Membrane-bound Ribosomes of Myeloma Cells V. Subcellular Distribution of Immunoglobulin mRNA Molecules

BERNARD MECHLER

Department of Biochemistry, University of Cambridge, Cambridge CB2 1QW, England. Dr. Mechler's present address is the Department of Cell Biology, Biozentrum der Universität Basel, CH-4056 Basel, Switzerland.

ABSTRACT The subcellular distribution of the most abundant mRNA sequences, particularly those of the immunoglobulin heavy (Ig H) and light (Ig L) chain mRNA sequences, of MOPC 21 (P3K) mouse myeloma cells has been examined by translating the mRNA of various subcellular fractions in a messenger-dependent reticulocyte lysate (MDL) and by identifying Ig products with the use of a specific antiserum. Analyses of the distribution of the mRNA template activity and the translation products by SDS polyacrylamide gel electrophoresis reveal that ~85% of the mRNA present in the free ribosomal fraction is incorporated into polysomes and that the remainder is present as mRNP particles. On the endoplasmic reticulum (ER) the mRNA is found entirely in polysomes. In general, the size class of free (F) and membrane-bound (MB) polysomes corresponds to the size of their translation products. Thus, mRNAs coding Ig H (5.0×10^5 daltons in size) and Ig L (2.5×10^5 daltons in size) are incorporated into polysomes formed of 12 and 6 ribosomes, respectively. About 10% of the Ig mRNAs are not bound to membranes. A third of these are associated with mRNPs and the remainder incorporated into F polysomes of the same size as the Ig-synthesizing MB polysomes.

The mRNA molecules of eucaryotes are distributed between two different classes of polysomes that synthesize either soluble proteins when they are free (F) (29, 27, 7, 8), or secretory and integral membrane proteins when they are attached to the endoplasmic reticulum (ER) (24, 32, 23). The mechanism by which specific mRNAs are selected for translation on membrane-bound (MB) polysomes has not been fully elucidated. Two mechanisms have been proposed: (*a*) the specificity for membrane attachment might be determined by the N-terminal amino acid sequence of the nascent polypeptide chain (22, 2) or (*b*) the recognition mechanism might depend on the direct interaction of the 3′ end of the mRNA with membranes (21, 13, 20).

Previous molecular hybridization analysis has revealed striking differences in the number and abundance of mRNA sequences associated with F and MB polysomes in MOPC 21 (P3K) mouse myeloma cells in culture (17). The F mRNA population contains ~40 times more different sequences than the MB mRNA population, but the mRNA sequences on the ER are on the average more abundant. In these cells, the Ig H and Ig L mRNAs are among the most abundant sequences present on the ER and account for approximately one-third of the MB mRNA molecules. However, the Ig mRNAs are also found in reduced but non-negligible amounts in the soluble compartment.

In view of the importance of the observation of mRNA molecules coding for secretory protein in F polysomes, especially with respect to the problem of MB polysome formation, it was of interest to investigate the nature and size of the particles with which Ig mRNA was associated.

In this report, the subcellular mRNA distribution is examined by using first a cell fractionation procedure that allows the analysis of virtually all F and MB ribosomes present in the cytoplasm under conditions in which they are devoid of cross-contamination (18), by translating the mRNA contained in the various subcellular fractions in a messenger-dependent reticulocyte lysate (MDL) (26), and finally by identifying the Ig products with the use of a specific antiserum.

The results show that most of the cytoplasmic mRNAs are incorporated into polysomes and are translated as efficiently by F as by MB polysomes, because the density of ribosomes per mRNA molecule depends uniquely on the size of the

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synthesized polypeptide product. Furthermore, the untranslated mRNAs accumulate in the cytoplasm as F mRNP particles. Most of the Ig H and L mRNAs are associated with MB polysomes, and the relatively small fraction that is found in the F ribosomal fraction is either incorporated into F polysomes of the same size as the MB polysomes or present as mRNP particles. The absence of MB Ig mRNP particles suggests that the initiation of the translation of Ig mRNA occurs on F ribosomes.

