA polymerase I (Boehringer-Mannheim, Klenow fragment) for 15 min at 20 °C. The reaction was terminated by adding 95 μL of 1 mM EDTA and incubating at 65 °C for 5 min. *E. coli* tRNA (20 μg) was added, and the nucleic acids were recovered by three ethanol precipitations. This procedure routinely gave incorporation of $1–5 \times 10^5$ cpm.

Biological and Physical Containment. Work was conducted in facilities classified CII and CIII by the Australian Committee on Recombinant DNA (ASCORD), both classified P3 under National Institutes of Health (NIH) guidelines, in compliance with both ASCORD and NIH guidelines for recombinant DNA research.

Results

Construction of Duplex cDNAs. To favor production of long transcripts, cDNA was synthesized on Ig mRNAs with a high concentration of dNTPs and reverse transcriptase (Efstratiadis et al., 1976; Kacian & Myers, 1976). With κ mRNA templates, the yield of cDNA typically ranged from 30 to 55%, whereas the yield with various heavy chain mRNA templates was 15–30%. This difference presumably reflects the greater length of the latter templates and a lower yield of very long transcripts. To convert the cDNA to a duplex form, *E. coli* DNA polymerase I was added to a first-strand reaction mixture, which had been boiled to dissociate any residual mRNA (D. Kemp, personal communication; Wickens et al., 1978). The amount of second-strand synthesis varied from 20 to 80% of first-strand incorporation.

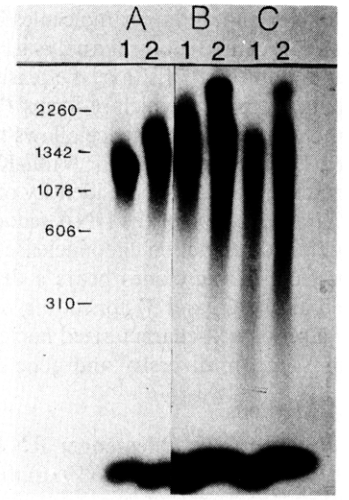

FIGURE 1: Gel electrophoretic analysis of cDNA made on three mRNA templates: (A1) MOPC 41A κ first-strand products; (A2) κ second strand; (B1) HPC 76 μ first strand; (B2) μ second strand; (C1) S107 α first strand; (C2) α second strand. The ^{32}P-labeled cDNA products, prepared as described under Materials and Methods, were precipitated with ethanol, dissolved in 12.5 μL of formamide, and resolved on a 3.75% acrylamide–0.67% bisacrylamide gel in phosphate-buffered formamide (Lehrach et al., 1977). The markers were a *Hae*III digest of φX174 DNA and a *Hin*dIII digest of λ DNA.

The size of products made in both reactions was evaluated by electrophoresis on polyacrylamide gels in formamide. Figure 1 shows that the great majority of the first-strand products made on a κ mRNA (A1), a μ mRNA (B1), and an α mRNA (C1) were more than 1000 nucleotides long. For the κ mRNA, this represents an excellent yield of full-length transcripts; indeed, there appears to have been partial synthesis of the κ second strand by reverse transcriptase. In any case, it is clear from Figure 1 that a substantial portion of the κ and μ first-strand products were extended by DNA polymerase I (A2 and B2), as was a smaller portion of the α cDNA (C2). A significant fraction of the μ second-strand products was more than 3000 nucleotides long (B2), as was a small amount of the α products (C2), while the longest κ products reached the expected 2000 nucleotides for a full-length closed κ duplex (A2).

That the expected duplex cDNA molecules had been formed was indicated by the resistance of the second-strand products to the single-strand specific nuclease S1. Over a range of S1 nuclease concentrations, generally less than 10% of the ^{3}H (second strand) counts in the duplex molecules was rendered acid soluble.

To increase the frequency of clones with long inserts, we enriched for the longest S1-treated duplex molecules by chromatography on A150 agarose beads (D. Kemp, personal communication), which provided useful separations in the size range 400 to 2000 bp. Since considerable amounts of small fragments were present, only fractions containing the largest DNA molecules were pooled for cloning.

Cloning of cDNA Sequences. We added deoxycytidylate tracts of 10 to 40 residues to duplex cDNA fractions from the agarose column, annealed the tailed cDNA to linear pBR322 DNA having terminal dG tracts of about 20 residues, and used the annealed molecules to transform the EK2 biocontainment

