Membrane-bound Ribosomes of Myeloma Cells

IV. mRNA Complexity of Free and Membrane-bound Polysomes

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ABSTRACT We have analyzed the sequence complexity, frequency distribution, and template activity of free (F) and membrane-bound (MB) polysomal mRNA populations of MOPC 21 (P3K) mouse myeloma cells. Using the technique of mRNA-cDNA hybridization, we find that F poly(A)$^+$ RNA, which represent 60% of total polysomal mRNA, consists of ~8,000 different mRNA sequences distributed in three abundance classes, while MB poly(A)$^+$ RNA (20% of total polysomal mRNA) includes only 230 mRNA species and almost completely lacks very infrequent mRNA species. Cross-hybridization indicates that MB mRNA sequences are also present in F mRNA, but in reduced concentrations. Translation of F and MB RNA fractions in a messenger-dependent reticulocyte lysate indicates that essentially all MB RNA contains poly(A), whereas 25% of F mRNA lacks poly(A). Furthermore, the use of a cDNA highly specific for the immunoglobulin light (Ig L) chain mRNA allows the determination of the subcellular content of this message. Ig L mRNA, representing ~5% of total polysomal poly(A)$^+$ RNA, is one of the most abundant MB mRNAs. 90% of Ig L mRNA is found in MB polysomes and 10% in F polysomes.

In animal cells, proteins are synthesized on polysomes either free in the cytoplasm or associated with the endoplasmic reticulum (ER). Evidence from numerous studies suggests that free (F) polysomes are the site of synthesis of soluble proteins (39, 40, 35, 10, 13), whereas membrane-bound (MB) polysomes are engaged in the synthesis of secretory proteins (29, 41) or integral membrane proteins (26, 7, 8). In certain cases, however, this segregation was found not to be absolute, as secretory proteins can be synthesized in detectable amounts by F polysomes (20). The presence of mRNA coding for secretory proteins in F polysomes could have some functional importance in the formation of MB polysomes. However, the mechanism by which particular messages are selected for translation on MB polysomes is still obscure.

The number of different mRNA sequences and their relative abundance in a population of RNAs can be measured by kinetic analysis of hybridization of poly(A)$^+$ RNA with its cDNA (3). In this report, we have used this technique to analyze the complexity of the mRNA sequences present in the F and MB ribosomal fractions of MOPC 21 (P3K) cells, and we have compared the sequence homology between these two mRNA populations. In addition, we have determined the distribution of immunoglobulin light (Ig L) mRNA by using two different approaches. One approach exploits the ability of reverse transcriptase to synthesize highly specific and radioactive DNA copies of the Ig L chain mRNA when a synthetically produced hexanucleotide (T_2G_3T) is used as a primer (34, 25). This Ig L cDNA was used to measure the Ig L mRNA content of F and MB polysomes. Another approach used cell-free protein synthesis and immunoprecipitation to characterize the Ig H and L mRNA content in F and MB mRNA populations. The results obtained from both approaches indicate that 90% of Ig H and L mRNA sequences are recovered with MB polysomes and ~10% with F polysomes.

MATERIALS AND METHODS

Cell Culture

All analyses were performed using MOPC 21 (P3K) mouse myeloma cells grown in suspension cultures in Dulbecco's Modified Eagle's Medium supple-

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mented with 10% horse serum. All cells were harvested during exponential growth from cultures not exceeding 5.0 × 10^5 cells/ml. When labeling was required, [^{3}H]uridine, [^{3}H]adenosine, or [^{3}H]oleic acid was added to the cultures 24 h before the collection of the cells (22, 23).

Preparation of F and MB Polysomes

To ensure maximum yield of F and MB polysomes, exponentially growing P3K cells were concentrated to 2 × 10^6 cells/ml and further incubated for 45 min at 37°C in prewarmed growth medium. At the end of the incubation, the cells were diluted in a fivefold volume of ice-cold Earle's saline medium, washed twice, and resuspended at a concentration of 5 × 10^8 cells/ml in ice-cold hypotonic buffer medium RSB (0.01 M KCl, 0.0015 M $MgCl_2$, 0.01 M Tris-HCl, pH 7.4). The cells were allowed to swell for 5 min and were then ruptured mechanically with 10 strokes of a tight-fitting (B) Dounce glass homogenizer (Kontes Glass Co., Vineland, N. J.). The homogenate was centriguged at 1,000 g for 2 min to sediment the nuclei; the supernate that formed the cytoplasmic extract fraction was retrieved. The nuclear pellet was resuspended with RSB in half the volume of the homogenate and formed the nuclear fraction. The cytoplasmic extract was further used for the separation of free ribosomal and microsomal fractions. In the case of the nuclear fraction, a supplementary microsomal fraction was obtained and pooled with the microsomal fraction prepared from the cytoplasmic extract.