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 supplemented with 10% horse serum. All cells were harvested during exponential growth from cultures not exceeding 5×10^5 cells/ml. To ensure maximum yield of polysomes, exponentially growing P3K cells were concentrated at 2×10^6 cells/ml and further incubated for 45 min at 37°C in prewarmed growth medium.

Preparation of F and MB Polysomes

At the end of the incubation, the cells were diluted fivefold in ice-cold Earle's saline solution, 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 directly used for the separation of F and MB polysomes according to a modification of our previously described method (18).

The homogenate was diluted fivefold in 2.5 M sucrose $TK_{150}M$ (0.15 M 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 1.2 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. Under these conditions, nuclei were found to sediment to the bottom of the tube and into the 2.5-M sucrose layer, whereas F polysomes were in the loading zone. Microsomes were located at the interface between the 2.05 and 1.3 M sucrose layers.

For the analysis of the sedimentation characteristics of the ribosomal particles contained in the free ribosomal and membrane fractions, 15-55% linear sucrose density gradients in $TK_{80}M$ (0.08 M KCl, 0.005 M $MgCl_2$, 0.05 M Tris-HCl, pH 7.4) were used. To dissolve the membranes, 0.5% sodium deoxycholate and 0.5% Brij 58 were added to the membrane fraction before loading on the 15-55% sucrose density gradient. Centrifugation was carried for 8.5 h at 4°C in a Spinco SW 27 rotor at 23,000 rpm. Gradients were collected in fractions of ~1 ml and the adsorbance profile was measured at 260 nm.

Extraction of RNA

Fractions from the sucrose gradients of F and MB ribosomal particles were diluted with 0.5 vol of distilled H_2O, and the RNA was extracted at room temperature with 0.1% SDS and an equal volume of phenol:chloroform (1:1). RNA was precipitated from the aqueous phase by the addition of 2.5 vol of ethanol and 0.1 vol of 2 M Na-acetate, pH 5.2 and recovered after 15 h at -20°C by centrifugation for 20 min at 6,000 rpm in a Sorvall SM 24 rotor DuPont Instruments-Sorvall, DuPont Co. (Newtown, Co.). After dissolution in distilled H_2O, aliquots of RNA were placed in translation assay tubes, in which the RNA was lyophilized.

In Vitro Protein Synthesis and Immunoprecipitation

Translation of lyophilized RNA in the messenger-dependent reticulocyte lysate was carried out as described by Pelham and Jackson (26). RNA in the reaction mixture was kept at concentrations below 1 mg/ml to prevent inhibitory effect because of excess of RNA (17).

After incubation, the reaction mixtures were diluted 40-fold in 0.5% NP 40 net NET buffer (0.15 M NaCl, 0.005 M EDTA, 0.05 M 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 (Ig G fraction) using formaldehyde-treated *Staphylococcus aureus* to collect antigen-antibody complexes (10).

Polyacrylamide Gel Electrophoresis and Fluorography

Proteins were resolved by polyacrylamide gel electrophoresis according to Laemmli (12). The separation gel was 15 or 17.5% acrylamide and the stacking gel 3.6% acrylamide.

The procedure of Bonner and Laskey (3) was used to impregnate the gels with 2,5-diphenyloxazole and they were dried for 1 h at 100°C. Kodak X-omat film was presensitized according to the method of Laskey and Mills (14) and used for the fluorography.

Materials

[^{35}S]methionine was prepared by ion exchange chromatography from a protein hydrolysate of [^{35}S]sulfate-labeled *Escherichia coli* preparation.

