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Purification and Translation of an Immunoglobulin λ Chain Messenger RNA from Mouse Myeloma†

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ABSTRACT: Here we describe the 500-fold purification of an mRNA encoding an immunoglobulin λ light chain derived from the mouse myeloma tumor, RPC-20. Purification involves the isolation of membrane-bound polysomes, oligo(dT)-cellulose chromatography, and sucrose gradient centrifugation under conditions favoring denaturation of polynucleotide complexes. The mRNA purified in this way directs the cell-free synthesis of a polypeptide which is five or six amino acids longer than the mature form of RPC-20 light chain. In addition to directing the synthesis of a precursor-like polypeptide, the mRNA migrates on electrophoresis as a band containing approximately 1150 nucleotides, about 500 more than required to encode the mature form of the light chain.

Considerable insight has been gained into the genetic representation of immunoglobulin light chains from studies using purified κ light chain mRNAs and their complementary reverse transcripts (Faust et al., 1974; Honjo et al., 1974; Rabbitts, 1974; Stavnezer et al., 1974). These studies indicate that the κ constant region is represented relatively few times in the mouse genome. However, because of extensive sequence diversity in the variable regions of mouse κ chains and uncertainties in predicting the extent of cross-hybridization between κ gene sequences, it has not been possible to calculate unambiguously the number of variable region sequences in the mouse genome. This is so despite several recent studies which provide important hints of a relatively small or unique number of κ variable region genes (Leder et al., 1974a,b; Tonegawa et al., 1974a,b; Rabbitts and Milstein, 1975; Rabbitts et al., 1975).

Since quantitation of the variable region gene sequences has strong predictive value in terms of distinguishing between the germ line and somatic mutation hypotheses (Gally and Edelman, 1972; Wigzell, 1973; Cohn et al., 1974; Hood et al., 1974), it remains an important research goal. In this respect, the λ class of mouse light chain offers rather special advantages (to be discussed in the following article, Honjo et al., 1976) for determining the genetic representation of variable gene sequences. In the present report we describe the purification, characterization, and translation of an mRNA encoding an immunoglobulin λ chain derived from the RPC-20 myeloma tumor. This mRNA is employed in an accompanying study for the quantitation of λ chain constant and variable region genes (Honjo et al., 1976).

Materials and Methods

(*a*) *Preparations of Myeloma Polysomes and Myeloma mRNA.* Dr. M. Potter kindly supplied the myeloma tumors, which were grown, harvested, and stored as described (Swan et al., 1972). The polysomes were prepared according to a slightly modified procedure of Swan et al. (1972). One hundred grams of dissected tumor were homogenized as described (Swan et al., 1972), except that buffer A contained 50 mM Tris-HCl, pH 7.8, 25 mM KCl, 5 mM $MgCl_2$, 10^{-4} M cycloheximide, 0.1% diethyl pyrocarbonate, and 0.88 M sucrose. The homogenate was centrifuged through a discontinuous sucrose gradient composed of 7 ml of 1.5 M sucrose and 15 ml of 1.75 M sucrose, each containing buffer A lacking diethyl pyrocarbonate. After centrifugation at 25 000 rpm (81 500*g*) for 60 min in a Beckman SW27 rotor, the polysomes, which had been concentrated in the 1.5 M sucrose layer, were harvested. The RNA was extracted, followed by two successive purifications on oligo(dT)-cellulose and two sucrose gradient centrifugations, as described by Honjo et al. (1974). The second oligo(dT)-cellulose chromatographic step was modified slightly. The column was washed with 0.25 M KCl–0.01 M Tris-HCl (pH 7.5) instead of 0.18 M KCl–0.01 M Tris-HCl (pH 7.5).

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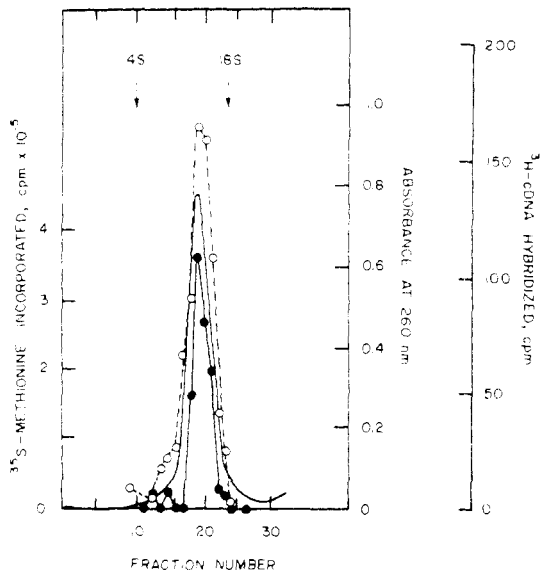

