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Synthesis and Isolation of DNA Complementary to Nucleotide Sequences Encoding the Variable Region of Immunoglobulin κ Chain†

Janet Stavnezer*,‡ and J. Michael Bishop

ABSTRACT: We have prepared radioactive DNAs complementary to nucleotide sequences encoding the C ($cDNA_{\kappa C}$) and V ($cDNA_{\kappa V}$) regions of the immunoglobulin κ chain produced by the mouse myeloma MOPC 41. Our procedure exploited two technical innovations. First, we used random oligodeoxynucleotides to initiate transcription of DNA from κ chain messenger RNA by RNA-directed DNA polymerase. Since initiations occurred at various sites along the messenger RNA, this procedure circumvented problems encountered when DNA synthesis was initiated on oligo(dT) bound to poly(A) at the 3′ terminus of the messenger. Second, we fractionated $cDNA_{\kappa C}$ from $cDNA_{\kappa V}$ by molecular hybridization with messenger RNA for the κ chain produced by the NP2 variant of mouse myeloma; this RNA contains a deletion affecting the entire V region and therefore hybridizes with DNA complementary to the C region, but not with DNA complementary to the V region. We characterized $cDNA_{\kappa C}$ and $cDNA_{\kappa V}$ by molecular hybridization with RNAs from a series of mouse myelomas synthesizing κ chains with different V regions. $cDNA_{\kappa C}$ hybridized extensively with all of the RNAs tested. By contrast, the divergence of the V genes for the various κ chains was manifest in the extent and rate of hybridization with $cDNA_{\kappa V}$ and in the thermal stabilities of the hybrids. We estimate that a specific $cDNA_{\kappa V}$ can anneal appreciably with only a relatively small fraction of the DNA encoding identified V regions in mice. Consequently, the use of molecular hybridization to enumerate genes for the V region of κ chains can provide only minimum values which may be well below the total number of κ chain V genes.

The constant (C)[1] and variable (V) regions of immunoglobulin (Ig) proteins are encoded in separate genes which must be joined to produce an Ig molecule. C regions are shared by many different antibody molecules and the number of C region genes is limited accordingly. By contrast, V regions display great diversity and the mechanism which generates this diversity remains in dispute; either the entire repertoire of V genes is encoded in germ-line DNA, or a smaller number of genes undergoes somatic mutation during development of the immune system (Williamson, 1976).

Molecular hybridization can be used to study the genesis of diversity in the V region (Leder et al., 1974; Tonegawa et al., 1974; Rabbitts and Milstein, 1975; Rabbitts et al., 1975; Farace et al., 1976; Honjo et al., 1976a; Storb and Marvin, 1976; Tonegawa, 1976) and the transposition of Ig genes required to produce complete Ig proteins (Hozumi and Tonegawa, 1976). In order to facilitate these studies, we have prepared and separated radioactive DNAs complementary to at least a portion of the C region ($cDNA_{\kappa C}$) and the V region ($cDNA_{\kappa V}$) in the messenger RNA ($mRNA_{\kappa}$) for the κ light chain produced by the mouse myeloma MOPC 41 (Figure 1). Our procedure exploited two technical innovations. (i) The transcription of DNA from $mRNA_{\kappa}$ by RNA-directed DNA polymerase was initiated on random oligodeoxynucleotides (Goulian et al., 1973; Taylor et al., 1976). In this procedure, initiations occur at various positions along the mRNA; consequently, a portion of the transcripts will have initiated within the V region and will contain no DNA complementary to the C region. Previously described procedures for transcribing $mRNA_{\kappa}$ used oligo(dT) to initiate DNA synthesis at the 3′ end of the RNA template; under these circumstances, the V region is transcribed infrequently and incompletely, and the resulting transcripts from the V region are covalently linked to DNA transcribed from the C region (Stavnezer et al., 1974; Rabbitts and Milstein, 1975; Schechter, 1975; Farace et al., 1976; Honjo et al., 1976a; Smith and Huang, 1976). (ii) We separated $cDNA_{\kappa V}$ from $cDNA_{\kappa C}$ by molecular hybridization with RNA from the mouse myeloma variant NP2 (Kuehl and Scharff, 1974). The V region is deleted from all of the $mRNA_{\kappa}$ in this variant (Kuehl et al., 1975): consequently, only $cDNA_{\kappa C}$ can hybridize to the RNA, and separation of hybridized from unhybridized DNA by fractionation on hydroxylapatite (HAP) provides both $cDNA_{\kappa C}$ and $cDNA_{\kappa V}$. We also performed a further selection by hybridization with RNA from a mouse myeloma (TEPC 15) whose κ chain is highly diverged from that of MOPC 41 (Gray et al., 1967; Barstad et al.,

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[1] Abbreviations: C and V regions, constant and variable regions of immunoglobulins, respectively; Ig, immunoglobulin; $mRNA_{\kappa}$, messenger RNA for Ig κ chain; cDNA, DNA complementary to $mRNA_{\kappa}$; $cDNA_{\kappa C}$, DNA complementary to portion of $mRNA_{\kappa}$ coding for the C region; $cDNA_{\kappa V}$, DNA complementary to portion of $mRNA_{\kappa}$ coding for the V region; C_Rt, product of concentration of RNA nucleotides in mol/L and time in s; HAP, hydroxylapatite; T_m, temperature required to denature 50% of a duplex nucleic acid; $NaDodSO_4$, sodium dodecyl sulfate; PB, buffer made of equimolar NaH_2PO_4 and Na_2HPO_4, pH 6.8; V_{κ} genes, genes for V regions of κ chains.

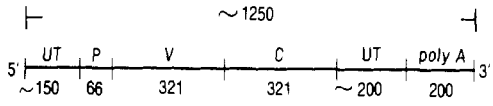

FIGURE 1: Diagrammatic representation of $mRNA_\kappa$ (Milstein et al., 1974). The numbers refer to the length in nucleotides of the $mRNA_\kappa$ and the regions within it. The lengths of the untranslated regions are approximate. The number of nucleotides coding for the precursor peptide was determined from the amino acid sequence of the *MOPC 41* precursor (Schechter and Burstein, 1976). The lengths of the regions coding for the V and C regions were derived from the protein sequences of κ chains. UT = untranslated region; P = nucleotides coding for the peptide present in the κ chain precursor.

1974). Again, $cDNA_{\kappa V}$ was isolated as DNA which failed to hybridize to the RNA.