Separation of free ribosomal and microsomal fractions was performed according to a modification of our previously described method (22). The cytoplasmic extract or the nuclear fraction was diluted fivefold in 2.5 M sucrose $TK_{150}M$ (0.15 KCl, 0.005 M $MgCl_2$, 0.05 M Tris-HCl, pH 7.4) and layered over 2 vol of 2.5 M sucrose $TK_{150}M$. Two layers of sucrose $TK_{150}M$ solutions were successively added, one with 2.05 M sucrose and a second with 1.3 M sucrose. The gradients were centrifuged for 5 h at 4°C in a Spinco SW 27.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 25,000 rpm. The bottom of the tube was punctured, 30-drop fractions were collected into glass tubes, and the adsorbance at 260 nm was measured. Free ribosomes were present essentially in the load zone of the gradient, whereas microsomes floated at the interface between the 2.05 and 1.3 M sucrose layers.

To purify the polysomes from both ribosomal and microsomal fractions, F ribosomes and the microsomal fraction that had been treated with 0.5% sodium deoxycholate and 0.5% Brij 58 were layered on 15-30% sucrose $TK_{80}M$ (0.08 M KCl, 0.005 M $MgCl_2$, 0.05 M Tris-HCl, pH 7.4) density gradients containing a 4-ml cushion of 69% sucrose $TK_{80}M$ for 8.5 h of centrifugation at 27,000 rpm in a Spinco SW 27 rotor. Gradients were collected and the adsorbance profile was measured at 260 nm. Polysomes containing two or more ribosomes were concentrated at the interface between the 30 and 69% solutions. The F and MB polysomal fractions were diluted with 1 vol of distilled water and then extracted.

Extraction of RNA

The polysomal fraction was added to an equal volume of hot SDS buffer (1% SDS, 0.2 M NaCl, 0.02 M Tris-HCl, pH 7.4, 0.04 M EDTA) in a large tube in a boiling water bath at 100°C. After 120 s, the tube was plunged into ice water and the contents were cooled to 30°C and then digested with proteinase K (0.5 mg/ml) for 10 min. The digest was adjusted to 0.1 M in Tris-HCl, pH 9.0 and 1% SDS and then extracted three times at room temperature with an equal volume of phenol:chloroform (1:1). RNA was precipitated from the aqueous phase by addition of 2.5 vol of ethanol and 0.1 vol of 2 M Na-acetate, pH 5.2.

Isolation of poly(A)$^+$ RNA

Total F or MB polysomal RNA (20 OD_{260} U/ml) dissolved in binding buffer (0.5 M LiCl, 0.01 M Tris-HCl, pH 7.4, 0.002 M EDTA, 0.1% SDS) was boiled for 2 min, cooled to room temperature, and was passed over a 1-ml oligo (dT)-cellulose column (Collaborative Research Inc., Waltham, Mass.), equilibrated with the same buffer. The column was then washed thoroughly with binding buffer followed by an additional washing step with 0.15 M LiCl, 0.01 M Tris-HCl, pH 7.4, 0.002 M EDTA, 0.1% SDS. This washing step eluted 50% of the material from the column. The eluted material was found, on the basis of SDS-sucrose gradients, to be made of ribosomal RNA and was devoid of template activity in the translational assay (*vide infra*). The RNA remaining bound to oligo (dT)-cellulose was eluted with low salt buffer (0.01 M Tris-HCl, pH 7.4, 0.002 M EDTA, 0.1% SDS). The RNA was then precipitated by the addition of 0.1 vol of 2 M Na-acetate, pH 5.2 and 2.5 vol of ethanol and recovered after 15 h at −20°C by centrifugation for 20 min at 12,500 rpm in a Sorvall SS34 rotor (DuPont Instruments-Sorvall, DuPont Co., Newtown, Conn.). It was dissolved in distilled H_2O and the concentration of poly(A)$^+$ RNA was determined by ultraviolet adsorbance at 260 nm (1.0 OD/ml taken as 40 μg/ml of RNA) and by titration with radioactive poly(U) (3).

The size distribution of poly(A)$^+$ RNA was determined by analysis on 5-23% linear sucrose density gradients containing 0.1 M LiCl, 0.01 M Tris-HCl, pH 7.4, 0.004 M EDTA, and 0.1% SDS for 3 h (7.5°C) at 50,000 rpm in a Spinco SW 50.1 rotor (23). No intact 28S ribosomal RNA could be detected in either F or MB poly(A)$^+$ RNA.

Synthesis of cDNA

cDNA was prepared from the respective poly(A)$^+$ RNA fractions using avian myeloblastosis virus reverse transcriptase (34). The Ig L cDNA was prepared using 13S Ig L mRNA template plus the olignucleotide T_2G_3T as a primer (34). The cDNA was purified according to Rabbitts et al. (34).

Hybridization Reactions

RNA-cDNA hybridizations were carried out at 70°C in 0.24 M sodium phosphate buffer, pH 6.8, containing 0.05% SDS. The mixture of RNA-cDNA and buffers were prepared at appropriate concentrations of RNA in siliconized tubes and the hybridization reactions were performed under paraffin oil. Aliquots were removed and diluted in 0.5 ml of 0.012 M NaCl, and the amount of cDNA in hybrid was assayed using S_1 nuclease (3).