FIGURE 1: Second sucrose density gradient centrifugation of RPC-20 mRNA. The SG 1 fraction of RPC-20 mRNA (1.2 A_{260} units) was centrifuged as described (Honjo et al., 1974). Closed circles, solid line: protein synthetic activity; open circles, dotted line: hybridization with RPC-20 [^{3}H]cDNA; solid line: absorbance at 260 nm.

(*b*) *Cell-Free Protein Synthesizing Systems and Assays.* Translation of λ chain mRNA was carried out with the wheat germ cell-free system (Roberts and Paterson, 1973; Efron and Marcus, 1973). The preparation of the wheat germ extract was according to Roberts and Paterson (1973) except that the preincubation was omitted. Sucrose gradient fractions were assayed in 50-μl reaction mixtures containing 1.5 mM ATP,[1] 0.3 mM GTP, 16.5 mM creatine phosphate, 400 μg of creatine kinase, 3 mM DTT, 15 mM Hepes buffer (pH 7.6), 120 μM (each) of amino acids, and 5 μM radioactive amino acid ([^{3}H]leucine or [^{35}S]methionine, as indicated) were incubated for 2 h at 23 °C. Reactions were stopped by the addition of 20 μl of RNase A (1 mg/ml) and incubated for 20 min at 37 °C. Material prepared for tryptic peptide analysis was synthesized in 50 μl reactions containing 2 mM ATP, 0.4 mM GTP, 9 mM creatine phosphate, 0.5 μg of creatine kinase, 0.3 mM DTT, 20 mM Hepes buffer (pH 7.6), 30 μM each of nonradioactive amino acids, and 30 μM of radioactive amino acids (the specific activity of [^{3}H]leucine was made 22 Ci/mmol by lyophilization of 33 μl of 55 Ci/mmol specific activity material to dryness, followed by dissolving in 4 μl of 0.25 mM nonradioactive leucine).

(*c*) *Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis.* [^{35}S]Methionine-labeled products were synthesized in the wheat germ cell-free system. After RNase digestion, 50 μl of reducing buffer containing 0.13 M Tris-HCl (pH 6.8), 4% sodium dodecyl sulfate, 10% 2-mercaptoethanol, 24% glycerol, and 0.01% bromophenol blue were added. [^{3}H]-Leucine-labeled products were precipitated with 20% Cl_3CCOOH and washed sequentially with 5% Cl_3CCOOH, ethanol–ether, and ether solutions. All mixtures and standards were heated for 2 min at 100 °C and applied to a slab gel (20% acrylamide) as described (Blattler et al., 1972).

(*d*) *Tryptic Peptide Analysis.* Double-sized protein synthesis reaction mixtures labeled with [^{3}H]leucine were applied to preparative sodium dodecyl sulfate–polyacrylamide gels and electrophoresed. The putative precursor light chain band was identified (cf. Figure 2), eluted, and together with [^{14}C]leucine-labeled authentic RPC-20 protein, digested with trypsin as described (Roberts et al., 1975). After lyophilization, the sample was dissolved in dimethyl sulfoxide, acidified, applied to a Technicon-P column at 37 °C, and eluted with a pyridine acetate gradient between 0.01 M pyridine–0.45 M acetic acid, pH 3.1, and 1 M pyridine–0.45 M acetic acid, pH 5.5. Fractions (3.2 ml) were collected and counted in 9 ml of Aquasol in a liquid scintillation counter.

(*e*) *Polyacrylamide Gel Electrophoresis in Formamide.* RNA was electrophoresed on 3.5% polyacrylamide gels in formamide, containing 20 mM sodium phosphate, pH 6.5, modified from Staynov et al. (1972). Lyophilized RNA samples were dissolved in 20 mM sodium barbital buffered formamide, pH 6.5 (containing 20% sucrose and 0.05% bromophenol blue), and layered on the gels through an overlay of 5 mM sodium barbital buffered formamide, pH 6.5. Electrophoresis was carried out at 100 V for 5 h at room temperature. Gels were stained with "Stains-all" (Eastman Kodak Co.).