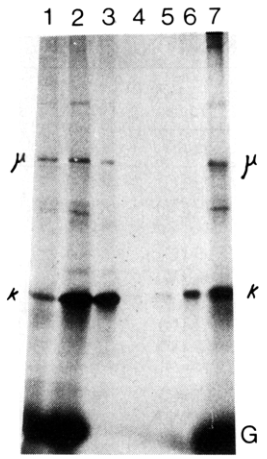

FIGURE 2: Hybrid arrest of κ mRNA translation by cloned DNA. Linear pM41K1 DNA was hybridized with a mixture of HPC 76 μ and κ mRNAs (Paterson et al., 1977), and translation products were analyzed by electrophoresis on a 10% polyacrylamide gel slab containing $NaDodSO_4$. Track 1 shows products from the hybrid; track 2, from the melted hybrid; and track 7, from the unhybridized mRNA mixture. Tracks 3–6 show immunoprecipitated products; track 3, those from the unhybridized mRNA mixture, precipitated with anti-κ antibody; track 4, as in track 3 but with nonimmune serum; track 5, products from the hybrid, with anti-κ antibody; and track 6, from the melted hybrid with anti-κ antibody.

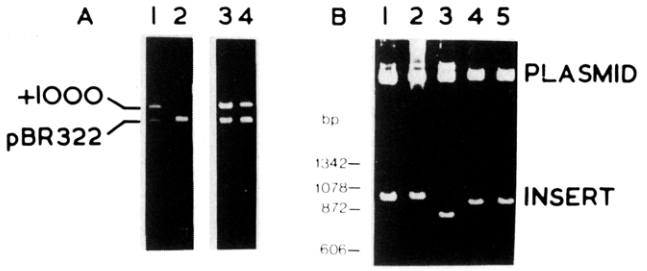

FIGURE 3: Sizing of insert sequences in cDNA clones. (A) Plasmid DNA, prepared by the rapid heat-shock method, was digested with *Eco*RI or *Pst*I and electrophoresed on a 1% agarose slab gel containing 0.072 M Tris, 0.06 M NaH_2PO_4, 2 mM EDTA, pH 7.8, and 1 μg/mL ethidium bromide. *Eco*RI digests of DNA from clones pS107κ4 and pH76κ14 are shown in tracks 1 and 3, respectively, and *Pst*I digests in tracks 2 and 4. The released *Pst*I fragments have been run off the gel. Linearized pM41κ1 DNA (which has an insert of 960 bp) and pBR322 DNA provided size markers. Linear pBR322 DNA was included in all tracks as an internal size marker. (B) Purified plasmid DNA (1.5 μg), digested with *Pst*I, was electrophoresed on a 2% agarose gel: (track 1) pS107κ4; (2) pM603κ2; (3) pH76κ10; (4) pB61κ21; (5) pM173κ14. The *Hae*III fragments of φX174 DNA provided size markers.

strain *E. coli* χ1776 (Curtiss et al., 1977). We achieved efficient, reproducible transformation of χ1776 only by using the $MnCl_2$ procedure described by Villa-Komaroff et al. (1978). Typically we obtained about 100 tetracycline-resistant transformants from 0.1 pmol of cDNA. Lower numbers were obtained from cDNA preparations to which less than 20 dC residues had been added, as assessed by [^{3}H]dCTP incorporation. Since only about 5 colonies were obtained from the tailed vector alone (0.1 μg), it was clear that most transformants involved plasmids with inserts. Insertion into the *Pst*I site should inactivate the pBR322 ampicillin gene (Bolivar et al., 1977), and we did find that about 62% of the transformants were ampicillin sensitive. This test, however, did not always give clear-cut results in our hands, so we did not use it to exclude any transformants.

Identification of Ig κ Clones. To detect κ clones, transformants were screened by colony hybridization (Grunstein & Hogness, 1975). The probe was [^{32}P]cDNA made on our best preparation of κ mRNA, that from MOPC 41A. Evidence regarding the identity and purity of that mRNA based upon fingerprinting, translation, nucleotide sequencing, and kinetic hybridization has been presented previously (Cory et al., 1976; Bernard et al., 1977; Gough & Adams, 1978). Only clones that exhibited reproducibly strong hybridization were selected for further study.

To verify that we were selecting κ clones, we tested whether hybridization of DNA from one clone to a mixture of κ and μ mRNAs could arrest translation of the κ mRNA (Paterson et al., 1977). Track 7 in Figure 2 shows the products from the unhybridized mixture of κ and μ mRNAs. Comparison with the hybrid-arrested sample (track 1) indicates that pM41κ1 DNA suppressed most κ messenger activity but not μ (or globin) activity, while track 2 confirms that melting the hybrid freed κ activity. As expected, the polypeptide programmed by the released mRNA was precipitable by anti-κ antibody (track 6), while much less was precipitated from the arrested sample (track 5). The immunoprecipitation exhibited specificity because no products were precipitated by a nonimmune serum (track 4), nor was globin precipitated by the anti-κ antibody (track 3), although this antibody preparation did precipitate some μ chain. Hybrid arrest was also demonstrated with the *Pst*I released insert from pM41κ1 (not shown).

Selection of Clones Carrying the Longest Inserts. To determine which clones contained the longest inserts and which were excisable with *Pst*I, we prepared plasmid DNA from 4–5-mL cultures by a rapid heat-shock procedure, based on that of Curtiss et al. (1977). Figure 3A shows representative tracks from a gel on which 24 clones had been analyzed. Tracks 1 and 3 show plasmids digested with *Eco*RI, which does not cut within these inserts, run together with an internal pBR322 marker. The mobilities of the linearized plasmids indicated that they contained inserts of 1000 ± 100 bp. (For screening, this procedure was preferable to sizing *Pst*I fragments because less DNA was required.) *Pst*I converted one of these plasmids to pBR322 size (track 2), indicating that the insert had been excised, while the other (track 4) was merely linearized, indicating that only one *Pst*I site had been cleaved. The frequency of clones with excisable inserts was only 50% with one batch of vector, but nearly 90% with a subsequent batch.

For detailed study we selected a clone bearing a long, excisable insert representative of each of the seven κ mRNAs studied. To measure insert lengths accurately, purified plasmid DNA digested with *Pst*I was electrophoresed on a 2% agarose gel (Figure 3B). Of the five clones examined on this gel, four have inserts between 950 and 1000 bp long (Table III). While pH76κ10 (track 3) appears to be shorter (850 bp), it also contains a 160-bp fragment not detectable on this gel.

Orientation of Inserts within pBR322. By taking advantage of a *Kpn*I site within several of the κ V regions (see below), we could orient the cDNA sequences with respect to pBR322, following the rationale in Figure 4A. As pBR322 lacks a *Kpn*I site, a *Bam*HI + *Kpn*I digest of the chimeric plasmid yields fragments L and S. If an insert has its V region proximal to the pBR322 *Bam*HI site, as in Figure 4A, fragment S will be 1300–1400 bp long, i.e., 1123 bp of pBR322 DNA (Sutcliffe, 1978) plus about 200 bp of insert, while the opposite orientation will give a fragment S about 2000 bp long. It is clear from Figure 4B that three of the clones are oriented as in Figure 4A, while the clone in track 3 (pM41κ1) has the opposite orientation. The orientation of pM173κ14, which lacks a *Kpn*I site, was determined by a similar analysis of an *Hpa*II digest resolved on a 5% polyacrylamide gel (Table III).

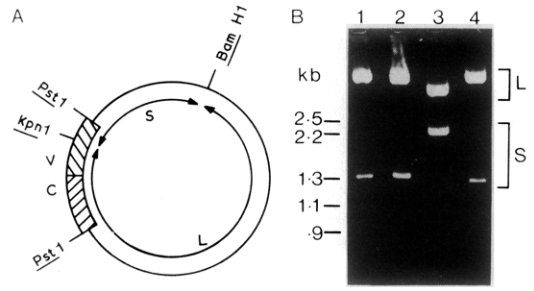

FIGURE 4: Orientation of insert sequences with respect to the plasmid. (A) Schematic representation of a chimeric plasmid with a 1000-bp insert (hatched region) which has a *Kpn*I site within the V region and proximal to the plasmid *Bam*HI site. *Bam*HI + *Kpn*I digestion produces two fragments, L and S. (B) Plasmid DNA (1.5 μg) from five clones was digested with *Bam*HI and *Kpn*I and electrophoresed on a 2% agarose gel: (track 1) pS107κ4; (2) pH76κ10; (3) pM41κ1; (4) pB61κ21. The *Hin*dIII fragments of λ DNA and the *Hae*III fragments of ϕX174 DNA provided size markers.