In Vitro Protein Synthesis and Immunoprecipitation

Translation of RNA in the mRNA-dependent reticulocyte lysate was carried out as described by Pelham and Jackson (31). Incubation was done at 30°C for up to 1 h.

After incubation, the reaction mixtures were diluted 40-fold in 0.5% NP 40 NET buffer (0.15 M NaCl, 0.005 M EDTA, 0.05 Tris-HCl, pH 7.4) and centrifuged for 90 min at 100,000 g in a Spinco Ti 50 rotor. The supernates were immunoprecipitated with a rabbit anti-mouse Ig antiserum (IgG fraction) using formaldehyde-treated *Staphylococcus aureus* to collect antigen-antibody complexes (17).

Polyacrylamide Gel Electrophoresis and Fluorography

Proteins were resolved by polyacrylamide gel electrophoresis according to Laemmli (18). The separate gel was 17.5% acrylamide and the stacking gel was 3.6% acrylamide.

The procedure of Bonner and Laskey (5) was used to impregnate the gels with 2,5-diphenyloxazole, followed by drying for 1 h at 100°C. Kodak X-omat film XR-5 was presensitized according to the method of Laskey and Mills (19) and used for the fluorography.

Materials

[^{3}H]uridine, [^{3}H]adenosine, and [^{3}H]oleic acid were purchased from Amersham Radiochemical Centre. [^{35}S]methionine was prepared by ion exchange chromatography from a protein hydrolysate of [^{35}S]sulfate-labeled *E. coli* preparation.

RESULTS

Properties of F and MB poly(A)$^+$ RNA

To estimate the complexity of an mRNA population, it is necessary to know the average molecular weight of the mRNA. This was determined by sedimenting in sucrose gradients the poly(A)$^+$ RNA fractions, purified by oligo (dT)-cellulose chromatography, from F and MB polysomes of P3K cells which have been labeled for 24 h with [^{3}H]uridine. These results are summarized in Table I. The F-labeled poly(A)$^+$ RNA molecules exhibit a heterogeneous distribution with a mean mol wt of 660,000 daltons, whereas the mean mol wt of MB mRNA is ~550,000 daltons. Although these figures are broad average values, they are close to those reported for mRNA from other tissues (3, 9, 12, 36, 1) and will be used to calculate the complexities of F and MB messages.

The content of F and MB mRNA in P3K cells was quantitated both by optical density measurements at 260 nm and by hybridization to [^{3}H]poly(U), assuming that the poly(A) moiety

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TABLE I
Properties of F and MB Poly(A) + RNA

Fraction	Polysomal RNA*	Polysomal poly(A)+RNA*	No. average molecular weight of RNA‡	No. of poly(A)+ RNA molecules per cell§
	pg	*pg*	*daltons*	
Free	13.9 ± 3.0	0.142 ± 0.043	660,000	127,500
MB	3.4 ± 0.6	0.039 ± 0.013	550,000	42,500

* RNA content was calculated according to the yield of RNA measured by ultraviolet adsorbance at 260 nm, the relationship 1 mg of RNA is 25 OD_{260} U, and the number of cells from five independent harvests of P3K cells (~10^9 cells per harvest). The amount of poly(A)+ RNA is the average value of determinations made from optical density measurements and by titration with [^{3}H]poly(U).

‡ RNA size was determined by velocity centrifugation. F poly(A)+ RNA showed a modal distribution around 18S and MB poly(A)+ RNA a modal distribution around 16S.

§ $\frac{\text{Amount of F or MB mRNA per cell in grams} \times 6.10^{23}}{\text{Molecular weight of F or MB mRNA in daltons.}}$

contains an average 100 residues (thus comprising ~4% of the length of F mRNA and 5% of the length of MB mRNA). Table I shows that the content of poly(A)+ RNA is ~0.18 pg per cell, assuming complete recovery of F and MB polysomes (22). From the average molecular weight of F and MB poly(A)+ RNA, we have calculated that the number of poly(A)+ RNA molecules is ~170,000 molecules per P3K cell of which 42,500 are associated with MB polysomes and 127,500 with F polysomes. Thus, there are three times more F than MB polysomal poly(A)+ RNA molecules.

The existence of poly(A)$^-$ mRNA in various cells (24, 27) indicated the need to determine whether mRNA purification by oligo (dT) chromatography results in a selective loss of some mRNA sequences, particularly in the F RNA population. Different amounts of total poly(A)+ and poly(A)$^-$ RNA fractions of F and MB polysomes were translated in a messenger-dependent reticulocyte lysate (MDL) cell-free protein synthesis system (31), and their products were analyzed by SDS-gel electrophoresis (Fig. 1). As judged by TCA precipitation of incorporated [^{35}S]methionine, >95% of the template activity of the MB mRNA was recovered in the poly(A)+ RNA fraction, whereas in the F RNA ~70% of the template activity was found in the poly(A)+ RNA fraction and 30% in the poly(A)$^-$ RNA fraction (data not shown). The poly(A)$^-$ RNA of F polysomes appears to be enriched for higher molecular weight proteins when compared to the unfractionated F RNA. When this poly(A)$^-$ RNA was recycled over the affinity column, it passed quantitatively through the column. This translation experiment shows that the MB mRNA contains essentially two messages that are more abundant than any others in the population (with the possible exception of a third message coding for the polypeptide indicated pF), whereas the number of RNA species of nearly equal abundance is larger in the F population. At this level of sensitivity, it appears that for both polysomal fractions the poly(A)+ RNA sequences are highly representative of the sequences present in the unfractionated RNA. It therefore seems reasonable to assume that this is also the case for the sequences which are not detected in this assay. This experiment shows also that excess of polysomal RNA and poly(A)+ RNA causes inhibition of protein synthesis only at concentrations higher than 1 mg/ml and 50 μg/ml, respectively.