RESULTS

Distribution of mRNA Molecules in F and MB Ribosomes

P3K cells were broken by Dounce homogenization and the postnuclear supernate was fractionated to yield free ribosomal and membrane fractions on a discontinuous sucrose density gradient, under conditions that have been found to prevent adsorption of F polysomes to membranes (18). In P3K cells, 80% of the polysomes are found free in the cytoplasm and the remainder attached to the ER (18, 17). The F and detergent-liberated MB ribosomal particles were then sedimented on 15-55% sucrose density gradients (Figs. 1 *a* and 2 *a*). The RNA from each individual fraction of both sucrose gradients was extracted and translated in MDL. The mRNA template activity was estimated by measuring the [^{35}S]methionine incorporation. In the F ribosomal fraction, this activity was associated (85%) with polysomal structures (dimers, trimers, etc.) and was also present in a region of the sucrose gradient that contains native

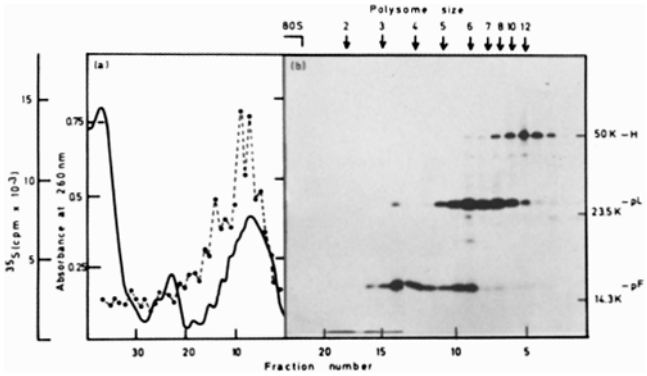

FIGURE 1 Distribution of the in vitro products directed by mRNA extracted from MB ribosomal particles. A membrane fraction, which was prepared from 5×10^7 P3K cells as described in Materials and Methods, was treated with 0.5% sodium deoxycholate and 0.5% Brij 58 and sedimented on a 15-55% linear sucrose density gradient. (*a*) The RNA from each individual fraction of such gradients was extracted with SDS and phenol-chloroform and 50% of the RNA of each fraction was used to direct protein synthesis in 10 μl MDL containing [^{35}S]methionine. After incubation the [^{35}S]methionine incorporation was measured in 1-μl aliquot of each in vitro reaction and the remainder analyzed in a separated track of a 15% polyacrylamide slab gel containing SDS (*b*). (*a*) Sedimentation profile and template activity. Sedimentation is from left to right. (—) optical density at 260 nm; (●---●) cpm (^{35}S). (*b*) In vitro products.

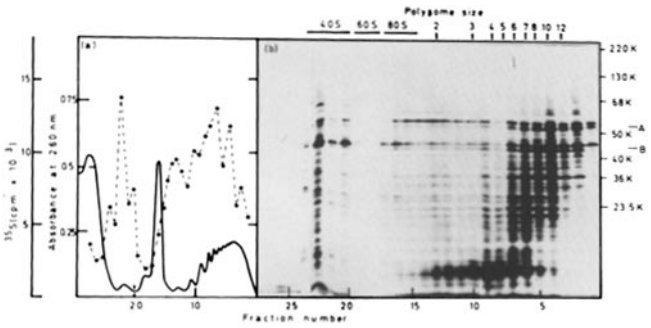

FIGURE 2 Distribution of the in vitro products directed by mRNA extracted from F ribosomal particles. The polypeptides synthesized in MDL by mRNA extracted from the F ribosomal particles which had been sedimented under the conditions described in Fig. 1 *a* were analyzed on a 15% polyacrylamide slab gel containing SDS. The lines marked *A* and *B* indicate the position of the 53,000- and 44,000-dalton polypeptides discussed in the text. (*a*) Sedimentation profile and template activity. Sedimentation is from left to right. (—) optical density at 260 nm; (●---●) cpm (^{35}S). (*b*) In vitro products.

ribosomal subunits not directly engaged in protein synthesis. It has been demonstrated that most of the messages that cosediment with native ribosomal subunits are in the form of mRNP particles (30). By contrast, on the membranes, all the detectable mRNA activity was found associated with polysomal structures.