In this communication, we describe the purification and characterization of $cDNA_{\kappa C}$ and $cDNA_{\kappa V}$, and we evaluate the relative abilities of these cDNAs to hybridize with $mRNA_\kappa$ from a variety of myelomas which produce divergent κ chains. Our results indicate that a specific $cDNA_{\kappa V}$ can anneal appreciably with only a relatively small fraction of the DNA encoding identified κ chain V regions in mice. Consequently, the use of molecular hybridization to enumerate genes for the V region of κ chains can provide only minimum estimates of the total number of V_κ genes.

Materials and Methods

Reagents and Solutions. Reagents were obtained and glassware was treated as in Stavnezer et al. (1974) except as noted. RNA-directed DNA polymerase from avian myeloblastosis virus was obtained from M. A. Chirigos (National Cancer Institute, Bethesda, Md.), restriction endonuclease *Hin*dIII from H. Boyer, and ^{32}P-labeled SV 40 DNA from B. Polisky. Solutions were treated with 0.1% diethylpyrocarbonate and autoclaved when possible. Myelomas were obtained as follows: NP2 from W. Marzluff; Pc3936A from M. Weigert; MOPC 31c, MOPC 149, W3082, MOPC 384, and TEPC 15 from Litton-Bionetics.

Isolation of $mRNA_\kappa$. $mRNA_\kappa$ was isolated from frozen MOPC 41 myelomas (Stavnezer et al., 1974) as described previously with the following modifications. In order to facilitate the purification of $mRNA_\kappa$, some of the myeloma tumors used for the mRNA preparation were labeled by intraperitoneal injection of the tumor-bearing mice with approximately 5–10 mCi of ^{32}P in two doses, 18 and 4 h before the mice were killed. Approximately 20 g of myelomas was thawed, minced, and homogenized in 100 mL of cold 250 mM sucrose–100 mM NaCl–1.5 mM $MgCl_2$–4 mM Tris-HCl, pH 7.4, containing 200 μg of cycloheximide/mL. The nuclei were pelleted at 6000*g* for 5 min, and the supernatant solution was centrifuged at 16 000*g* for 25 min to pellet the microsomes. The pellets were rapidly homogenized in 17 mL of 2% sodium dodecyl sulfate–500 μg of heparin/mL–5 mM Tris-HCl, pH 7.4. An equal volume of 20 mM Na_2EDTA was added and then proteinase K (EM Reagents) to 200 μg/mL. After incubation at 37 °C for 0.5 h, RNA was extracted three times with phenol–chloroform.

After ethanol precipitation, the mRNA was adsorbed twice to an oligo(dT)-cellulose column (Collaborative Research, T-3) in 0.5% sodium dodecyl sulfate–0.5 M NaCl–10 mM Tris-HCl, pH 7.4, and eluted with 1% sodium dodecyl sulfate in H_2O. The mRNA was centrifuged as before, except prior to loading on the gradient it was heated at 80 °C for 2–4 min in 1% $NaDodSO_4$–10 mM Na_2EDTA–10 mM Tris-HCl, pH 7.4. The $mRNA_\kappa$ (13–14 S) was located by its ability to direct κ chain synthesis in the reticulocyte cell-free system (Stavnezer and Huang, 1971; Stavnezer et al., 1974). The $mRNA_\kappa$ was then further purified by electrophoresis in a 2.3% polyacrylamide gel, cross-linked with ethylene diacrylate (Bishop et al., 1967). The $mRNA_\kappa$ was eluted from the gel slices by soaking in 1–2 mL of 1% $NaDodSO_4$–100 μg of heparin/mL–40 μg of yeast RNA/mL–10 mM Na_2EDTA–10 mM Tris-HCl, pH 7.4, overnight at room temperature. After removing the supernatant, the slices were incubated 6–7 h more with 1 mL more of the same solution. After phenol–chloroform extraction of the $mRNA_\kappa$, the heparin and yeast RNA carrier were removed either by (i) centrifugation in a 5–20% sucrose gradient in the SW 65 rotor for 2.5 h at 64 000 rpm at 23 °C, or by (ii) adsorption of the $mRNA_\kappa$ to oligo(dT)-cellulose. The $mRNA_\kappa$ was ethanol precipitated with a carrier of 1 mg of rCTP or rUTP.

The purity of each preparation of $mRNA_\kappa$ was assessed by the kinetics of hybridization between $mRNA_\kappa$ and DNA complementary to $mRNA_\kappa$ (cDNA). The reaction appeared to have a single component with a $C_rt_{1/2}$ (C_rt = concentration of RNA nucleotides in mol/L × time in s) varying between 6×10^{-4} and 10^{-3} (corrected to standard conditions; Britten and Smith, 1970). These results conform to the value of $C_rt_{1/2}$ predicted for an RNA the size of $mRNA_\kappa$ (400 000 daltons; Stavnezer et al., 1974) by comparison with the kinetics of hybridization of purified polio RNA with polio cDNA (unpublished data of the authors) and of globin mRNA with globin cDNA (Honjo et al., 1976b).

Preparation of Primers for $cDNA_\kappa$ Synthesis. Oligodeoxynucleotide primers for $cDNA_\kappa$ synthesis were prepared by two methods.

Method 1. Calf thymus DNA was treated with DNase as described by Goulian et al. (1973) with the following modifications. Calf thymus DNA (10 mg/mL) (Sigma) was digested with DNase (175 μg/mL) for 1 h at 37 °C in 10 mM $MgCl_2$–20 mM Tris-HCl, pH 7.4, then adjusted to 10 mM EDTA–0.1 M NaOH, heated at 68 °C for 10 min, neutralized, and extracted with phenol–chloroform. Oligonucleotides having a chain length of 9 to 13 were isolated on Sephadex G-50 (using the calibration described by Hohn and Schaller, 1967), lyophilized, and incubated in 0.6 N NaOH for 2 h at 37 °C.

Method 2. *E. coli* DNA primers were prepared as above except the oligonucleotides were not treated with base or fractionated on Sephadex; after extraction with $NaDodSO_4$ and phenol–chloroform, the entire digest was ethanol precipitated, dissolved in H_2O, and boiled.