These F and MB poly(A)+ RNA fractions were used as templates for the synthesis of highly radioactive complements

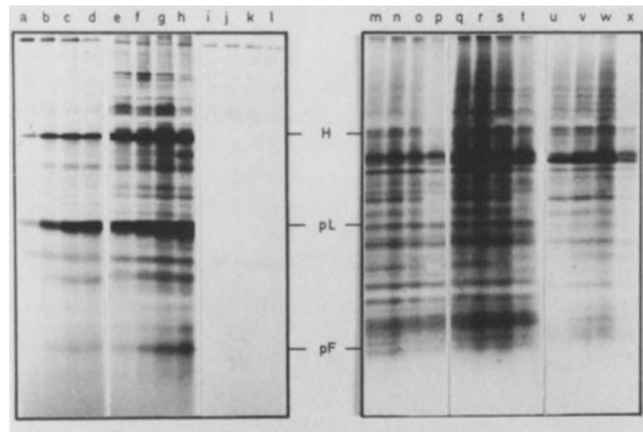

FIGURE 1 In vitro products directed by F and MB RNA fractions. Fluorogram of [^{35}S]methionine-labeled polypeptides synthesized in MDL and analyzed on 17.5% polyacrylamide slab gel containing SDS. The arrows marked *H*, *pL*, and *pF* indicate the respective positions of Ig H chain (50,000 daltons in size), the precursor form of Ig L chain (25,000 daltons in size), and the precursor form of a third secreted protein (15,000 daltons in size). The reactions, which were 10 μl in volume, contained (*a*) 2, (*b*) 5, (*c*) 10, and (*d*) 20 μg of unfractionated MB RNA; (*e*) 0.08, (*f*) 0.2, (*g*) 0.4, and (*h*) 0.8 μg of poly(A)+ RNA; (*i*) 1, (*j*) 2.5, (*k*) 5, and (*l*) 10 μg of MB poly(A)$^-$ RNA; (*m*) 5, (*n*) 10, (*o*) 20, and (*p*) 40 μg of unfractionated F RNA; (*q*) 0.1, (*r*) 0.25, (*s*) 0.5, and (*t*) 1 μg of F poly(A)+ RNA; (*u*) 2.5, (*v*) 5, (*w*) 10, and (*x*) 20 μg of poly(A)$^-$ RNA; half of each reaction was applied to the gels and the fluorograms were exposed for 1 d.

using reverse transcriptase from avian myeloblastosis virus (3). For the purposes of our experiments, we have assumed that the relative abundance of cDNA copies reflects the frequency distribution of the different classes of mRNA. (Detailed discussion of this aspect can be found elsewhere [3]). For both F and MB fractions, the average size of cDNA corresponded to single-stranded molecules of 400 nucleotide length and the cDNA preparations were almost completely sensitive to single-strand specific S_1 endonuclease.

Complexity of F and MB poly(A) + RNA

The complexity of an mRNA population can be estimated by comparing the kinetics of hybridization of cDNA to an excess of its template mRNA and the kinetics of cDNA hybridization to a standard mRNA of known complexity (3). The reaction is followed by expressing the percentage of cDNA converted to double-stranded form as a function of the logarithm of Rot (RNA concentration in moles per liter × times in seconds). Ideally, the Rot value sufficient to hybridize 50% of the cDNA ($Rot_{1/2}$) is proportional to the complexity of the mRNA. In our experiments we have used, as a standard, rabbit globin mRNA that consists of two different mRNA molecules, one α-chain and one β-chain mRNA with an overall mol wt of 4.32×10^5 daltons. Under our annealing conditions, the kinetics of hybridization of rabbit globin cDNA to globin mRNA exhibits a $Rot_{1/2}$ of 6.17×10^{-4} mol/liter · s (33). From this result, it can be calculated that pure mRNA species of mol wt 5.5 and 6.6×10^5 daltons will hybridize to their respective cDNA copies with $Rot_{1/2}$ of 7.8×10^{-4} (MB mRNA) and 9.4×10^{-4} (F mRNA). These values were used to calculate the complexities of F and MB poly(A)+ RNA populations.