The translational products labeled with [^{35}S]methionine were analyzed by one-dimensional SDS polyacrylamide gel electrophoresis to determine the location of the more abundant messages present in both ribosomal fractions (Figs. 1 *b* and 2 *b*). These gels show a reasonably good correlation between the polysomal size and the size of the protein products in both MB and F polysomes. On the membranes (Fig. 1 *b*), three products of highly abundant mRNAs were observed. These mRNAs code for products with the mobility of Ig H (5.0×10^5 daltons in size), the precursor form of Ig L (pL: 2.5×10^5 daltons in size), and the precursor form of a secreted protein with a size slightly larger than half the size of the pL polypeptide (pF: $\sim 1.5 \times 10^5$ daltons in size). These three proteins were associated with three distinct classes of polysomes that had on the average 12, 6, and 3-4 ribosomes, respectively. As shown in Fig. 2 *b*, a similar correlation applies to F polysomes that synthesize a great variety of abundant proteins. About 25 conspicuous polypeptide bands can be seen. As a rule, one can therefore estimate that any F or MB polysomes contain approximately one ribosome per message unit corresponding to a 4,000-dalton polypeptide. Similar values have been described for other systems (31, 11, 25). Therefore, a message coding a 40,000-dalton protein is on the average loaded with 10 ribosomes. However, two exceptions to this rule can be distinctly observed in F polysomes, where two classes of messages present a visible bimodal polysomal distribution: one class codes for a 53,000-dalton protein(s) (A) that are translated by polysomes containing either 2 or 12 ribosomes; the other directs the synthesis of a 44,000-dalton protein(s) (B) and is associated with one or 10 ribosomes. On the basis of their abundance and the molecular weight of their translation products, these two classes of messages are thought to code for tubulin and actin, respectively.

The mRNA sequences present in the free ribosomal fraction of P3K cells as mRNP particles appear to contain a representative sample of F and MB polysomal sequences with a particular enrichment for the messages which had a bimodal distribution in the F polysomes.

As indicated before, no evident mRNA activity above the endogenous background of the MDL could be found in association with MB native ribosomal subunits. Because previous experiments had revealed that the membrane fraction contained native 40S ribosomal subunits that had a high turnover and were presumably attached to the ER through an mRNA molecule (19, 20), it was of interest to analyze in more detail whether the membrane fraction contained small numbers of initiation complexes formed of newly made mRNAs coding for secretory proteins and associated with native 40S ribosomal subunits. Such newly initiated messages are expected to be present in very small number compared to the total number of messages in MB polysomes, because they would only account for the replacement of mRNA and for the mRNA increment caused by cell growth. The experiment shown in Fig. 3 indicated that all the messages for secretory proteins present on the membranes were indeed in polysomes and none in mRNP particles or initiation complexes. The few H and pL chains that could be detected after a long exposure of the gel (Fig. 3 *c*) are of such an intensity that could be considered to correspond entirely to small quantities of contaminating polysomes. This interpretation is strengthened by the fact that there was no detectable peak of Ig H or pL template activity in the region of the native 40S ribosomal subunits in the gradient (fractions 2 and 3) although two other polypeptides seemed to be coded by genuine mRNA components sedimenting in this region. These polypeptides (X and Y with mol wt of 46,000 and 20,000) appeared to be minor components and could be of mitochondrial or viral origin (18).

Distribution of Ig H and Ig L mRNA in the F Ribosomal Particles

Molecular hybridization analysis had shown that ~10% of Ig mRNAs were recovered in the F ribosomal fraction (17). The location of the Ig mRNAs among the F ribosomal particles was further investigated by in vitro translation of the mRNA extracted from individual fractions of a sucrose density gradient of F polysomes (not shown) and by analysis of the immunoprecipitated Ig products on an SDS polyacrylamide gel. Fig. 4 shows that both Ig mRNAs were present in F mRNP particles and in F polysomes with approximately a third in mRNP

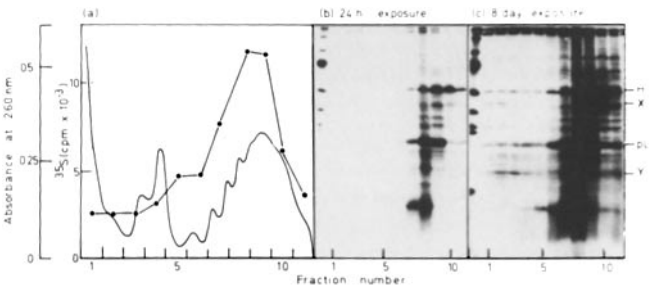

FIGURE 3 In vitro products directed by mRNA extracted from MB native ribosomal subunits. Fluorograms at two exposure times: 24 h (*b*) and 8 d (*c*) of the polypeptides synthesized in MDL by mRNA extracted from the MB ribosomal particles that have been sedimented in a sucrose density gradient (*a*) as described in Fig. 1 *a*, and analyzed on a 17.5% polyacrylamide slab gel containing SDS. (—) optical density at 260 nm; (●—●) cpm (^{35}S). The lines marked *X* and *Y* indicate the positions of the 46,000- and 20,000-dalton polypeptides discussed in the text.

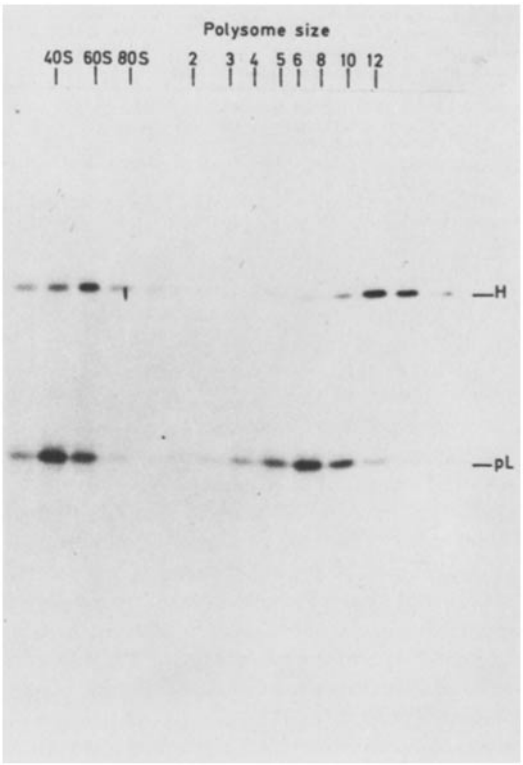

FIGURE 4 Distribution of the in vitro immunoglobulin polypeptides directed by mRNA extracted from F ribosomal particles. The F ribosomal particles were prepared and sedimented as in Fig. 1 *b*. 50% of the extracted RNA from each individual fraction of the gradient was used to direct protein synthesis in 40 μl MDL containing [^{35}S]methionine. After 1-h incubation the Ig products were immunoprecipitated as described in Materials and Methods, resuspended in gel sample buffer, boiled 3 min, and electrophoresed in a 17.5% polyacrylamide slab gel containing SDS. The figure is a fluorogram of the gel. *H* and *pL* indicate the positions of the Ig H chain (50,000 daltons in size) and the precursor form of the Ig L chain (25,000 daltons in size).

particles and two-thirds in polysomes. These polysomal Ig mRNAs were in F polysomes of the same size as the MB Ig-synthesizing polysomes.

DISCUSSION

The results presented in this study indicate a good qualitative correlation between the number of messages estimated by molecular hybridization technique (17) and the number of polypeptides detected in the present translation assay; it was previously found that the MB mRNA population consisted of three abundant sequences and, indeed, three major polypeptides are seen by translation of MB polysomal mRNA. With F polysomes, the exact number of abundant messages (13 according to hybridization data) is more difficult to estimate on the basis of the number of major polypeptide bands. The 25–30 major bands observed on the gel might correspond to a larger number of polypeptides, of which only a portion represent abundant mRNA products, the remainder due to comigration of several proteins encoded by less abundant messages. Furthermore, some of the polypeptides might be coded by mRNA depleted in poly(A) which have been eliminated in the molecular hybridization analysis. This poly(A)$^-$ mRNA represents ~25% of the mass of F mRNA (18).

The correlation between the polysomal size and the size of the protein products confirms that, in animal cells, mRNA molecules are monocistronic (11) and shows that, under standard conditions of growth, most of the abundant mRNA sequences are translated with the same efficiency by both types of polysomes. One can assume that the same is true of the less abundant messages that were not detected in the assay and thus of most messages incorporated into polysomes.

This study shows also that a proportion of the cytoplasmic mRNA is present in mRNP particles, as has been already observed in various types of cultured animal cells (for review see references 15 and 16). These mRNP particles contain numerous mRNA sequences that are presumably of different origins: some mRNP particles might originate from disassembled polysomes of mitotic cells (6) that represent ~5% of the cell population at any time; some might represent mRNAs that exceed the translational capacity of the cells or are newly made mRNAs that have not yet been translated; and finally some others might arise from mRNAs characterized by a low efficiency of initiation. An effect analogous to this postulated low efficiency of initiation might well be observed for the two more abundant mRNA sequences of the mRNP fraction; these two messages, which code for actin and tubulin-like proteins, are associated with polysomes containing too few ribosomes relative to the large size of the polypeptides they synthesize. The bimodal distribution might correspond to one or more of the various forms of actin and tubulin mRNAs present in eukaryotic cells (9, 4).

The major aim of this work was to obtain further insight into the mechanism of MB polysome formation. Two mechanisms have been proposed for the selective translation of specific mRNA by MB polysomes. In terms of protein synthesis, they differ operationally in the subcellular sites at which the initiation of translation of an mRNA which will subsequently form an MB polysome takes place. If the specificity for membrane attachment is determined by the nascent polypeptide chain (22, 2), the initiation should occur on a free ribosome. However, if the recognition depends on a specific polynucleotide sequence that binds directly to the membranes (21, 13, 20), the translation should start immediately on the ER. As a prerequisite to the study of the mechanism of entry of Ig mRNA into MB polysomes, I have investigated the subcellular distribution of Ig mRNA with a particular emphasis on the detection of F and MB mRNP particles in cells actively engaged in protein synthesis.

All the results presented here are consistent with the hypothesis that the specificity for membrane attachment resides in the nature of the polypeptide chain. All nonfunctional Ig mRNA molecules are found free in the cytoplasm. It has not been possible to detect significant amounts of Ig mRNP particles on the membranes of P3K cells actively engaged in protein synthesis. However, it has to be noted that the absence of detectable MB Ig mRNP particles does not eliminate formally the possibility that some transitory elements, i.e., initiation complexes made of mRNA/40S with a high turnover were present on the ER (see below).

The presence of Ig mRNA molecules in F polysomes is of uncertain significance. Two series of arguments indicate that these Ig mRNAs are probably not precursors of MB Ig-synthesizing polysomes although, if the hypothesis of the polypeptide leader sequence is correct, one should find in this fraction the newly translated Ig mRNA that has not yet synthesized a nascent chain long enough to interact with the membranes. First, the Ig mRNAs are translated by F polysomes of the same

size as the MB polysomes. If these F polysomes were precursors of MB polysomes, they should be of smaller size: monomers, dimers, or trimers. Second, the proportion of Ig mRNA translated by F polysomes, ~10% of the MB Ig-synthesizing polysomes, is far too high to represent precursor polysomes that are expected to occur at a frequency corresponding to the frequency of mRNA replacement and mRNA increase caused by the cell growth. