FURTHER CHARACTERIZATION OF THE mRNA CODING FOR IMMUNOGLOBULIN LIGHT-CHAIN

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SUMMARY. The highly purified (> 95%) mRNA coding for immunoglobulin light (L)-chain yields on acrylamide gels a discrete 15.5S band and a "shoulder" ranging in size from 15.5 to 9.5S. The "shoulder" was isolated and found to be fragmented mRNA as judged from: 1. hybridization kinetic analysis, using the complementary-DNA to the L-chain mRNA; 2. capacity to form aggregates, similarly to the intact 15.5S mRNA. Partial cleavage of the mRNA probably occurs during mechanical disruption of the myeloma cells. Fragments with intact 3' end are selected due to binding via the poly(A) moiety to oligo(dT)-cellulose, i.e., the fragments should be deficient at the 5' end where mRNA translation is initiated. In agreement, the fragmented mRNA is essentially untranslatable in a cell-free system.
The size of the L-chain mRNA is rather uncertain. A value of 15.5S is obtained from migration in acrylamide gels made in water or formamide, a value of 12S is obtained from sucrose gradient centrifugation.

The biological purity of the mRNA coding for Ig L-chain isolated from myeloma polysomes by means of the double antibody technique was calculated to be over 95%. This value was derived from the estimation of contamination of the pure mRNA by non-L-chain mRNAs (1), from the extent of precipitation of myeloma and non-myeloma polysomes each with anti-L-chain and non-L-chain antibodies (1,2), and from amino acid sequence analyses of the total cell-free products programmed by the mRNA (3). Chemical purity, from rRNA and tRNA, was found to be over 95% (1). Nonetheless, on polyacrylamide gels the L-chain mRNA exhibits two components: a discrete band of 15.5S (40-50%), and a diffuse "shoulder" ranging in size from 15.5 - 9.5S (60-50%). In the present report it is shown that the 15.5 - 9.5S material is composed of fragmented L-chain mRNA which is presumably deficient at the 5' end.

MATERIALS AND METHODS. The mRNA coding for M-321 Ig L-chain was prepared from mouse myeloma polysomes specifically precipitated with antibodies to the L-chain followed by chromatography on oligo(dT)-cellulose (1,2). Over 95% of the total cell-free products programmed by the mRNA corresponded to the M-321 L-chain protein (1,3). The synthesis of the cDNA to the M-321 mRNA with the aid of the avian-myeloblastosis-DNA polymerase, and its characterization, were described (4). RNA-DNA hybridizations were performed at 68° in 0.02 M Na-phosphate (pH 7.0), 0.3 M NaCl, 2 mM EDTA, and 0.1% SDS. Hybrids were assayed by Staphylococcal nuclease (5).

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*Reprints requests mail to Rehovot, Israel. Abbreviations: cDNA, complementary DNA; Crt, product of RNA concentration (moles of nucleotide per liter) and time (sec); DMSO, dimethylsulfoxide; Ig, immunoglobulin; L-chain, light chain.