Fig. 2 compares the kinetics of hybridization of F and MB mRNA with their respective cDNAs. There is an obvious

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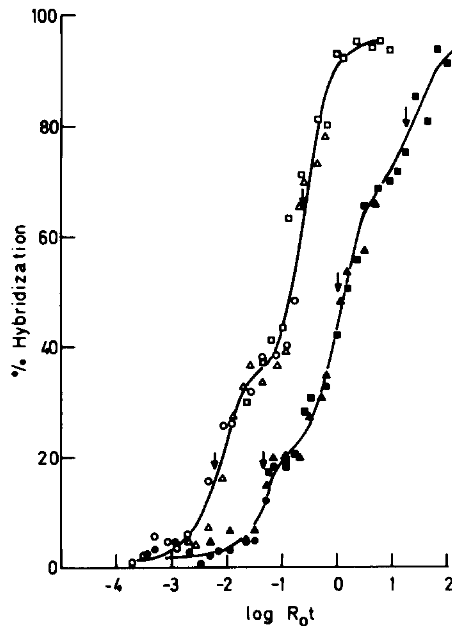

FIGURE 2 Hybridization of F and MB cDNA to template mRNA. The hybridization reactions contained F mRNA at concentrations of 1 μg/ml (●), 20 μg/ml (▲), and 200 μg/ml (■), and MB mRNA at concentrations of 0.6 μg/ml (○), 6 μg/ml (△), and 60 μg/ml (□). They were performed with 0.3 ng of cDNA (2,000 cpm) per point. 10-μl aliquots were removed and assayed by single-strand specific nuclease as described in Materials and Methods. Rot values are the product of concentration of RNA, in moles of nucleotides per liter, times the duration of the reaction in seconds. (○, △, □) MB mRNA-driven reaction with MB cDNA. (●, ▲, ■) F mRNA-driven reaction with F cDNA.

major difference in the kinetics of hybridization of F and MB mRNA; the cDNA hybridization with MB mRNA proceeded more quickly than the corresponding hybridization with F mRNA, indicating that the MB mRNA population is much less complex than the free. Complete hybridization of globin cDNA and mRNA occurs over two log units, while MB and F mRNA hybridized over four and five log units, respectively. Thus both F and MB mRNA are composed of large and heterogeneous populations of mRNA sequences present at varying frequencies. These hybridization kinetics were quite reproducible between two different cDNA preparations for each type of mRNA and between different mRNA preparations.

The F mRNA hybridization curve exhibits roughly three transitions, possibly corresponding to the three abundance classes described in HeLa cells (3). Making this assumption, it is possible to calculate the number of different mRNA sequences in each class and the number of copies of each mRNA per cell (3). The results of these calculations are shown in Table II. The general conclusion which can be drawn is that the F mRNA population of P3K cells contains ~8,000 different messages, while the MB mRNA population includes only 230 different mRNA species. The MB mRNA appears to be distributed in two abundance groups, of which one group is formed by three very abundant mRNA species (each present 5,250 times per cell). This group contains 40% of the MB mRNA population or 8% of the total message in the cytoplasm of P3K cells. As shown later, this group represents the hybridization of Ig mRNA sequences which account for ~10% of the total protein synthesis of P3K cells (unpublished results). The other group consists of ~225 different messages, each present 120 times per cell. This frequency is very similar to that of the middle-abundance group of F mRNA. This analysis of the complexity of F and MB messages reveals clearly that the mRNA sequences are differently distributed between F and MB polysomes. In particular, the MB polysomes contain many fewer distinct mRNA sequences than the F polysomes and are characterized by an almost complete absence of very low abundance mRNA sequences.

Comparison of the F and the MB mRNA Populations

The F and MB mRNA populations were compared by annealing the mRNA of one fraction to the cDNA of the second fraction. This comparison indicated the proportion of the sequences that are common to both mRNA populations and evaluated their frequency among the heterologous population. For example, if the MB sequences, which are the less numerous of the two mRNA populations, are also present in the F mRNA population but reduced in abundance, the cross-hybridization between MB cDNA and F mRNA would occur to the same extent as in the homologous hybridization but at increased Rot value. Fig. 3 shows the cross-hybridization obtained by annealing F and MB cDNA to their reciprocal template mRNA. Approximately 80% of the MB cDNA cross-hybridized to F mRNA, indicating that most of the MB sequences are present in the F mRNA. The shift in the Rot curve of ~1.3 to 2.0 log units suggests that the MB sequences are no longer present at the same concentration in the F mRNA population as in the MB polysomes, but are 20–100 times less abundant. In addition, a comparison of this heterologous curve to the F and MB homologous curves (the broken lines derived from Fig. 2) shows that the first transition of the cross-hybridization curve reaches the same 40% levels as in the MB cDNA-mRNA hybridization curve. This analogy suggests that the most highly abundant MB sequences are among the 50 most frequent F messages.

About 50% of the F cDNA hybridized to MB mRNA. Although the abundance class of the cross-hybridizing cDNA cannot be determined from the data presented, the shift in the Rot curve suggests that a substantial proportion of the F mRNA sequences is present in the MB polysomes at a concentration 5- to 10-fold less than in the F polysomes themselves.

Ig L mRNA Content of F and MB Polysomes

We have measured the Ig L mRNA concentrations in F and MB polysomes by hybridizing their poly(A)$^+$ RNA with a cDNA strictly specific for Ig L mRNA. The comparison of the kinetics of hybridization of this cDNA with MB poly(A)$^+$ RNA with the kinetics of hybridization of Ig L cDNA with its pure template mRNA as a standard would allow the determination of the concentration of Ig L mRNA sequences in these RNA populations. Ig L cDNA was prepared by using a synthetically produced hexanucleotide (T_2G_3T) as a primer so that pure transcript of the Ig L mRNA were generated, although the mRNA template population was not purified to homogeneity (34, 25). However, the heterogeneity of the Ig L mRNA preparation prevented its use as a kinetic standard for its hybridization. We circumvented this problem by calculating a theoretical $Rot_{1/2}$ of 5.7×10^{-4} mol/liter · s on the basis of our earlier measurment of the kinetics of annealing of rabbit globin $\alpha + \beta$ mRNA with its cDNA, taking into account the molecular weight difference between globin and Ig L mRNA.

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TABLE II
Sequence Complexity of F and MB Poly(A)$^+$ RNA from P3K cells

Fraction	Transition	P*	Observed $Rot_{1/2}$	Corrected $Rot_{1/2}$	No. of sequences‡	Molecules of each sequence per cell§
			mol/liter·s	*mol/liter·s*		
Free	1	0.23	0.050	0.012	13	2,284
	2	0.50	0	0.50	530	121
	3	0.27	25	6.75	7,180	5
MB	1	0.37	6.3×10^{-3}	2.37×10^{-3}	3	5,254
	2	0.63	0.28	0.176	225	119

* Fraction of hybridizable F or MB cDNA, was determined by estimation utilizing line or plots of the data (3).
‡ Taking the molecular weight of one sequence of F mRNA equivalent as 6.6×10^5 daltons and MB mRNA as 5.5×10^5 daltons.
§ $\frac{\text{Amount of F or MB mRNA per cell in grams} \times P \times 6.10^{23}}{\text{Molecular weight of F or MB mRNA} \times \text{number of sequences}}$.

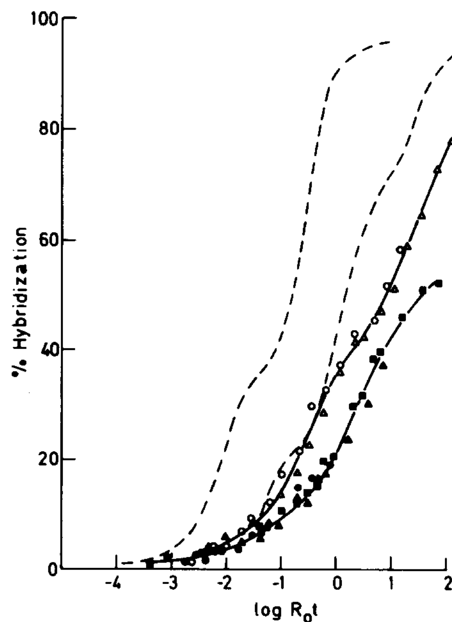

FIGURE 3 Reciprocal hybridization of F and MB cDNA to template mRNA. The hybridization reactions contained F mRNA at concentrations of 8 (○) and 360 μg/ml (△) and MB mRNA at concentrations of 1.5 (●), 15 (▲), and 150 μg/ml (■). (○, △) F mRNA-driven reaction with MB cDNA. (●, ▲, ■) MB mRNA-driven reaction with F cDNA. The dashed lines represent the homologous hybridizations as indicated in Fig. 2.

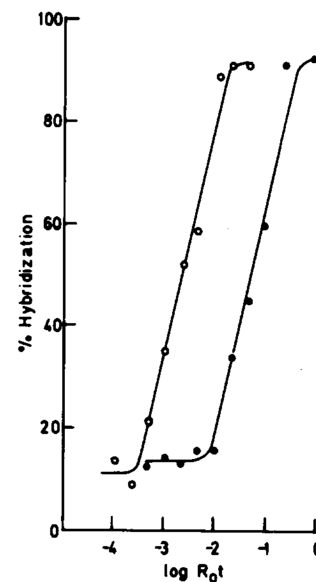

FIGURE 4 Hybridization between F and MB mRNA with Ig L cDNA. F mRNA at a concentration of 2 μg/ml (●) and MB mRNA at a concentration of 0.4 μg/ml (○) were hybridized with a cDNA specific for Ig L mRNA using T_2G_3T as a primer. The hybridization reactions were performed with 0.06 ng of cDNA (400 cpm) per point.