Preparation of $cDNA_\kappa$. DNA complementary to $mRNA_\kappa$ was synthesized in a reaction mixture containing 80 mM Tris-HCl (pH 8.1), 12.5 mM $MgCl_2$, 0.2% 2-mercaptoethanol, 100 μg of actinomycin D/mL (Calbiochem), 0.01% NP-40 (Shell Co.), 1.5 mM each of dCTP, dATP, and dGTP, 0.05–0.5 mM [^3H]dTTP (47 Ci/mmol) (Amersham), $mRNA_\kappa$ (10 μg/mL), RNA-dependent DNA polymerase from avian myeloblastosis virus (10 μg/mL), and one of the following: $oligo(dT)_{12-18}$ (4 μg/mL) (Collaborative Research), calf DNA primers (700 μg/mL), or *E. coli* DNA primers (330 μg/mL). After incubation for 2–4 h at 37 °C (oligo(dT) primer) or at room temperature (calf or *E. coli* DNA primers), the single-stranded cDNA was isolated by RNase digestion and fractionation on an HAP column in 0.12 M phosphate buffer (PB) at 60 °C (Stavnezer et al., 1974). The single-stranded cDNA was precipitated with 0.2% cetyltrimethylammonium bromide as described by Stehelin et al. (1976), except that the nucleic acid carrier was 500 μg of yeast RNA; the precipitate was collected by centrifugation for 30 min at 16 000*g*. The pellets were dissolved in 1 M NaCl–0.6 N

NaOH, incubated at 37 °C for 2 h to eliminate RNA and traces of RNase, and ethanol precipitated. The specific activity of the cDNA was 16 000 cpm/ng. The size of the cDNA was determined by sedimentation in alkaline sucrose (Stavnezer et al., 1974) or by electrophoresis in polyacrylamide gels made up in 98% formamide (Maniatis et al., 1975). The molecular weight in alkaline sucrose was calculated according to Prunell and Bernardi (1973), whose formula was derived for DNAs similar in size to the cDNA. We have validated their formula (J.S., unpublished data) using coelectrophoresis of 5.0S DNA (Stavnezer et al., 1974) with DNA fragments produced by digestion of SV 40 DNA with the restriction endonuclease *Hin*dIII. The size of the DNA fragments has been determined by electron microscopy (Danna and Nathans, 1971). Electrophoresis was performed in 4% polyacrylamide gels made up in 98% formamide (Maniatis et al., 1975).

Isolation of Cellular RNAs. RNAs were isolated from cytoplasmic fractions prepared from either packed cells or frozen myeloma tumors (Stavnezer et al., 1974). The cytoplasm was treated with 200–500 μg of proteinase K/mL–1% $NaDodSO_4$–10 mM EDTA, pH 7, and extracted with phenol–chloroform. After ethanol precipitation, the RNA was incubated with DNase (RNA:DNase, 200:1) for 45 min at room temperature and then extracted with $NaDodSO_4$ and phenol–chloroform. Total cellular RNA was isolated by method 2 in Stavnezer et al. (1974).

Nucleic Acid Hybridization. Hybridization of RNA with DNA was carried out under mineral oil in 0.6 M NaCl–0.002 M EDTA–0.04 M Tris-HCl, pH 7.4, at 68 °C (stringent conditions), or in 1.5 M NaCl–0.005 M EDTA–0.1 M Tris-HCl, pH 7.4, at 59 °C (nonstringent conditions) (Rice and Paul, 1972; Stavnezer et al., 1974). In order to minimize the effect of trace amounts of nucleases, carrier nucleic acids were added so that reactions included at least 1 mg of RNA/mL and 4 μg of DNA. To eliminate any possible hybridization with labeled poly(dT) in the cDNA, unlabeled poly(dT) was included in all reactions in at least 15-fold excess of the amount of poly(A) estimated to be present in the reactions. Hybridization performed under stringent conditions was assayed with either S_1 nuclease (Sullivan et al., 1973) or by fractionation on HAP columns in 0.12 M PB at 60 °C (Stavnezer et al., 1974). Hybridization performed under nonstringent conditions was assayed on HAP columns in 0.14 M PB at 45 °C. In both cases the double-stranded nucleic acids were eluted with 0.4 M PB. The results were expressed as a function of C_rt. The value of C_rt was corrected to standard conditions for reactions in 0.6 M NaCl at 68 °C by multiplying by 5 (Britten and Smith, 1970); no correction factor for reactions in 1.5 M NaCl at 59 °C has been established. The results were corrected for background with unhybridized cDNA (14% for $cDNA_{\kappa C}$ and 2–4% for $cDNA_{\kappa V}$).

Thermal Denaturation of Hybrids. The products of the hybridization were loaded onto HAP columns (≤100 μg of RNA/mL bed volume) in 0.14 M pb at 45 °C. Single-stranded DNA was eluted, the column flow was stopped, and the temperature of the water bath was raised the desired increment. When water inside a column of identical size achieved the desired temperature, the single-stranded DNA was again eluted. After elution at 90 °C, columns were washed with 0.4 M PB to remove any remaining hybrid.

Results

Synthesis of cDNA Using Random Primers. In preliminary tests, we found that oligonucleotides produced by digestion of either calf thymus DNA or *E. coli* DNA with DNase I can serve as primers for the initiation of DNA synthesis with

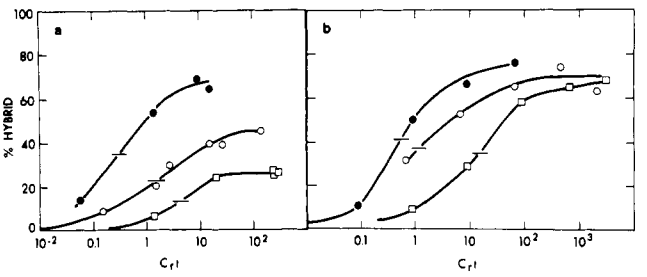

FIGURE 2: (a) Hybridization between cDNA primed with calf oligodeoxynucleotides and total cell RNA from three myelomas. Complementary DNA (0.03 ng) was incubated with various excess amounts of myeloma cell RNAs in volumes of 50 μL under stringent conditions for times ranging from 1 to 21 h. The samples were then assayed by hydrolysis with S_1 nuclease. (●—●) MOPC 41 RNA; (○—○) 66.2 RNA; (□—□) NP2 RNA. (b) Hybridization of cDNA primed with oligo(dT) to total RNA from three myelomas. The hybridizations for 14.5 h and assays were performed as in a. The $C_rt_{1/2}$ values are indicated by the horizontal line on each curve. (●—●) MOPC 41 RNA; (○—○) 66.2 RNA; (□—□) NP2 RNA.

$mRNA_\kappa$ as template. Maximum DNA synthesis was obtained at DNA/RNA ratios (mass) of 60:1 for calf thymus DNA and 40–50:1 for *E. coli* DNA; at these ratios of primer/template, the amount of DNA synthesized was 20–40% of the amount of RNA template. The yield of cDNA obtained with random primers was 50–100% of the yield obtained with $oligo(dT)_{12-18}$. The yield at room temperature was 50% greater than at 37 °C, even when the reaction was initiated at room temperature and then shifted to 37 °C after 10 min. The size of the cDNA synthesized at room temperature and at 37 °C was heterogeneous, with an average chain length of about 200–300 nucleotides, determined by sedimentation in alkaline sucrose gradients and by electrophoresis in polyacrylamide gels in 98% formamide.

To determine whether the cDNA initiated with random primers contained sequences complementary to the V region of $mRNA_\kappa$, the cDNA was hybridized with total cellular RNA obtained from three myelomas producing different κ chains (Figure 2a); (i) MOPC 41, the source of the $mRNA_\kappa$ used as template; (ii) 66.2, which produces a κ chain differing from the MOPC 41 κ chain by 49% in the first 63 amino acids of the V region (Gray et al., 1967; Smith, 1973); and (iii) NP2, which produces $mRNA_\kappa$ from which the V region has been deleted (Kuehl and Scharff, 1974; Kuehl et al., 1975; Kuehl, Smith, and Rose, unpublished data). The final level of hybridization obtained with NP2 RNA was about 35% of that obtained with RNA from MOPC 41, and the amount of hybridization obtained with RNA from the myeloma 66.2 was 64% of the amount with MOPC 41 RNA. When these same RNAs were hybridized with cDNA primed with oligo(dT), the final levels of hybridization were virtually identical (70–76%) (Figure 2b); the rates of hybridization differed because these myelomas contain different amounts of $mRNA_\kappa$ (Stavnezer et al., 1974; Kuehl et al., 1975). Therefore, cDNA initiated with random primers contained DNA not represented by complementary nucleotide sequences in NP2 and 66.2 cellular RNAs; we concluded that cDNA probably contained nucleotide sequences homologous to at least part of the V gene for the MOPC 41 κ chain. The proportion of V region-specific nucleotide sequences in the cDNA initiated with random primers varied among preparations, and cDNA initiated with *E. coli* primers yielded more $cDNA_{\kappa V}$ than did cDNA initiated with calf primers (13% with *E. coli* primers; 7% with calf primers).

Purification of $cDNA_{\kappa C}$ and $cDNA_{\kappa V}$. $cDNA_{\kappa V}$ was sepa-

TABLE I: Purification of $cDNA_{\kappa V}$.

Step in purification	DNA (cpm × 10^{-6}) recovered as: Single strand	Hybrid	Recovery of $cDNA_{\kappa V}/cDNA_{\kappa V'}$ in step (%)	Cumulative recovery of $cDNA_{\kappa V}/cDNA_{\kappa V'}$ (%)
1. Single-stranded cDNA (662 ng, 10.59 × 10^6 cpm) primed with *E. coli* oligodeoxynucleotides hybridized with 4.75 mg of MOPC 41 RNA to C_rt = 180 (stringent conditions)	2.88	7.71		
2. cDNA recovered as hybrid in step 1 hybridized with 16.4 mg of NP2 RNA to C_rt = 160 (nonstringent conditions) (sometimes this step was repeated with unhybridized cDNA recovered from this step)	1.01	6.70	13	13
3. Single-stranded $cDNA_{\kappa V}$ from step 2 hybridized with 260 μg of TEPC 15 RNA to C_rt = 19 (nonstringent conditions)	0.58	0.43	57	7.4

TABLE II: Specificity of $cDNA_{\kappa C}$, $cDNA_{\kappa V}$, and $cDNA_{\kappa V'}$.

RNA	% hybridization[a] Before selection	After selection $cDNA_{\kappa C}$	After selection $cDNA_{\kappa V}$[b]	After selection $cDNA_{\kappa V'}$[c]
MOPC 41 (C_rt = 6)	76	91	84	84
NP2 (C_rt = 84)	59	87	28	8

[a] All hybridizations were carried out to 80 times the $C_rt_{1/2}$ of the reaction, at a $mRNA_\kappa$ sequence:cDNA ratio of 160. The input of $mRNA_\kappa$ was computed from the $C_rt_{1/2}$ of the hybridization (Stavnezer et al., 1974). [b] Obtained by selection with NP2 RNA. [c] Obtained by selection with NP2 and TEPC 15 RNAs.

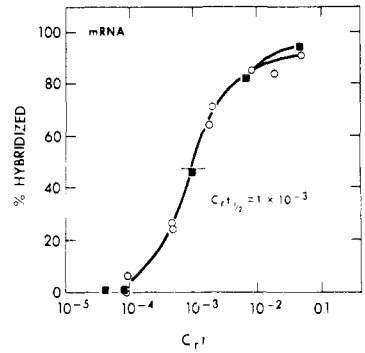

FIGURE 3: Hybridization between purified $mRNA_\kappa$ from the myeloma MOPC 41 and either $cDNA_{\kappa C}$ or $cDNA_{\kappa V}$. Reaction mixtures (20 μL) containing 1 ng of $mRNA_\kappa$ and 0.05 ng of either $cDNA_{\kappa C}$ or $cDNA_{\kappa V}$ were incubated under stringent conditions for various times (45 min to 18.5 h). The amount of hybridization was assayed on HAP columns at 60 °C. The $C_rt_{1/2}$ of the reaction is indicated by the horizontal line on the curves: (○—○) $cDNA_{\kappa V}$; (■—■) $cDNA_{\kappa C}$.

rated from $cDNA_{\kappa C}$ by hybridization with RNA from NP2 myeloma followed by fractionation of the hybridized DNAs on HAP. The procedure employed three cycles of hybridization and fractionation (Table I).

(1) The cDNA was hybridized with cytoplasmic RNA from MOPC 41 in the presence of excess poly(dT) and the hybrids were isolated by adsorption to HAP. This step removed labeled poly(dT) produced by transcription of poly(A) and selected DNA capable of hybridizing to $mRNA_\kappa$.

(2) The DNA recovered as hybrids in step 1 was treated with NaOH to hydrolyze the MOPC 41 RNA and then hybridized with cytoplasmic RNA from the myeloma NP2. The hybridized and unhybridized DNAs were separated by fractionation on HAP. On occasion, this step was repeated with the unhybridized DNA.

(3) The DNA which failed to hybridize in step 2 was hybridized with RNA from myeloma TEPC 15, followed by fractionation on HAP. The tumor TEPC 15 produces a κ chain which is extensively diverged from the κ chain of MOPC 41 in the V region (60% difference in the first 35 amino acids) (Gray et al., 1967; Barstad et al., 1974). We designated the DNA which hybridized with NP2 RNA in step 2 as $cDNA_{\kappa C}$, the DNA which did not hybridize with NP2 RNA as $cDNA_{\kappa V}$, and the DNA which did not hybridize with TEPC 15 RNA in step 3 as $cDNA_{\kappa V'}$.

We anticipated that some of the cDNA would be composed of chains initiated within the C region but extending into the V region. Two measures were taken to facilitate removal of this DNA from the preparation of $cDNA_{\kappa V}$. First, the hybridizations with NP2 RNA and TEPC 15 RNA were performed under nonstringent conditions in order to stabilize short duplexes and therefore permit the hybridization of cDNA containing short portions complementary to the C region of $mRNA_\kappa$. Second, in steps 2 and 3 we found it preferable to use a special grade of HAP (DNA grade of Bio-Gel HTP) because it has a relatively high affinity for short DNA:RNA hybrids (unpublished observation of J.S.).

Specificity of $cDNA_{\kappa C}$ and $cDNA_{\kappa V}$. We evaluated the purity of $cDNA_{\kappa C}$, $cDNA_{\kappa V}$, and $cDNA_{\kappa V'}$ by hybridization with RNAs from the myelomas MOPC 41 and NP2 (Table II); the reactions were carried out under conditions designed to drive the hybridizations to completion. $cDNA_{\kappa C}$ hybridized to the same extent (87–91%) with RNAs from both myelomas and therefore represented nucleotide sequences shared by the $mRNA_\kappa$s of the two tumors. By contrast, $cDNA_{\kappa V}$ reacted extensively (84%) with MOPC 41 RNA but poorly (28%) with NP2 RNA. $cDNA_{\kappa V'}$ also reacted extensively with MOPC 41 RNA (84%), whereas the reaction with NP2 RNA was very small (8%).

$cDNA_{\kappa C}$, $cDNA_{\kappa V}$, and $cDNA_{\kappa V'}$ hybridized to purified $mRNA_\kappa$ from MOPC 41 at identical rates (Figure 3; data for $cDNA_{\kappa V'}$ not illustrated). By contrast, the limited reactions between NP2 RNA and either $cDNA_{\kappa V}$ or $cDNA_{\kappa V'}$ were slower than the reaction between NP2 RNA and $cDNA_{\kappa C}$ (Figure 4h). We attribute the limited reactions between NP2 and $cDNA_{\kappa V}/cDNA_{\kappa V'}$ to residual C region DNA covalently linked to V region DNA, and we suggest that the rate of the reactions was retarded because the C region DNA was relatively short (see Discussion).

We recovered 13% (after correction for losses) of cDNA as

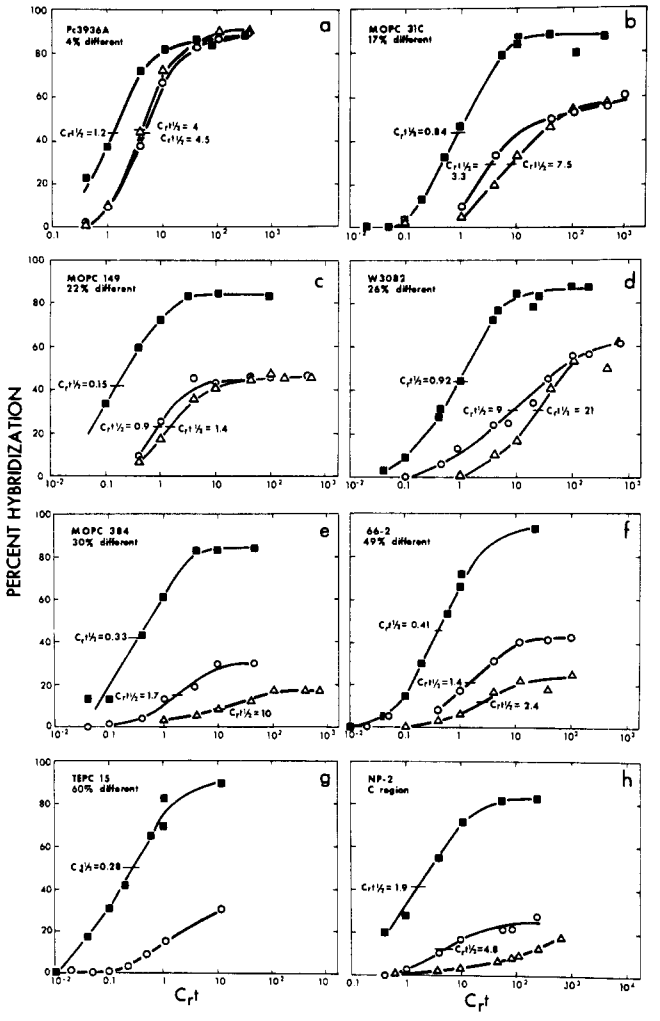

FIGURE 4: Hybridization of RNA from mouse myeloma cells with $cDNA_{\kappa c}$, $cDNA_{\kappa v}$, and $cDNA_{\kappa v'}$. Total cellular or cytoplasmic RNAs were hybridized with cDNA (ratio of $mRNA_\kappa$:cDNA was at least 20:1) for various times up to 41 h. Hybridizations and assays were performed under nonstringent conditions (see Materials and Methods). The source of the RNA and the divergence from MOPC 41 in the known portions of the κ V region amino acid sequence are given in each panel. The number of amino acid residues which have been sequenced in each region is given in Table III. MOPC 41 κ chain has been sequenced completely (Gray et al., 1967). Values of $C_rt_{1/2}$ are indicated by the horizontal line on each curve. (O—O) $cDNA_{\kappa v}$; (Δ—Δ) $cDNA_{\kappa v'}$; (■—■) $cDNA_{\kappa c}$.