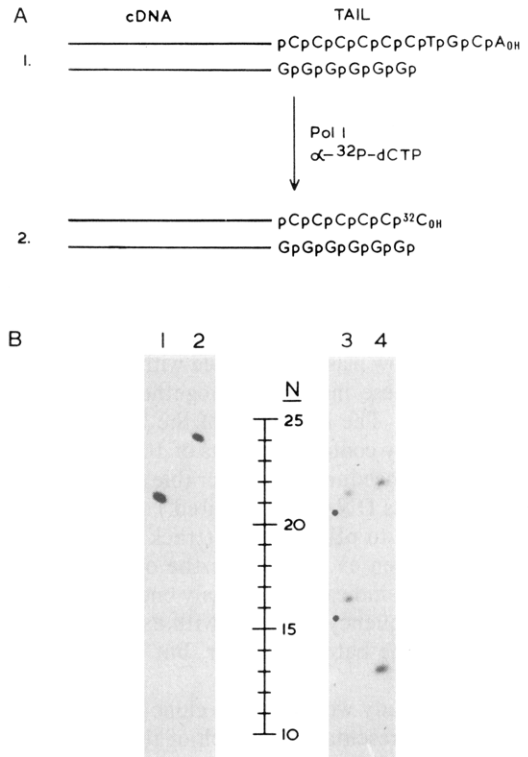

FIGURE 5: Sizing of the oligo(dG·dC) tails. (A) Method for labeling the 3′-terminal dC residue. The end of a cDNA insert released by *Pst*I is shown in 1. The 3′-exonuclease activity of DNA polymerase I (Brutlag & Kornberg, 1972) removes the four unpaired residues and exchanges the terminal dC residue with [α-^{32}P]dCTP, resulting in the labeled molecule shown in 2. (B) Sizing of labeled depurination products by fractionation on a 0.35 mm thick 12% polyacrylamide gel in 7 M urea, 90 mM Tris-borate, and 2.5 mM EDTA: (track 1) pH76κ10 (160-bp *Pst*I fragment); (2) pH76κ10 (850-bp *Pst*I fragment); (3) pB61κ21; (4) pS107κ4. The gel was autoradiographed at −80 °C. Samples from a sequencing experiment, electrophoresed on neighboring tracks, provided precise size markers, and the scale is given in nucleotides (N). The positions of faint bands visible in the original autoradiograph are indicated by dots to the left of the appropriate tracks.

Lengths of the Oligo(dG·dC) Tails. To size the oligo(dG·dC) tails in each clone, the 3′-terminal deoxycytidylate residues of purified insert fragments were labeled by using the 3′-exonuclease activity of DNA polymerase I (Brutlag & Kornberg, 1972), as illustrated in Figure 5A. The labeled DNA was depurinated (Ling, 1972), and pyrimidine tracts were fractionated on a 12% polyacrylamide gel, alongside samples from a sequencing experiment to permit precise sizing (Figure 5B). Since the pH76κ10 cDNA sequence has an internal *Pst*I site, its insert is purified as two fragments; each has a single tail and yielded a single pyrimidine band (tracks 1 and 2). Inserts with two tails yielded two major bands (tracks 3 and 4). Often faint bands were seen at a position one residue shorter than the major band (see track 3). We think these products result from slight overdigestion by the 3′-exonuclease activity, so that the 3′-terminal dC residue in some molecules is lost. The lengths of the pyrimidine tracts, which represent maximum lengths for the tails as they include any adjacent pyrimidine residues, are given in Table III.

Table I: Restriction Data Used To Determine the Regions of the mRNA Contained within κ *c*DNA Clones

clone	length of fragments (bp)[a] *Hpa*I	*Hin*cII	length of sequence beyond V[b]	C[c]
pM603κ2	489 (C)	489	114	222
	489 (V)	316		
		171		
pM41κ1	510 (C)	454	79	243
	454 (V)	337		
		176		
pM173κ14	492 (C)	444	69	225
	444 (V)	315		
		175		
pM21κ1	533 (C)	430	55	266
	430 (V)	360		
		171		
pB61κ21	498 (C)	405	30	231
	405 (V)	333		
		169		
pH76κ10	523 (C)	349	93[d]	256
	308 (V)	308		
		170		
pS107κ4	524 (V)	524	149	184
	451 (C)	271		
		172		

[a] The restriction fragments were sized on 5% polyacrylamide gels (Materials and Methods). Assignments of the *Hpa*I fragments to C and V region halves of each sequence were made by comparison of the two digests (see text). [b] The length of the sequence beyond the 5′ end of the V region is calculated by subtracting 375 bp (equivalent to 125 amino acids) from the length of the *Hpa*I V fragment. [c] The length of the sequence beyond the 3′ end of the C region is calculated by subtracting 267 bp (equivalent to the remaining 89 amino acids of the C coding region) from the length of the *Hpa*I C fragment. [d] The length of the extra 160-bp V region *Pst*I fragment is included (see Table III).

mRNA Sequences Represented in Clones. We could determine the positions within the mRNA sequence to which each cloned sequence extends simply by digestion with two restriction enzymes (Table I). *Hpa*I cleaves all the κ sequences uniquely at a position known to correspond to amino acid residue 125, near the beginning of the C region, while *Hin*cII cuts at both that site and at a position corresponding to amino acid residues 180–181 in the C region (Hamlyn et al., 1978; Seidman et al., 1978b; Strathearn et al., 1978). The V region *Hpa*I fragment is thus common to *Hpa*I and *Hin*cII digests. Taking the κ chain to be 214 amino acids in length (Gray et al., 1967; Svasti & Milstein, 1972), there will be 267 bp from the *Hpa*I site to the 3′ end of the C coding region ((214–125) × 3) and 375 bp to the 5′ end of the V coding region (125 × 3). Hence, subtracting 267 bp from the size of the C-region fragment from each clone gives the length of the sequence beyond C, and subtracting 375 bp from the V-region fragment gives the distance beyond the V region. Table I indicates that all the clones contain substantially more than the V and C coding regions.