When this Ig L cDNA was hybridized with MB poly(A)$^+$ RNA, a single transition was observed with a $Rot_{1/2}$ value of 3×10^{-3} mol/liter · s (Fig. 4). This transition corresponds to the fastest hybridization component observed when MB cDNA was hybridized to its template mRNA (Fig. 3) although the rate of hybridization is slightly faster than the average rate for the most frequent class of MB mRNA. This result confirms that one of the three most abundant MB messages codes for Ig L. Ig L cDNA also annealed with F poly(A)$^+$ RNA, but at an increased Rot value ($Rot_{1/2} = 7.5 \times 10^{-2}$ mol/liter · s). Using these data, we can calculate (Table III) that $\sim 8 \times 10^3$ Ig L mRNA molecules per cell are present in MB polysomes and 1×10^3 molecules in F polysomes. Therefore, F polysomes contain ~11% of Ig L mRNA.

We have also examined the content of Ig L mRNA sequences by translating the various F and MB RNA fractions in a messenger-dependent protein synthesis system. Unlike the experiment illustrated in Fig. 1, the synthesized Ig polypeptides were specifically immunoprecipitated from aliquots containing equal amounts of labeled products and analyzed by gel electrophoresis and fluorography as shown in Fig. 5. The densitometric quantitation (not shown) revealed that the ratio of the two polypeptides synthesized by MB poly(A)$^+$ RNA to those synthesized by F poly(A)$^+$ RNA was about 25 to 1 for both Ig H and L chains. Because there is three times as much F as MB poly(A)$^+$ RNA, F polysomes contain ~12% of the Ig H and L chain template activity. This is consistent with the results of the above hybridization experiment.

TABLE III
Estimate of the Number of Ig L mRNA Molecules Present in F and MB Polysomes

Fraction	$Rot_{1/2}$	Fraction Ig L mRNA × 10^2	No. of Ig L mRNA molecules per cell*
	mol/liter·s		
F	7.5×10^{-2}	0.76	969
MB	3.0×10^{-2}	19	8,075

* Number of F or MB poly(A)$^+$ RNA molecules per cell (see Table I) × fraction Ig L mRNA.

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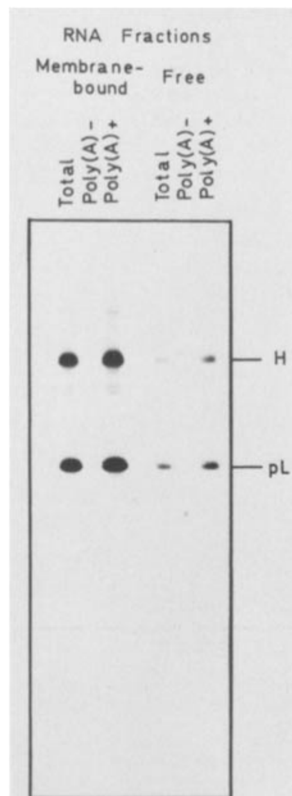

FIGURE 5 In Vitro synthesis of Ig H and Ig L from F and MB RNA fractions. F and MB RNA fractions were prepared from exponentially growing P3K cells and separated into poly(A)$^+$ and poly(A)$^-$ fractions as described in Materials and Methods. These RNAs were used at optimal concentrations (as defined in Fig. 1) to stimulate protein synthesis in MDL. The [^{35}S]methionine incorporation was measured from each independent reaction. An equal amount of [^{35}S]methionine-labeled product was removed from each corresponding F and MB RNA incubation and the Ig H and L were specifically immunoprecipitated using *Staphylococcus aureus*, strain A, as immunoadsorbent and analyzed on 17.5% polyacrylamide slab gel containing SDS.

This experiment also shows that absence of translatable Ig H and L mRNA sequences in F and MB polysomal poly(A)$^-$ RNA fractions and the enhancement of their translation when they have been purified by affinity chromatography. Because P3K cells contain equimolar amounts of polysomes synthesizing Ig H and L chains (B. Mechler, unpublished observation), the presence of an excess of synthesized Ig L chains does not reflect an imbalance in the number of sequences for these two polypeptides, but corresponds to a greater cell-free translation efficiency of the Ig L mRNA.

In conclusion, these molecular hybridization and cell-free protein synthesis experiments demonstrate that ~90% of both Ig mRNAs are present on the endoplasmic reticulum and ~10% associated with free ribosomal particles in P3K cells.

DISCUSSION

In this report we have measured the complexity and the frequency of poly(A)$^+$ RNA sequences recovered in F and MB polysomes and we have also determined the content of Ig H and L mRNA sequences present in these two mRNA populations. The significance of these results depends critically on the yield and the purity of the polysomal fractions we have studied. Modification of a cell fractionation procedure previously described for P3K cells (19) allows us to recover 95% of the ribosomes present in P3K cytoplasm and to obtain a yield of MB polysomes similar to that of F polysomes (22). Furthermore, to detect possible cross-contamination occurring during the preparation of the two fractions we have performed a variety of controls: labeling of membrane lipids with [^{3}H]oleic acid, addition of [^{3}H]adenosine-labeled F polysomes, or [^{3}H]adenosine-labeled microsomes to unlabeled cell extracts followed by an analysis of the radioactivity distribution in the resulting F ribosomal and membrane fractions. We could find no evidence of cross-contamination (result not shown) and we conclude that the purity of both fractions approaches 98%. However, it is possible that the cell homogenization could by itself displace from the membranes some polysomes which would then be recovered in the F ribosomal fraction.