(*f*) *Preparation of cDNA.* [^{3}H]cDNA was made with the RPC-20 mRNA as a template and purified to obtain a preparation of uniform size (820 bases long) as described by Honjo et al. (1974) (cf. Honjo et al., 1976). [^{3}H]cDNA complementary to mouse globin mRNA was synthesized and purified as above up to the alkaline sucrose gradient step. Globin cDNA was 570 bases long.

(*g*) *Hybridization of* [^{3}*H*]*cDNA to mRNA.* The hybridization reactions were carried out as described (Honjo et al., 1974). The hybrid formed was assayed by S_1 nuclease digestion (Ando, 1966; Sutton, 1971; Ross et al., 1973).

(*h*) *Thermal Denaturation of Nucleic Acid Hybrids.* Thermal denaturation of hybrids was carried out either on a hydroxylapatite column or by S_1 nuclease digestion as described by Honjo et al. (1974).

(*i*) *Other Materials.* [^{3}H]Leucine and [^{35}S]methionine were obtained from New England Nuclear and Amersham/Searle, respectively. Crown brand wheat germ is a product of Centennial Mills, Portland, Ore. The sources of other reagents used in this study have been indicated (Aviv and Leder, 1972; Ross et al., 1972, 1973).

Results

(*a*) *Purification of RPC-20 mRNA.* Purification of λ chain mRNA was carried out according to the procedure employed previously for κ chain mRNA (Honjo et al., 1974). Membrane-bound polysomes were isolated from RPC-20 tumors and used as a source of λ chain mRNA. RNA derived from the polysomes was applied to an oligo(dT)-cellulose column and poly(A)-containing mRNA was eluted. After two successive oligo(dT)-cellulose chromatographic steps (dT 1 and dT 2), the mRNA preparation obtained was centrifuged in a sucrose density gradient (SG 1) under denaturing conditions (Honjo et al., 1974). The resulting fractions were assayed for their abilities to direct protein synthesis in the wheat germ cell-free system and to hybridize with [^{3}H]cDNA complementary to previously purified RPC-20 mRNA. Peak fractions were subjected to a second similar sucrose gradient centrifugation (SG 2) and analyzed in a similar manner. The results are shown in Figure 1. The absorbance peak at $s_{20,w}$ = 13–14 S coincides with the protein synthetic activity and hybridization peaks. Fractions 19–21 were combined and dialyzed against distilled water. In some cases the preparation was precipitated with 0.1 volume of 2.5 M NaOAc, pH 5.0, and 2 volumes of ethanol. This mRNA preparation was stored in liquid nitrogen.

[1] Abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ATP, adenosine 5′-triphosphate; GTP, guanosine triphosphate.

TABLE I: The Extent of Purification of RPC-20 mRNA as Estimated by Hybridization Kinetic Analysis.

RNA Fraction[a]	$C_rt_{1/2}$ (mol s l.$^{-1}$)	Purification (Fold)[b]	Total RNA (A_{260})	Immunoglobulin mRNA (A_{260})[c]
Polysomal RNA	2.5×10^{-1}	1	2730	5.1
dT 1	1.1×10^{-2}	22.7	62.6	2.7
dT 2	3.3×10^{-3}	75.8	7.9	1.1
SG 1	1.2×10^{-3}	208	1.2	0.67
SG 2	4.7×10^{-4}	532	0.4	0.4

[a] dT 1 and dT 2 designate 1st and 2nd oligo(dT)-cellulose chromatographic steps. SG 1 and SG 2 indicate 1st and 2d sucrose density gradient centrifugation steps. [b] Purification fold was calculated from $C_rt_{1/2}$ values. [c] The amount of immunoglobulin was calculated with reference to the purity of the SG fraction.

The extent of purification at each step was estimated by hybridization kinetic analysis using a previously prepared RPC-20 [^{3}H]cDNA probe as described below (Table I). The $C_rt_{1/2}$ value was reduced from 2.5×10^{-1} mol s l.$^{-1}$ in polysomal RNA to 4.7×10^{-4} mol s l.$^{-1}$ in the SG 2 material, corresponding to a 532-fold purification. The final yield of the RPC-20 mRNA was 7.8%.

(*b*) *Translation of RPC-20 mRNA in Vitro.* The proteins synthesized in response to the RPC-20 mRNA in a wheat germ cell-free system were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and compared with an authentic RPC-20 light chain protein (Figure 2). RPC-20 mRNA directs synthesis of a protein of approximately 23 750 daltons, which would correspond to five or six more amino acids than are contained in the authentic RPC-20 protein (23 123 daltons) (Apella, 1971).

The tryptic peptides derived from the product of a protein synthesis reaction have been compared with those from authentic RPC-20 protein by Technicon-P column chromatography (Figure 3). Each standard RPC-20 peptide coeluted with a peak derived from the in vitro product. There was one additional peak (fraction 13) in the in vitro product that was absent in the in vivo product, a result consistent with a peptide cleavage step involved in the processing of mature λ light chains in vivo. Such a mechanism previously has been suggested to be involved in κ light chain biosynthesis (Swan et al., 1972; Milstein et al., 1972; Mach et al., 1973; Schechter, 1973).

(*c*) *Formamide–Polyacrylamide Gel Electrophoresis of RPC-20 mRNA.* A major band increases in prominence as a function of purification (Figure 4A). This band migrates as an approximately 400 000-dalton polynucleotide, equivalent to about 1150 nucleotides in length as judged by comparison to 28 and 18S ribosomal rRNA and mouse globin α and β mRNA's (Figure 4B).

(*d*) *Reverse Transcription and Hybridization Kinetic Analysis of RPC-20 mRNA.* The purified RPC-20 mRNA serves as an efficient template for the RNA-dependent DNA polymerase-catalyzed synthesis of [^{3}H]cDNA (see Honjo et al., 1976). This [^{3}H]cDNA provides a further means of assessing the purity of the RPC-20 light chain mRNA using hybridization kinetic analysis. By comparing the $C_rt_{1/2}$ values obtained using the mRNA and probe to similar values obtained from standards of known purity, the relative purity of the RPC-20 mRNA preparation can be determined (Bishop, 1969; Birnsteil et al., 1972; Honjo et al., 1974). The hybridization

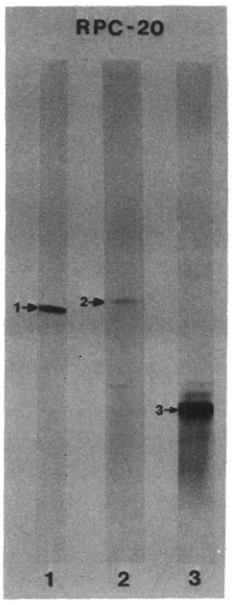

FIGURE 2: Polyacrylamide gel electrophoresis of in vitro synthesized product. Slot 1, in vivo synthesized RPC-20 light chain; slot 2, in vitro synthesized product directed by RPC-20 mRNA; slot 3, in vitro control, directed by globin mRNA.

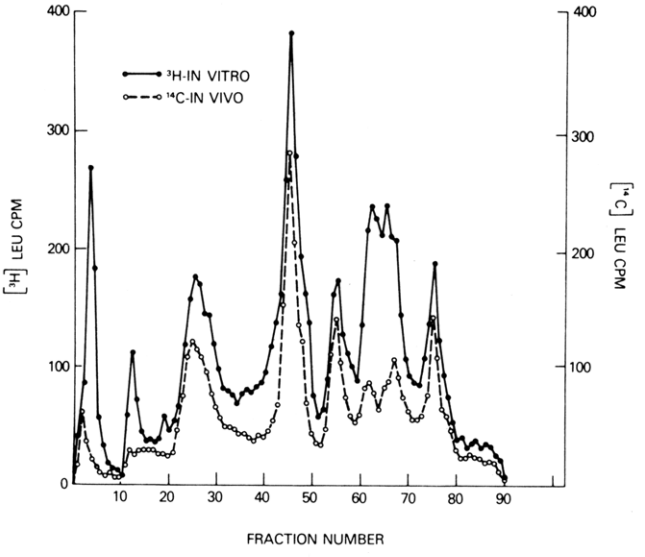

FIGURE 3: Technicon-P column chromatography of tryptic peptides derived from proteins synthesized in response to SG 2 RPC-20 mRNA. Open circles, dotted line: peptides from in vivo RPC-20 protein; closed circles, solid line: peptides from an in vitro reaction mixture containing SG_1 RPC-20 mRNA.