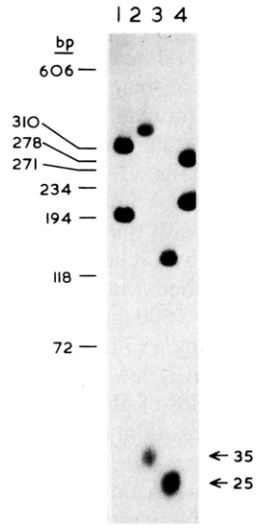

FIGURE 6: Identification of terminal *Mbo*II fragments. End-labeled insert DNA, digested with *Mbo*II, was fractionated on a 0.35 mm thick 5% polyacrylamide gel and autoradiographed at −80 °C: (track 1) pM173κ14; (2) pM603κ2; (3) pS107κ4; (4) pB61κ21. The *Hae*III fragments of φX174 DNA provided size markers.

Restriction Endonuclease Maps. Cleavage maps of the cloned κ sequences, shown in Figure 7, were derived by digesting the purified insert DNA with restriction enzymes, both singly and in pairwise combinations. Excellent resolution of the products was achieved using 0.35 mm thick 5% polyacrylamide gels, on which bands containing as little as 20 ng of DNA could be detected routinely, a sensitivity significantly higher than that obtained with conventional 2–4 mm thick gels. The unique *Hpa*I site (Table I) served as a reference point in all sequences. For example, *Hae*III cut the pM41κ1 insert once, yielding fragments of 672 and 287 bp. This site could be related to the unique *Hpa*I site by analyzing a *Hae*III + *Hpa*I digest, which yielded the original *Hpa*I V region fragment (454 bp), the smaller *Hae*III fragment (287 bp), and a new 207-bp fragment. Hence, the *Hae*III site lies within the C region, 207 bp from the *Hpa*I site, and presumably corresponds to amino acid 196, where a *Hae*III site occurs in two other κ sequences (Hamlyn et al., 1978; Seidman et al., 1978b). By a similar argument, the unique *Kpn*I cleavage site was placed 378 bp to the left of the *Hpa*I site, just into the precursor peptide region, where a *Kpn*I site at amino acid residue −3 is consistent with the known amino acid sequence of the MOPC 41A precursor peptide (Burstein & Schechter, 1977). These three unique sites served as reference points for further mapping.

To confirm assignments of terminal fragments and ensure that small terminal fragments had not been overlooked, we also examined restriction fragments of insert DNA terminally labeled with [α-^{32}P]dCTP as described above (Figure 5A). For example, Figure 6 displays the terminal *Mbo*II fragments of four inserts. Two gave the expected fragments, but pS107κ4 and pM603κ2 DNA each yielded a small, previously undetected, *Mbo*II fragment. From other data, the 25-bp fragment from pS107κ4 can only fit at the 5′ terminus (Figure 7). The 35-bp fragment from pM603κ2 DNA, however, must be at the 3′ terminus, since the large (365 bp) fragment can be placed with confidence at the 5′ end, because it contains the *Kpn*I sites (Figure 7). It follows that the nucleotide sequence within the 3′-terminal region of this clone must differ from that of the other six κ clones, which lack this 3′-terminal *Mbo*II site (see Discussion).

The complete amino acid sequences of three of the κ chains studied are known. As listed in Table II, in two cases (pM41κ1 and pM21κ1) all of the restriction sites which fall within the coding region of these clones can be related unambiguously to a particular amino acid residue. Similarly, restriction sites occurring within regions of known amino acid sequence (see Table III) in two other clones (pM603κ2 and pB61κ21) can be related to a particular position within that sequence. However, the restriction sites within the V region of pM173κ14

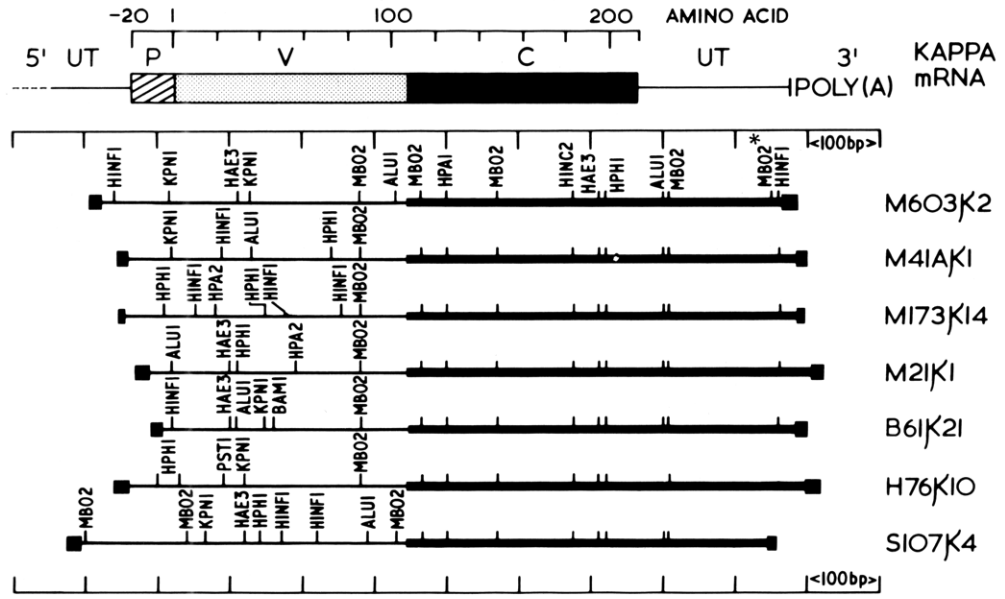

FIGURE 7: Restriction endonuclease maps of κ cDNA clones. The κ mRNA at the top of the diagram has 211 untranslated nucleotides at the 3′ end (Hamlyn et al., 1978), 642 nucleotides encoding the constant (C) and variable (V) regions, and approximately 60 nucleotides encoding the precursor peptide (P). The C region halves of the cloned cDNA sequences are illustrated by the thick lines and the V region halves by the thin lines. The blocks at the ends of the cDNAs represent the oligo(dG-dC) tails (Table III). Restriction sites were mapped by determining their positions with respect to unique sites within the sequence (see Results). The lengths of terminal fragments produced by all of the restriction enzymes shown were determined by digestion of end-labeled DNA as described in Figure 6. For the enzymes *Alu*I and *Hin*fI, only the terminal fragments were determined (but note that pS107κ4 has only one *Alu*I site, pM21κ1 only one *Hin*fI site, and all *Hin*fI sites were determined in pM173κ14). *Hin*fI and *Alu*I sites were not mapped in pH76κ10. The 3′-terminal *Mbo*II site in pM603κ2 (*) appears to be a cloning aberration (see Discussion).

Table II: Correlation between Restriction Enzyme Recognition Sites and Amino Acid and Nucleotide Sequences[a]

region	pM41κ1		pM21κ1	
precursor[b]	*Kpn*I	−3	*Alu*I	−3
variable[c]	*Hin*fI	20	*Hae*III	25
	*Alu*I	31	*Hph*I	30 (3′)[e]
	*Hph*I	73 (5′)[e]	*Hpa*II	53
	*Mbo*II	81 (3′)[e]	*Mbo*II	82 (3′)[e]
constant[c]	*Mbo*II	117 (5′)[e]		
	*Hpa*I	125		
	*Mbo*II	149 (3′)[e]		
	*Hin*cII	181		
	*Hae*III	196		
	*Hph*I	203 (5′)[e]		
3′ noncoding[d]	*Alu*I	*179*		
	*Mbo*II	*159* (3′)[e]		
	*Hin*fI	*12*		

[a] The restriction sites shown in Figure 7 are given in order from the left (5′) end. The positions of the recognition sites are given in amino acid residues from the N terminus, except in the 3′ noncoding region, where the italicized numbers indicate nucleotides from the 3′ end of the mRNA, excluding the poly(A) sequence. [b] The amino acid sequence of the MOPC 41A precursor is from Burstein & Schechter (1977). The amino acid sequence of the MOPC 21 precursor peptide has not been determined in this region; the *Alu*I site fits with the restriction map of an independent MOPC 21 κ cDNA clone, determined by Strathearn et al. (1978). [c] The amino acid sequences of the variable and constant regions are referenced in Table III. The positions of the recognition sites are given in amino acid residues from the N terminus. [d] The 3′ noncoding sequence is from Hamlyn et al. (1978); the positions of the recognition sites are given in nucleotides from the 3′ terminus. [e] The enzymes *Mbo*II and *Hph*I cleave the DNA strand 8 bp from an asymmetric recognition site. The direction of the cleavage site (with respect to the mRNA) is given in parentheses.

do not fit well with the published MOPC 173 amino acid sequence (Schiff & Fougereau, 1975). Moreover, nucleotide sequence analysis of pM173κ14 (N. M. Gough, unpublished experiments) has established the sequence of 68 amino acid residues within the V region, of which 17 differ from the published MOPC 173 κ sequence. The reason for this discrepancy is not clear. The number of differences makes it highly unlikely that they result from either mutations occurring within the cell line or from cloning aberrations, particularly as the changes do not yield an atypical V_κ amino acid sequence. Indeed, 14 of the 17 different amino acids are the same as residues within the MOPC 41A κ amino acid sequence, and none result in termination codons. Similarly, in clone pS107κ4, the 5′-terminal *Kpn*I, *Hae*III, *Hph*I, and *Hin*fI sites are not consistent with the known sequence for the 41 N-terminal amino acid residues of the S107 κ chain (Barstad et al., 1974).