Complexity of F and MB mRNA Sequences

The hybridization data presented in this report confirm that F and MB polysomes contain populations of mRNA sequences which differ in number and abundance. There appear to be ~200–250 different mRNA sequences in MB polysomes and ~40 times more in F polysomes, although inaccuracy of the determination of the $Rot_{1/2}$ value of the third transition in the F mRNA may lead to an approximately two-fold error in the number of F mRNA sequences (for further discussion on the precision of this method, see discussion in references 3, 12, and 32). The relative small number of mRNA sequences on the ER may well reflect the specialized function of the ribosome association to the ER membranes in the synthesis of proteins intended for secretion (29, 41) or integral membrane proteins (26). It appars therefore that the majority of P3K mRNA sequences are contained in F polysomes.

It should be noted that the F but not the MB polysomes contain a fraction of mRNA sequences that do not bind to oligo (dT)-cellulose and presumably are not polyadenylated (24) or contain very short sequences of poly(A) (14). Measurement of their template activity indicates that these poly(A)$^-$ mRNA molecules represent ~25% of the F mRNA. Correspondingly, ~25% of the F mRNA fails to bind to the oligo (dT)-cellulose. Other investigators have found similar amounts of poly(A)$^-$ mRNA sequences among total cellular messages of HeLa cells (16) and chick myoblast (30).

As in a variety of other tissues and cell types, the cytoplasmic mRNA sequences are present at differing levels of abundance ranging from a few copies to as many as 6,000 copies per cell. Although the distribution of these sequences in three abundance classes (3, 2, 36) has recently been questioned (32), the concept of abundance classes is a useful one, albeit on oversimplification. The F polysomal mRNA sequences of P3K cells can be classified, from these considerations, as falling into three abundance classes that reflect the presence of three mRNA classes of high, middle, and low abundance. In contrast, the MB mRNA-cDNA hybridization curve exhibits only roughly two transitions that correspond to high and middle abundance classes. The class of low abundance is virtually nonexistent in MB polysomal mRNA; however, the absence of this latter class might be only apparent, because small variations in the hybridization curve near saturation would be undetectable. In any case, such low abundance class species would be present at

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a concentration of less than one copy per cell. The comparison of the frequences of F and MB mRNA sequences reveals that in the high abundance classes the MB messages are more abundant than the corresponding class of F mRNA. By contrast, in the case of the middle abundance class, F and MB sequences have the same frequency.

Relationship between F and MB Polysomal mRNA

Various investigators have studied the distribution of Ig mRNA sequences between F and MB polysomes and, using different techniques, have reached different conclusions regarding the extent of the segregation of Ig mRNA. Either Ig mRNA was found to be completely absent from the F polysomes (4, 6) or a substantial proportion of Ig mRNA (up to 30%) was found associated with the F polysomes (20, 28). This uncertainty is mirrored in investigations on the segregation of other specific messages coding, e.g., albumin (15, 37, 38, 40, 43) or viral glycoproteins. In cells infected by vesicular stomatitis virus, essentially all the viral glycoprotein mRNA was found in MB polysomes (11), whereas in the case of the related Sindbis virus this mRNA was almost equally distributed between F and MB polysomes (21, 42).

To study the mRNA distribution between F and MB polysomes, we have determined the extent of homology of the two mRNA populations and then we have used two approaches to estimate their Ig mRNA content. Our data show that essentially all the MB mRNA are present in significant concentrations, the most abundant sequences by a 25-fold factor, and the less abundant ones by a 100-fold factor. However, the actual content in the F polysomes of these sequences is significantly higher because 75% of the P3K poly(A)$^+$ RNA molecules is associated with F polysomes. With this correction, ~10% of the most abundant MB sequences and 2% of the less abundant ones are found in the F polysomal fraction, indicating that the purity of the F polysomes reaches at least 98%. On the basis of previously mentioned control experiments using the addition of prelabeled purified F polysomes of P3K cells during homogenization, we estimated that the purity of the MB polysomes reaches a similar value. However, the comparison of the hybridization curves between F cDNA and F mRNA of MB mRNA suggests that the purity of the MB polysomes may in fact be substantially lower.

The use of Ig L [^{3}H]cDNA confirms that one of the most abundant mRNA sequences is indeed Ig L mRNA and indicates that 11% of this mRNA is present in F mRNA. A similar result is obtained by estimating the amount of Ig synthesized from F and MB RNA fractions in a cell-free system. It is clear that both Ig H and L mRNA sequences are present in a relatively high proportion within the F mRNA, yet their presence in the F fractions appears not to result from cross-contaminating membranes. Of course, the possibility could not be eliminated that some displacement of Ig-syntheszing polysomes from the ER membranes could have occurred during cell homogenization. However, in light of the fact that only 2% of the less abundant class of MB sequences are found in F polysomes, either the Ig-synthesizing polysomes are more readily detached from ER membranes during homogenization or Ig mRNAs are indeed bona fide components of the F polysomal fraction.

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