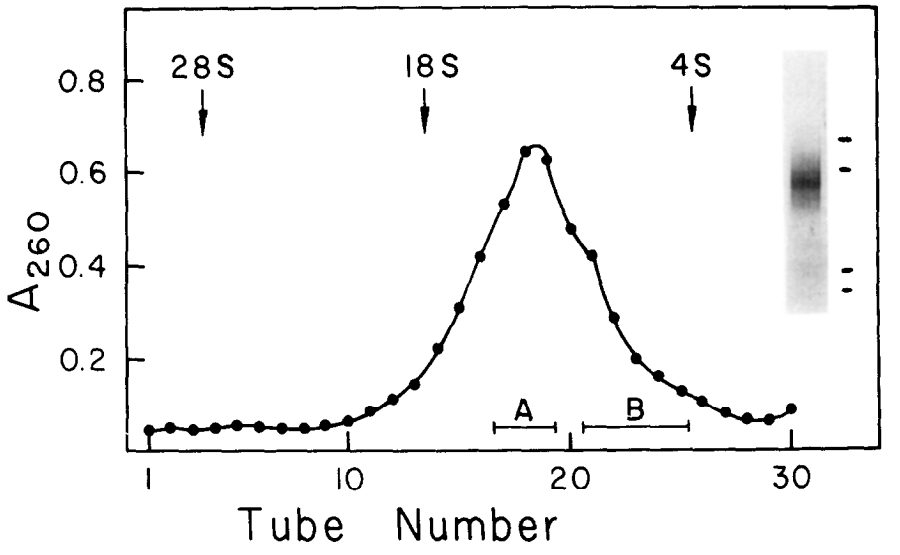

Fig. 1. Sucrose gradient centrifugation of unfractionated M-321 mRNA. The mRNA was layered onto a 15-30% sucrose gradient containing 5 mM Tris·HCl (pH 7.5) - 0.5 mM EDTA - 0.2% sodium dodecyl sulfate, and spun (36,000 rpm/16 hr) at 21° in the Spinco SW 41 rotor. Horizontal bars indicate tubes pooled to yield F-A and F-B. Arrows indicate the position of RNA markers. Insert: gel electrophoresis in aqueous medium (1.7% acrylamide - 0.5% agarose, ref. 14) of the unfractionated mRNA, bars on the side denote the positions of 28, 18, 5 and 4S RNA markers.

RESULTS. The profile of the M-321 L-chain mRNA obtained by sucrose gradient centrifugation is given in Fig. 1. Two fractions were pooled: A- from the peak, B- from the lower molecular weight region. After alcohol precipitation these fractions were analysed on acrylamide gels made in 98% formamide (Fig. 2). The gels were stained and scanned. The results (Table I) show that in comparison to the unfractionated sample, fraction-A (F-A) was enriched with the 15.5S band, but it still contained considerable amounts (42%) of molecules ranging in size from 15.5 - 11.5S. Fraction-B (F-B) was mainly composed of a heterogeneous population of molecules ranging in size from about 12-9.5S (97%) and a small amount (about 3%) of the 15.5S discrete band.

Nucleotide sequences of the L-chain mRNA were estimated in the mRNA fractions by hybridization kinetic experiments. The M-321 cDNA annealed with the three RNA preparations with comparable $Crt_{1/2}$ values, and at saturation 96% of the cDNA were hybridized (Fig. 3, Table II). The hybrids scored were specific for L-chain mRNA (4), and did not involve duplex formation with fragmented rRNA or with poly(A) segments. The M-321 cDNA did not anneal with rRNA at Crt values 100,000 folds higher than the $Crt_{1/2}$ measured, or with globin mRNA which contains poly(A), or with poly(A)-rich RNA of mouse liver (Fig. 3). The differences in $Crt_{1/2}$ values can be attributed to size differences in the mRNA prepar-

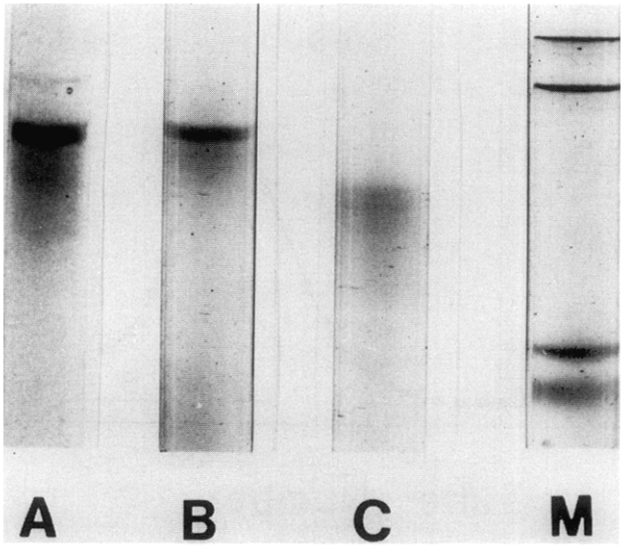

Fig. 2. Gel electrophoresis in a dissociating medium of M-321 L-chain mRNAs. The samples are: A, unfractionated M-321 mRNA; B, Fraction-A mRNA; C, Fraction-B mRNA; M, 28, 18, 5, and 4S RNA markers. The gels (3.2% acrylamide in 98% formamide) were run at 10 v/cm for 3 hr (15) and developed with "stain all" (14).