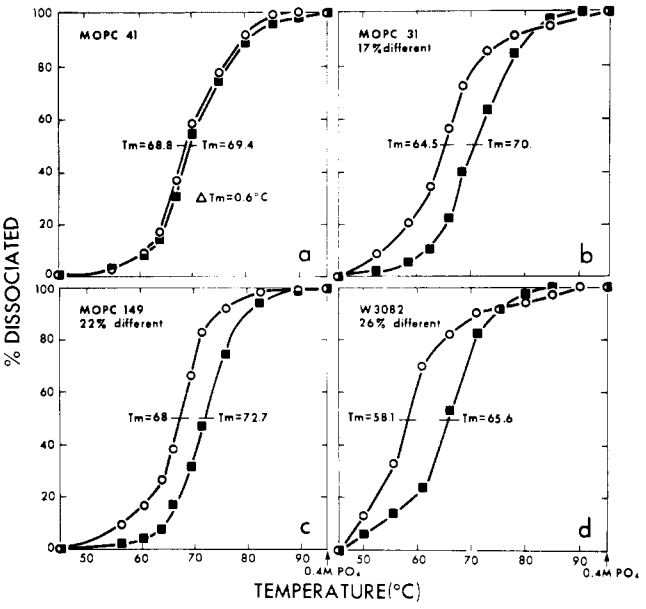

FIGURE 5: Thermal denaturation DNA:RNA hybrids of $cDNA_{\kappa c}$ and $cDNA_{\kappa v}$ with myeloma cell RNAs. Hybrids were formed under nonstringent conditions (see Methods). Reactions were carried to values of C_rt at least 100-fold greater than $C_rt_{1/2}$. Sufficient cellular RNA was used to obtain more than a 100-fold excess of $mRNA_\kappa$:cDNA. Hybrids of $cDNA_{\kappa c}$ and $cDNA_{\kappa v}$ with each myeloma cell RNA were denaturated simultaneously on two columns in the same water bath. The T_m's are indicated by the horizontal line on each denaturation curve. (O—O) $cDNA_{\kappa v}$; (■—■) $cDNA_{\kappa c}$.

$cDNA_{\kappa v}$ and 7.4% as $cDNA_{\kappa v'}$. The sizes of $cDNA_{\kappa c}$, $cDNA_{\kappa v}$, and $cDNA_{\kappa v'}$ were heterogeneous with an average chain length of 175, 135, and 120 nucleotides, respectively.

The Effect of Divergence in the V Regions on Hybridization with $cDNA_{\kappa v}$ and $cDNA_{\kappa v'}$. We characterized our cDNAs by molecular hybridization with RNAs from a series of mouse myelomas which synthesize κ chains with different V regions.[2] $cDNA_{\kappa c}$ hybridized to similar extents with all of the RNAs tested (Figure 4). By contrast, hybridizations between either $cDNA_{\kappa v}$ or $cDNA_{\kappa v'}$ and cellular RNAs containing $mRNA_\kappa$ divergent from that of MOPC 41 were incomplete (Figure 4 and Table III); divergence in κ chain genes was estimated from changes in the amino acid sequence of the gene product (see Table III).

The divergence of the V genes for the various κ chains was also manifest in the rate of hybridization with either $cDNA_{\kappa v}$ or $cDNA_{\kappa v'}$ (Figure 4 and Table III) and in the thermal stabilities of the hybrids (Figure 5 and Table IV). In these analyses, hybridization with $cDNA_{\kappa c}$ was used to standardize rates of reaction and measurements of thermal stability. We assumed that the divergence of nucleotide sequences among C regions of $mRNA_\kappa$s is not sufficient to affect significantly the parameters we measured (Stavnezer et al., 1974; Schechter, 1975). We also assumed that the myelomas synthesize only complete $mRNA_\kappa$s. One of the myelomas we studied (66.2) has been shown to synthesize a C_κ fragment and $mRNA_\kappa$ deleted in the V region, in addition to complete κ chains and $mRNA_\kappa$. However, the amount of the fragments synthesized is less than the amount of complete κ chains and $mRNA_\kappa$ (Kuehl and Scharff, 1974; Kuehl et al., 1975), and hence the presence of the fragment does not significantly affect the results. Other myelomas have also been found to synthesize C region fragments, but always in small amounts (10–20% of the amount of complete light chains) (Kuehl and Scharff, 1974; Schubert and Cohn, 1970). As shown above, $cDNA_{\kappa c}$, $cDNA_{\kappa v}$, and $cDNA_{\kappa v'}$ all reacted with MOPC 41 RNA at identical rates (Figure 3), but the rate with either $cDNA_{\kappa v}$ or $cDNA_{\kappa v'}$ was diminished relative to that with $cDNA_{\kappa c}$ when hybridizations were carried out with cellular RNAs containing $mRNA_\kappa$ divergent from that of MOPC 41 (Figure 4 and Table IV). With few exceptions, the greater the difference between the V regions of κ chains synthesized by MOPC 41 and the test myelomas, the lower the rate of hybridization of the V region cDNAs. The effect of divergence on rate of hybridization with the more diverged RNAs was appreciably greater with $cDNA_{\kappa v'}$ than with $cDNA_{\kappa v}$ (Table III), and the reactions with RNA from myelomas synthesizing κ chains whose V regions were very different from MOPC 41 were unexpectedly rapid ($cDNA_{\kappa v}$ hybridized with MOPC 384 and 66.2 RNAs, and $cDNA_{\kappa v'}$ hybridized with 66.2 RNA). We attribute these

[2] One of tumors tested (Pc3936A) was derived from an NZB mouse; the other tumors were from BALB/c mice. According to available data, the C regions of κ chains produced by NZB and BALB/c mice are not appreciably different (E. Loh, personal communication).

TABLE III: Hybridization of $cDNA_{\kappa V}$/$cDNA_{\kappa V'}$ with Myeloma Cell RNAs.

RNA	No. of residues sequenced from NH_2 terminus	Difference from MOPC 41 κ chain[a]	$cDNA_{\kappa V}$ Reduction in rate of hybridization (V/C)[h]	$cDNA_{\kappa V}$ Rel extent of hybridization (V/C)	$cDNA_{\kappa V'}$ Reduction in rate of hybridization (V/C)[h]	$cDNA_{\kappa V'}$ Rel extent of hybridization (V/C)
Pc3936A	26[b]	4%	3.7	1.0	3.3	1.0
MOPC 31c	23[c]	17%	3.9	0.68	8.9	0.67
MOPC 149	23[c]	22%	6.5	0.55	9.3	0.55
W3082	23[d]	26%	9.8	0.71	23	0.71
MOPC 384	23[c]	30%	5.2	0.36	30	0.20
66.2	63[e,f]	49%	3.4	0.45	5.9	0.26
TEPC 15	35[g]	60%	nt	nt	nt	nt
NP2		C region only	2.5	0.30	nt	0.10

[a] Computed as (number of positions with different amino acids ÷ number of amino acid residues sequenced)(× 100). [b] E. Loh, R. Riblet, M. Weigert, J. M. Hood, B. Black, and L. Hood, in preparation. [c] Hood et al. (1973). [d] Hood et al. (1974). [e] Smith (1973). [f] Excludes an N-terminal amino acid sequence which has no homologue in MOPC 41 κ chain. [g] Barstad et al. (1974). [h] Computed by taking $C_rt_{1/2}$ of $cDNA_{\kappa V}$ ÷ $C_rt_{1/2}$ of $cDNA_{\kappa C}$.

TABLE IV: Thermal Stability of RNA:DNA Hybrids.

Myeloma cell RNA[a]	T_m of hybrid with $cDNA_{\kappa V}$ (°C)	T_m of hybrid with $cDNA_{\kappa C}$ (°C)	ΔT_m (°C) ($T_{m_{\kappa C}} - T_{m_{\kappa V/\kappa V'}}$)	Average
MOPC 41	68.8[b]	69.4	0.6	0.65
	72.6[c]	73.3	0.7	
MOPC 31c (17%)	64.5[b]	70.0	5.5	4.6
	65.7[c]	69.6	3.9	
MOPC 149 (22%)	68.0[b]	72.7	4.7	
	65.8[b]	68.7	2.9	4.5
	63.2[b]	69.2	6.0	
W3082 (26%)	58.1[b]	65.6	7.5	

[a] Numbers in parentheses indicate extent of divergence from MOPC 41 in the N-terminal 23 amino acids. [b] $cDNA_{\kappa V}$. [c] $cDNA_{\kappa V'}$.

aberrant results to contamination of the cDNAs with C region nucleotide sequences (see Discussion).

We evaluated hybrids between $cDNA_{\kappa V}$ and RNA from various myelomas for mismatched base pairs by measuring the thermal stabilities of the hybrids (Figure 5 and Table IV). The measurements were standardized by comparing the T_m of hybrids with $cDNA_{\kappa V}$ ($T_{m_{\kappa V}}$) to the T_m of hybrids with $cDNA_{\kappa C}$ ($T_{m_{\kappa C}}$). Hybrids formed with MOPC 41 RNA had virtually identical $T_{m_{\kappa C}}$ and $T_{m_{\kappa V}}$ (Figure 5 and Table IV); the relative lengths of $cDNA_{\kappa C}$ (ca. 175 nucleotides) and $cDNA_{\kappa V}$ II (ca. 135 nucleotides) could account for the observed difference in T_m (0.6 °C) (Thomas and Dancis, 1973). Hybrids with RNA from myelomas whose κ chains diverge appreciably from the κ chains of MOPC 41 had larger differences between $T_{m_{\kappa C}}$ and $T_{m_{\kappa V}}$ (4–7 °C) (Table IV). Measurements with $cDNA_{\kappa V}$ and $cDNA_{\kappa V'}$ gave similar differences in T_m (Table IV). We conclude that divergence of nucleotide sequences encoding the V region of κ chains results in mismatched base pairs in hybrids formed between $mRNA_\kappa$ and $cDNA_{\kappa V}$ or $cDNA_{\kappa V'}$; divergence measured in this manner correlates approximately with divergence measured as changes in amino acid sequence (Table IV).

Discussion

Initiation of DNA Synthesis with Random Primers. The initiation of transcription of DNA from $mRNA_\kappa$ with calf or *E. coli* oligodeoxynucleotides does not appear to be completely random; the yield of $cDNA_{\kappa V}$ obtained using *E. coli* primers (13%) was greater than the yield obtained using calf primers (7%) and both types of primers gave more $cDNA_{\kappa C}$ than $cDNA_{\kappa V}$. If initiation were random over the entire $mRNA_\kappa$, about 30% of the DNA synthesized would be complementary to sequences not present in NP2 RNA. We suggest that the secondary structure of $mRNA_\kappa$ may favor initiation in the C region. Goulian et al. (1973) reported selectivity in the initiation of Dna synthesis when they used random primers prepared from one organism's DNA to initiate DNA synthesis with another organism's DNA as template for DNA polymerase I.

Specificity of $cDNA_\kappa$ Initiated on Random Primers. Random oligodeoxynucleotides can bind to both adenylated and unadenylated RNA and could therefore initiate DNA synthesis on any RNA contaminating our preparations of $mRNA_\kappa$. We have the following evidence that $cDNA_{\kappa C}$, $cDNA_{\kappa V}$, and $cDNA_{\kappa V'}$ are specific for nucleotide sequences encoding κ chain genes. (i) During the selection of $cDNA_{\kappa V}$/$cDNA_{\kappa V'}$ it is likely that cDNA transcribed from myeloma RNAs other than $mRNA_\kappa$ would hybridize with total cytoplasmic RNA from NP2 or TEPC 15 myelomas and would therefore be concentrated in the $cDNA_{\kappa C}$ and removed from $cDNA_{\kappa V}$/$cDNA_{\kappa V'}$. These contaminants should be detectable by hybridization with MOPC 41 $mRNA_\kappa$, since the contaminants would probably cause the $cDNA_{\kappa C}$ to hybridize with heterogeneous kinetics, differing from the kinetics of hybridization of $cDNA_{\kappa V}$/$cDNA_{\kappa V'}$. However, $cDNA_{\kappa C}$, $cDNA_{\kappa V}$, and $cDNA_{\kappa V'}$ hybridized to $mRNA_\kappa$ as single components (Figure 3 and unpublished data). The rates of the reactions (expressed as $C_rt_{1/2}$) were identical and conformed to the value predicted

from the complexity of $mRNA_\kappa$ (400 000 daltons) on the basis of results with poliovirus RNA (Stavnezer et al., 1974; unpublished data of the authors) and globin mRNA (Honjo et al., 1976b). (ii) The rates of hybridization between cDNA initiated with calf primers and RNAs from myelomas MOPC 41 and 66.2 (Figure 2a) were proportional to the relative amounts of κ chain protein synthesized by the tumors (Stavnezer et al., 1974). (iii) The extent and rate of hybridization between tumor RNAs and the V region cDNAs, and the thermal stabilities of the hybrids, reflected divergence in the V_κ genes (Figures 4 and 5; Tables III and IV).

Do $cDNA_{\kappa v}$ and $cDNA_{\kappa v'}$ Represent Translated Nucleotide Sequences? The $mRNA_\kappa$ of myeloma NP2 is deleted in the V region of κ chains but does encode the C region (Kuehl et al., 1975) and the amino-terminal peptide present in the polypeptide precursor of κ chains (Schmeckpeper et al., 1975; Schechter and Burstein, 1976; Kuehl, W. M., Smith, G. P., and Rose, S. M., unpublished data). The amino-terminal peptide of the κ chain fragment from NP2 differs in sequence from the amino-terminal peptide of MOPC 41 κ chain (Kuehl, Smith, and Rose, unpublished data; Schechter and Burstein, 1976), and hence the NP2 RNA may not hybridize with cDNA transcribed from this region of MOPC 41 $mRNA_\kappa$. It is not known whether the untranslated nucleotides at the 5′ terminus of $mRNA_\kappa$ (Milstein et al., 1974) are present in the $mRNA_\kappa$ from NP2. Consequently $cDNA_{\kappa v}$ could contain nucleotides complementary to a portion or all of the 321 nucleotides encoding the V region polypeptide, the 60–70 nucleotides encoding the amino-terminal peptide present in the polypeptide precursor of κ chains and the 150 (±50) untranslated nucleotides at the 5′ terminus of the messenger (Figure 1). However, the extent and rate of hybridization between myeloma RNAs and $cDNA_{\kappa v}$ or $cDNA_{\kappa v'}$, as well as the thermal stabilities of the hybrids, reflected the divergence in the amino acid sequence of the V regions of the κ chains (Tables III and IV).

C Region Nucleotide Sequences in $cDNA_{\kappa v}$ and $cDNA_{\kappa v'}$. Despite repeated selections by molecular hybridization, both $cDNA_{\kappa v}$ and $cDNA_{\kappa v'}$ reacted partially with NP2 RNA (ca. 22% and 8%, respectively) when assayed by fractionation on HAP; the rates of these reactions were appreciably slower than reactions between $cDNA_{\kappa c}$ and NP2 RNA (Figure 4h). By contrast, the reactions with NP2 RNA were less extensive (5% for $cDNA_{\kappa v}$) when hybridization was measured by hydrolysis with S_1 nuclease (unpublished data). We concluded that some molecules of $cDNA_{\kappa v}$ and $cDNA_{\kappa v'}$ consisted of DNA chains initiated within the C region but extending into the V region. Only the C region of these chains would base-pair with NP2 RNA, and their relatively short length could account for the retarded rate of hybridization with NP2 RNA. We do not know why these chains are detected in analytical hybridizations but are not eliminated in the preparative procedure. Selection of $cDNA_{\kappa v'}$ by hybridization with TEPC 15 RNA substantially reduces the contamination with C region nucleotide sequences (Tables II and III). We suggest that a relatively conserved portion of the V gene adjoins the C gene in $mRNA_\kappa$ of TEPC 15 and stabilizes duplexes formed with DNA chains containing covalently linked C and V region nucleotide sequences; these duplexes are then removed by the fractionation on HAP.

The presence of C region nucleotide sequences in $cDNA_{\kappa v}$ and $cDNA_{\kappa v'}$ may account for several aberrations in our data. (i) Hybridizations between $cDNA_{\kappa v}$ and RNAs from myelomas MOPC 384 and 66.2 were unexpectedly rapid, as was the reaction between $cDNA_{\kappa v'}$ and 66.2 RNA. We suggest that a major component of these reactions (which are limited in extent; see Figure 4) consists of hybridization between C region DNA and $mRNA_\kappa$; if so, the complementary nucleotide sequences in these reactions are well matched and the rates of hybridization will be diminished only by the chain length of the C region DNA. Since $cDNA_{\kappa v'}$ is less contaminated with C region DNA than is $cDNA_{\kappa v}$, the effect on rate of hybridization with $cDNA_{\kappa v'}$ becomes apparent only with the RNA from myeloma 66.2, which encodes the most divergent κ chain we studied. (ii) The effect of V_κ gene divergence on rates of hybridization was much greater for $cDNA_{\kappa v'}$ than for $cDNA_{\kappa v}$ (Table III). We attribute this to the fact that $cDNA_{\kappa v'}$ is less contaminated with C region DNA than is $cDNA_{\kappa v}$.

We presume that these artifacts could be reduced or eliminated by using S_1 nuclease to measure hybridization. However, assays with S_1 nuclease are too stringent to permit detection of the mismatched hybrids formed between $cDNA_{\kappa v}/cDNA_{\kappa v'}$ for MOPC 41 $mRNA_\kappa$ and RNAs from other myelomas.

The Effect of Mismatched Base Pairs on Rates of Hybridization. Extensive mismatching of base pairs in duplex DNA is required for a perceptible effect on rates of duplex formation; duplexes with T_m reduced by 10–20 °C (7–13% mismatching of bases; Ullman and McCarthy, 1973) reassociate approximately twofold slower than do perfectly matched duplexes (Bonner et al., 1973; Hutton and Wetmur, 1973; Marsh and McCarthy, 1974). Our data indicate a much larger effect of mismatching on formation of DNA:RNA hybrids; hybrids with T_m reduced by 7 °C formed at a rate tenfold slower than the rate for formation of fully homologous hybrids. We doubt but cannot prove that this discrepancy is due to structural differences between DNA duplexes and DNA:RNA hybrids. However, the mismatched base pairs in the hybrids we studied were probably clustered in hypervariable regions of the V genes, whereas the mismatched nucleotides were randomly spaced in the duplex DNAs studied previously (Bonner et al., 1973; Hutton and Wetmur, 1973; Marsh and McCarthy, 1974).

Enumeration of V Region Genes by Molecular Hybridization. Our data indicate that efforts to enumerate V genes for mouse κ chains are probably subject to large errors. Divergence in the V genes reduces both the extent and rate of hybridization with either $cDNA_{\kappa v}$ or $cDNA_{\kappa v'}$. These reductions are very large for V genes encoding κ chains which differ from the κ chain of MOPC 41 by 30% in the first 23 amino acids (Figure 4 and Table III), and over 70% of identified mouse κ chains are more than 30% divergent from the MOPC 41 κ chain (Hood et al., 1974). Moreover, the only mouse κ chains sequenced to date are from myelomas; consequently the variety of V_κ genes may considerably exceed current estimates (Hood et al., 1974).

We conclude that neither DNA nor RNA probes for a specific V_κ gene can react appreciably with the majority of V_κ genes in mice. Consequently, enumeration of V_κ genes by molecular hybridization provides only a minimum estimate which may be well below the total number of V_κ genes.

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