(Figure 5) between globin mRNA and its [^{3}H]cDNA, giving a $C_rt_{1/2}$ value of 3.7×10^{-4}, was used as a standard. Given that globin mRNA is an approximately 1:1 mixture of α and β globin mRNAs (Orkin et al., 1975) each of which is about 600 nucleotides long, the expected $C_rt_{1/2}$ value for RPC-20 mRNA (1150 nucleotides long) would be 3.6×10^{-4} (Honjo et al., 1974). The experimental $C_rt_{1/2}$ value determined for SG 2 RPC-20 mRNA is 4.7×10^{-4}. This value permits the calculation of a relative purity of $[3.6 \times 10^{-4}/4.7 \times 10^{-4}]100$ or about 76% for the SG 2 material.

The fidelity of hybridization between the SG 2 RPC-20 mRNA and its [^{3}H]cDNA was assessed by thermal denaturation of the hybrid from an hydroxylapatite column (Figure 6). The resulting melt profile is sharp with a T_m of 88 °C,

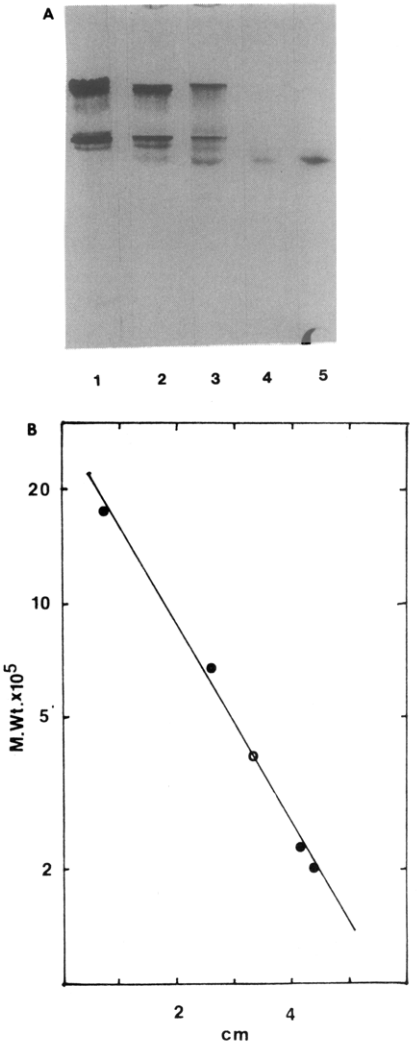

FIGURE 4: (A) Formamide–polyacrylamide gel electrophoresis of RPC-20 mRNA. Slot 1, RPC-20 membrane-bound polysomal RNA; slot 2, RPC-20 dT-1 RNA; slot 3, RPC-20 dT 2 RNA; slot 4, RPC-20 SG 1 RNA; slot 5, RPC-20 SG 2 RNA. (B) Comparative plot of the migration of RPC-20 mRNA (open circle) with respect to (in descending order) 28 and 18S ribosomal RNA's and mouse β and α chain globin mRNA's (closed squares) as a function of molecular weight (Orkin et al., 1975).

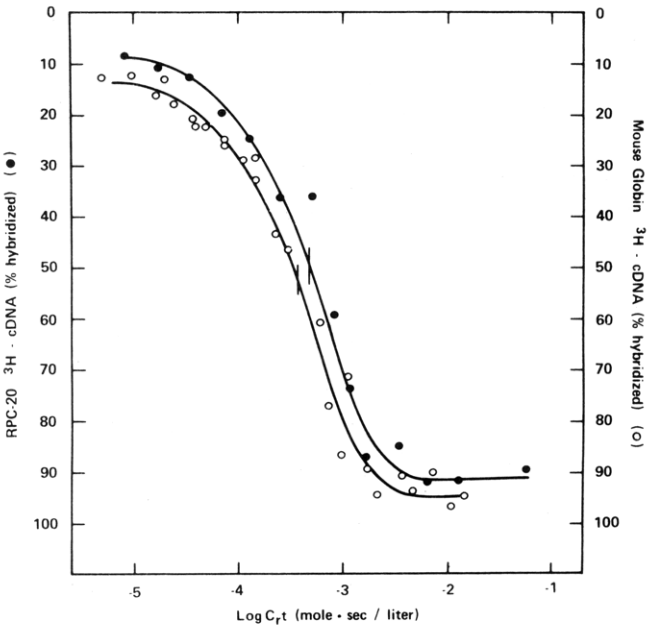

FIGURE 5: Comparative kinetics of annealing of RPC-20 and globin [^{3}H]cDNAs to their respective mRNAs. The mRNA was present in at least 20-fold excess over cDNA. Open circles: mouse globin [^{3}H]cDNA hybridized to mouse globin mRNA; closed circles: RPC-20 [^{3}H]cDNA hybridized to RPC-20 mRNA.

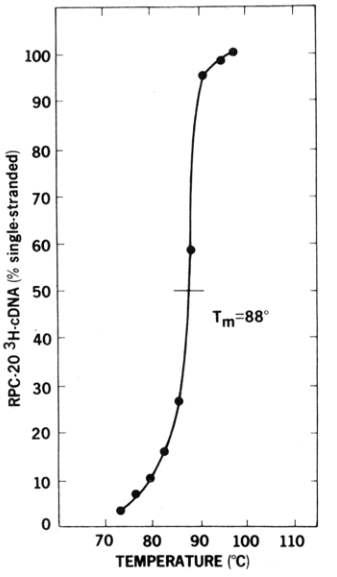

FIGURE 6: Thermal denaturation profile of the RPC-20 [^{3}H]cDNA–RPC-20 mRNA (SG 2) hybrid. The hybrid formed at a C_rt value of 1.0×10^{-2} was assayed for its thermal stability using hydroxylapatite column chromatography.

consistent with a congruent base pairing between probe and template.

Discussion

(*a*) *Cell-Free Translation of RPC-20 mRNA: a Possible λ Chain Precursor.* A number of studies have amply confirmed that in vitro synthesized κ light chain proteins are longer than the mature or excreted form of the κ light chain (Swan et al., 1972; Milstein et al., 1972; Mach et al., 1973; Schechter, 1973; Tonegawa and Baldi, 1973). In the case of certain κ subgroups, the lengths of the in vitro products exceed by some 20–50 amino acids those of the in vivo excreted light chain. In one well-documented instance, a portion of this additional length was shown to be a 20 amino acid long peptide at the amino terminal end of the in vitro synthesized protein (Schechter et al., 1975).

From the current study it seems that this phenomenon occurs among λ light chains as well (Figure 1). The additional molecular weight we observe could be accounted for by five or six additional amino acids. Preliminary studies (D. McKean, personal communication) indicate that the in vitro product is identical with the in vivo synthesized product in that both contain blocked N-termini, presumably pyrrolidonecarboxylic acid. If this is the case, it suggests that the additional amino acids at the λ chain occur at the C terminal end of the λ light chain.

(*b*) *Relative Purity of the RPC-20 mRNA.* While the RPC-20 mRNA runs as predominant band on formamide–polyacrylamide gel electrophoresis and directs the synthesis of the myeloma light chain, it obviously does not represent a homogeneous population of molecules with respect to size. Analysis by formamide–polyacrylamide gel electrophoresis suggests that the major band is contaminated by a minor proportion of both heavier and lighter RNAs. A more precise

estimate of the degree of sequence purity of the preparation has been obtained, however, using kinetic hybridization analysis in which RPC-20 cDNA is hybridized against the message preparation (Figure 5). Use of this technique and comparison of the $C_rt_{1/2}$ obtained with that of globin mRNA and its cDNA indicate that the RPC-20 mRNA is about 75% pure.

(*c*) *The Anatomy of the RPC-20 mRNA.* Formamide-polyacrylamide gel electrophoretic analysis indicates that the RPC-20 mRNA consists of approximately 1150 nucleotides. Since approximately 700 bases would be required to encode the RPC-20 in vitro polypeptide (putative precursor) and an additional 100–200 adenylic acid residues may be located at the 3′ end of the message, this would leave an untranslated sequence of about 250–350 bases. From what is known of κ chain mRNA (Milstein et al., 1974) one must assume that a portion of this untranslated region is at both the 3′ and 5′ terminal ends of the structural sequence. In this respect, the λ light chain mRNA is similar to both globin and other immunoglobulin mRNAs studied in which extensive nucleotide sequences exist in the message which apparently do not contain structural information. These sequences have considerable importance in the interpretation of hybridization studies using partial cDNA reverse transcripts (Honjo et al., 1976).

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