Discussion

Cloning Methodology. Although we were able to synthesize very large duplex cDNA in some experiments (Figure 1), size fractionation was crucial for obtaining clones with large inserts, and chromatography on agarose beads proved an effective enrichment method. Of the 50–200 clones we obtained in a typical transformation experiment, from 5 to 15 colonies usually hybridized well with the κ probe, and sizing plasmid DNA from these clones by a reliable "mini-lysate" procedure (see Materials and Methods) usually revealed several with inserts of about 700 to 1000 bp. Difficulties initially encountered in transforming χ1776 efficiently were overcome when we adopted the $MnCl_2$–low pH method (Villa-Komaroff et al., 1978), but our yields of plasmid DNA from this strain have consistently been less than 20% that from other *E. coli* K12 strains.

Insertion of duplex cDNA into the *Pst*I site of the vector pBR322 by the dG·dC tailing procedure (Bolivar et al., 1977) worked well, since the background of parental transformants with the tailed vector was extremely low, and with one batch of vector nearly 90% of the inserts could be excised with *Pst*I. The lengths of dG·dC tails, determined by a simple procedure involving labeling of 3′ termini and sizing of pyrimidine tracts (Figure 5), were between 11 and 23 bp (Table III). These values were about those expected from the amount of dCTP incorporated during tailing of the cDNA preparations, indicating that the cloning procedure does not preferentially select molecules with long tails. Indeed, we have found one recombinant with a tail of only seven residues (Adams et al., 1980).

Identity of the Clones. We had little difficulty in identifying the κ clones, because as a probe we had available cDNA made on the substantially purified, well-characterized MOPC 41A κ mRNA (Cory et al., 1976; Bernard et al., 1977). The clones selected gave reproducibly strong colony hybridization, and the following lines of evidence buttress their identification. (a) Our first isolate, pM41κ1, strongly hybridized to an insert fragment from the established κ cDNA clone K38 (Mach et al., 1977; Rochaix et al., 1978). (b) DNA from pM41κ1 specifically arrested translation of κ mRNA into the immunoprecipitable κ chain (Figure 2). (c) Electron microscopy indicated that complete R loops were formed between pM41κ1 DNA and MOPC 41A κ mRNA, whereas κ mRNAs of different isotypes gave R loops with V-region tails (B. Tyler, unpublished experiments). (d) Several clones containing other κ chain cDNAs hybridized with the κ insert from pM41κ1. (e) A sequence of about 50 residues determined from a 3′-terminal fragment of pH76κ10 (N. Gough, unpublished experiments) matches the known sequence in that region of several κ

Table III: Summary of Information on the κ cDNA Clones

plasmid	isotype[a]	known amino acid sequence (residues)	length of insert (bp) (a)[i]	length of insert (bp) (b)[j]	length of tails[l]	orientation[m]
pM603κ2	VK-8	1–41[d]	1000	980	20, 19	V
pM41κ1	VK-9	complete[e]	990	960	16, 14	C
pM173κ14	VK-10	complete[f]	950	935	11, 10	C
pM21κ1	VK-15	complete[g]	990	965	22, 20	*n*
pB61κ21	VK-21[b]	1–23[h]	950	915	21, 16	V
pH76κ10	*c*	none	850 + 160[k]	845 + 160[k]	23, 19	V
pS107κ4	VK-22	1–41[d]	990	970	21, 12	V

[a] Isotypes of κ chains are defined by Potter (1977). [b] McKean et al. (1978). [c] In Southern blot experiments pH76κ10 hybridizes to the same set of genomic restriction fragments as pB61κ21, suggesting that the H76 κ chain is related to the VK-21 isotype (S. Cory, unpublished experiments). [d] Barstad et al. (1974). [e] Gray et al. (1967). [f] Schiff & Fougereau (1975). [g] Svasti & Milstein (1972). [h] Hood et al. (1973). [i] Lengths of fragments excised with *Pst*I and sized on 2% agarose gels (Figure 3B). [j] Average of the sum of *Hpa*I and *Hae*III restriction fragments sized on 5% polyacrylamide gels. [k] The 160-bp *Pst*I fragment from pH76κ10 was sized on a 5% polyacrylamide gel. [l] The lengths of the oligo(dG·dC) tails were determined by depurination of terminally labeled insert DNA (Figure 5). [m] The region of the insert sequence (C = constant, V = variable) proximal to the *Bam*HI site of the plasmid is given (Figure 4). [n] Not done.

mRNAs (Hamlyn et al., 1978; Gough et al., 1979). (f) Finally, the restriction maps in Figure 7 clearly exhibit variable and constant regions, and many sites can be matched with known amino acid and nucleotide sequences (Table II).