TABLE I. Size and composition of L-chain mRNA preparations determined from electrophoretic mobility in formamide gels

L-chain mRNA	18S rRNA (%)	15.5S Band (%)	Shoulder (%)	Shoulder size range
Unfractionated	5	43	52	15.5 - 9.5S
Fraction-A	< 0.5	58	42	15.5 - 11.5S
Fraction-B	0	3	97	12. - 9.5S

The stained gels (Fig. 2) were scanned in a Gilford spectrophotometer at 570 nm, and composition determined from areas at the indicated S values.

ations. It was shown that the rate of hybridization of both DNA-DNA (6) and RNA-DNA (7) is proportional to $L^{0.5}$ (L, size of the polynucleotides). Accordingly, F-A which was enriched with the larger molecules (15.5S) hybridized at a faster rate (decreased $Crt_{1/2}$), F-B which was mainly composed of the smaller molecules (97%) hybridized at a slower rate (increased $Crt_{1/2}$). These results demonstrate that the nucleotide sequences in the three mRNA preparations are essentially the same.

It was of interest to measure the activity of the mRNAs in directing protein

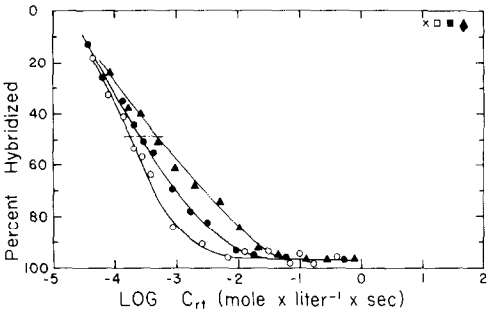

Fig. 3. Kinetics of hybridization of M-321 cDNA with various mRNA preparations. M-321 L-chain mRNAs annealed are: (●), unfractionated; (○), fraction-A; (▲), fraction-B. The RNA controls were: globin mRNA (×) and rRNA (□) of rabbit reticulocytes; poly(A)-rich RNA (■) and rRNA (◆) of mouse liver.

TABLE II. Properties of L-chain mRNA preparations

L-chain mRNA	Hybridization*		Activity in protein synthesis	
	$Crt_{1/2}$ (molexliter^{-1}xsec)	Saturation level (%)	S.A.† (pmole Leu/0.01A_{260})	Relative S.A.
Unfractionated	2.7×10^{-4}	96	4.8	1.00
Fraction-A	1.8×10^{-4}	96	6.8	1.41
Fraction-B	4.0×10^{-4}	96	0.35	0.07

* Hybridization parameters for annealing with the M-321 L-chain cDNA (Fig. 3).
† Specific activity calculated from kinetic slopes under conditions where the reaction was first order with respect to added mRNA (see Fig. 4)

synthesis in a cell-free system. The result was that F-A had increased activity (141%) whereas in F-B the activity was markedly reduced (7%) (Fig. 4, Table II). The reduced activity of F-B was not due to the presence of inhibitory molecules, since the addition of F-B did not inhibit protein synthesis directed by another mRNA (Table III). The capacity to stimulate protein synthesis was clearly associated with the 15.5S RNA. Activity was very poor in F-B which contained trace amounts of the 15.5S band. In the F-A mRNA and unfractionated mRNA, the increase in specific activity (factor of 1.41, Table II) was comparable to the increase in the weight fraction of the 15.5S mRNA (58/43 = 1.35, Table I).

It should be noticed that the mRNA sedimenting on sucrose gradient at 12S (Fig. 1) yield a prominent 15.5S band when analysed on acrylamide gels made either in aqueous medium (Fig. 1, insert) or in 98% formamide (Fig. 2). It has been previously shown that in the phenol extract of myeloma polysomes the L-chain mRNA is present in the form of aggregates of increasing size up to 35.5S, all of

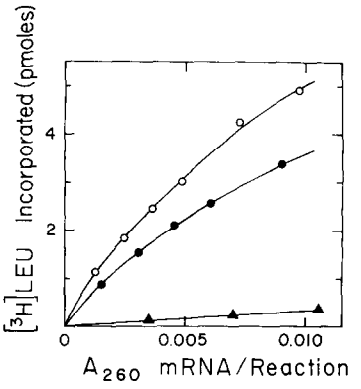

Fig. 4. Stimulation of protein synthesis in the Krebs II ascites cell-free system by increasing amounts of mRNA. mRNA added: (●), unfractionated; (○), fraction-A; (▲), fraction-B. Data represent net incorporation obtained by subtracting 0.38 pmoles for the minus mRNA control. Incorporation of [^{3}H]Leu into TCA precipitable material was measured after 1.5 hr at 36° (1).

TABLE III. The effects of F-B mRNA on the stimulation of protein synthesis

Fraction-B added (A_{260} RNA/reaction)	Net incorporation (pmoles [^{3}H]Leu)	
	found	calculated†
none*	1.55	
0.0035	1.79	1.67
0.0070	1.86	1.80

* The standard Krebs II ascites cell-free system contained 0.003 A_{260} units of unfractionated mRNA. Reaction conditions were as in Fig. 4.

† Incorporation derived from unfractionated mRNA plus the incorporation derived from F-B mRNA when added alone (see Fig. 4).

which can be reduced to the monomeric form (15.5S on acrylamide gels) by exposure to 98% formamide (1,8). The experiments quoted below show that the discrepancy in size mentioned above is also true for mRNA obtained from the aggregates. A sample of mRNA aggregates, ranging in size from about 22-26S (Fig. 5, insert A) was dissociated in 100% DMSO, diluted in water to 25% DMSO, and then analysed. In acrylamide gels made in aqueous medium the aggregates were reduced in size to the prominent 15.5S band and a "shoulder" of the fragemented mRNA (15.5-9.5S) (Fig. 5, insert B). By sucrose gradient centrifugation 3 peaks were observed at 28, 18 and 12S. The mRNA was located at the 12S peak by measuring the capacity of aliquots taken along the gradient to stimulate protein synthesis in the Krebs II ascites cell-free system, and by the fact that the 12S peak gave on

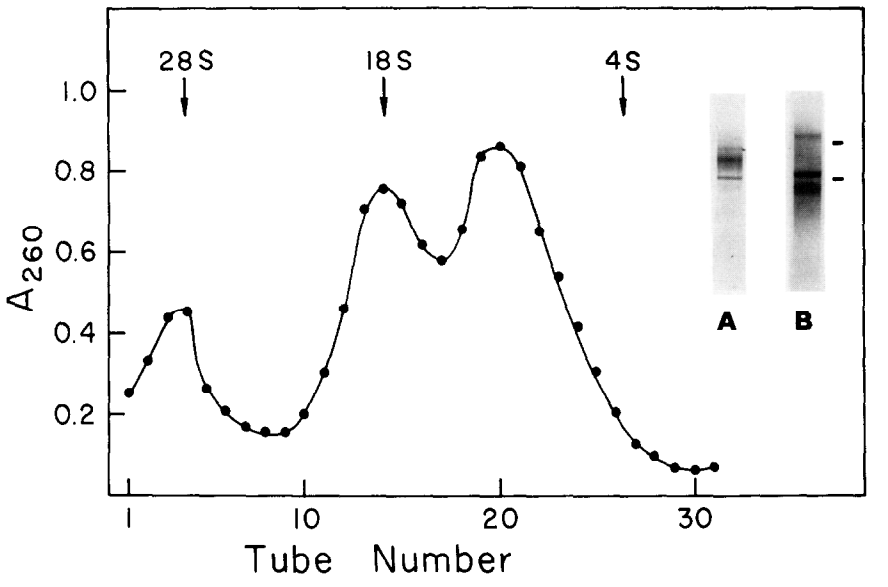

Fig. 5. Sucrose gradient centrifugation of M-321 L-chain mRNA aggregates dissociated by 100% DMSO. The aggregated mRNA was prepared from the 22S region of sucrose gradient of RNA extracted from immuneprecipitated myeloma polysomes (1). It was then dissolved in 100% DMSO, diluted in water to 25% DMSO and spun in the Spinco SW 41 rotor as detailed in Fig. 1. Arrows indicate the position of RNA markers. Insert: gel electrophoresis in aqueous medium (14) of aggregated mRNA before (A) and after (B) treatment with 100% DMSO. Bars on the side denote the positions of 28 and 18S rRNA markers.

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formamide gel a pattern which was indistinguishable from that shown in Fig. 2B (the 18 and 28S peaks are rRNA contaminants).

DISCUSSION. The hybridization kinetic experiments with the L-chain cDNA demonstrate that the 15.5 - 9.5S "shoulder" is fragmented L-chain mRNA. This is further corroborated by the fact that these fragments aggregate similarly to the intact 15.5S mRNA. It has been previously shown that the L-chain mRNA readily and preferentially forms aggregates, whereas non-L-chain mRNAs of the myeloma cell do not have this property (1,8). Aggregates reduced to the monomeric form, either by 98% formamide (1) or by DMSO (Fig. 5, insert B), contain the intact (15.5S) and fragmented (15.5 - 9.5S) mRNA species in the same ratio (about 1:1), irrespective of the size of the original aggregate (22 - 35.5S, ref. 1).

The use of antibodies for the specific precipitation of polysomes engaged in the synthesis of L-chains could have certainly led to the inclusion of fragmented mRNA in the final product. Broken polysomes, with broken mRNA, bear nascent L-chains, therefore they should be precipitated by antibodies to L-chain Partial degradation of the polysomes probably was not due to ribonuclease contamination in the antibodies, as mRNAs prepared from polysomes exposed to one or

two cycles of antibody purification had comparable activity (1). Partial degradation of the polysomes possibly was due to the use of Waring Blendor for homogenization of the myeloma cells. This rough procedure was used in order to process a large quantity of cells (100 gm) for preparing pure mRNA in milligram amounts (1,2).

The RNA extracted from the immune precipitated polysomes was chromatographed on oligo(dT)-cellulose (1). This had led to the selection of mRNA fragments having intact 3' end since the poly(A) moiety is located at this end. Consequently the fragments should be deficient at the 5' end where mRNA translation is initiated We have shown that the translation of M-321 L-chain mRNA is initiated from one site (9), resulting in the synthesis of the L-chain precursor which has N-terminal methionine (3). In agreement with the above, the fragmented mRNA had negligible activity in stimulating protein synthesis (Fig. 4, Table II).

The apparent molecular weight of the L-chain mRNA is about 440,000 (15.5S) when analysed by acrylamide gels made in dissociating (Fig. 2) or aqueous (Fig. 1, insert; Fig. 5, insert B) media, whereas by sucrose gradient centrifugation it is about 240,000 (12S) (Figs. 1,5). A similar discrepancy in size was observed with the ovalbumin mRNA (10). This may be attributed, at least in part, to the poly(A) segment in the mRNA. The apparent size of poly(A), and other homopolymers, was found to be larger when analysed by gel electrophoresis as compared to sucrose gradient centrifugation (11,12). The size of the M-321 L-chain mRNA should account for: 220,000 daltons to code the mature protein, 45,000 daltons to code the extrapieces in the precursor (20 amino acid residues at the N-terminus, about 25 residues at the C-terminus, ref. 1,9), and about 65,000 daltons for the poly(A) segment (uncorrected for uncertainty in sizing, ref. 13). This adds up to a minimal molecular weight of about 330,000. Evidently, the size determined by sucrose gradient centrifugation is too low. Considering the value from gel analyses (440,000), we are left with a putative untranslated segment of 110,000 daltons (4). However, it is not yet possible to determine to what extent (if at all) the size determined from acrylamide gels is overestimated. Consequently, the size of the putative untranslated segment in the L-chain mRNA is rather uncertain.

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