κ Sequences Represented in the Clones. The length of each insert sequence was determined both by sizing the *Pst*I fragment directly on an agarose gel and by summing the lengths of smaller restriction fragments derived from it, resolved on polyacrylamide gels (Table III). We think the slightly smaller estimates obtained from the latter procedure are more accurate, particularly as precise sizes are known for some of the smaller fragments by correlation with amino acid and nucleotide sequence data. In any case, a very substantial portion of the mRNA sequence is represented in each clone. Sizing of κ mRNAs under denaturing conditions has given length estimates of between 1200 and 1300 nucleotides, about 200 of which are the poly(A) tail (Brownlee et al., 1973). As all the clones have inserts between 915 and 1000 bp long (Table III) and none contains any substantial sequence complementary to the poly(A) tract (see below), each must contain most of the remaining 1000 to 1100 nucleotides of the mRNA.

The exact regions of the mRNA represented in each clone can be seen in the restriction maps (Figure 7). All of the clones have sequences extending beyond the V and C coding regions of the mRNA. Five clones contain virtually the entire 3′ noncoding sequence of 211 residues (Hamlyn et al., 1978), and the other two contain all except 30–40 bp. Nucleotide sequencing has confirmed that pH76κ10, which extends the furthest toward the 3′ end of the mRNA, contains the entire 3′ noncoding region, as well as 17 thymidylate residues complementary to the poly(A) tract of the mRNA (N. Gough, unpublished experiments).

5′ Noncoding Region. We can estimate the minimum length of the 5′ noncoding sequence in κ mRNAs from the mapping data on pS107κ4, the cloned sequence which extends furthest in that direction (Figure 7). The pS107κ4 sequence extends 149 bp (Table I) beyond the V region, about 60 bp of which would correspond to the 19–22 amino acids of the precursor peptide (Burstein & Schechter, 1978) and another 12 or 21 bp to the oligo(dG·dC) tail (Table III). Hence, the minimum length of the 5′ noncoding region in κ mRNAs is 149 − (60 + 21) = 68 nucleotides. R-looping data indicate that the region is unlikely to be much longer than this. Thus, even though pM41κ1 DNA contains little of the 5′ noncoding region, no 5′ mRNA tails could be visualized on hybridization with MOPC 41A κ mRNA, although a knob corresponding to the 200 nucleotides of poly(A) was very frequently seen (B. Tyler, unpublished experiments). Thus, the pM41κ1 sequence apparently extends to within 200 bp of the mRNA 5′ terminus. Hence, we estimate that the 5′ noncoding sequence in κ mRNAs is between 68 and 210 nucleotides long, and thus the complete κ mRNA sequence without poly(A) is estimated to be between 980 and 1120 nucleotides long.

The restriction maps reveal a sequence difference within the 5′ noncoding segment represented in clones pS107κ4 and pM603κ2, the only two clones that contain a significant portion of this region. Clone pM603κ2 contains a *Hin*fI site within this region, whereas there is no such site within the pS107κ4 sequence before the V coding region (Figure 7). However, any conclusion based upon a single restriction site must be tentative in light of the cloning aberration discussed below. Hence, 5′ noncoding sequences probably differ in different κ mRNAs.

An Apparent Cloning Aberration. The 3′-terminal *Mbo*II site in pM603κ2 is not present in the other six κ clones (Figure 7). To investigate this heterogeneity further, we have determined the sequence of the 3′-terminal 100 residues in the McPC 603 mRNA, as well as in four other κ mRNAs (MOPC 41A, MOPC 21, S107, and HPC 76), using a dideoxynucleotide procedure (Gough et al., 1979). The McPC 603 mRNA sequence was exactly the same as the others and did not contain an *Mbo*II site; there were, however, two positions near the expected site where a single base change could create an *Mbo*II site. Hence, this site may have been created during the synthesis of the cDNA or the cloning step. Copying errors by reverse transcriptase on synthetic polynucleotides have been documented (Battula & Loeb, 1975).

Evidence for a Unique C_κ Gene. With the exception of the pM603κ2 *Mbo*II site, the restriction maps of the seven κ sequences are identical through the C region (Figure 7) and hence provide no evidence for a multiplicity of mouse C_κ genes. The question of whether the mouse contains one or more C_κ genes has not been resolved, because the available nucleic acid hybridization data cannot distinguish between one and a few C_κ genes (Rabbitts & Milstein, 1977; Valbuena et al., 1978) and the slight differences in the published amino acid sequences for seven κ C regions may result from sequencing errors (Hamlyn et al., 1978; Smith, 1978). However, nucleotide sequences recently reported for a substantial portion of the C region in two κ mRNAs are identical (Hamlyn et al., 1978; Seidman et al., 1978b), as are sequences of about 100 nucleotides from the 3′ noncoding region of five κ mRNAs (Hamlyn et al., 1978; Gough et al., 1979). Our restriction mapping results thus provide additional support for the hypothesis that C_κ nucleotide sequences are identical and hence favor the existence of a unique C_κ gene in the mouse.

Acknowledgments

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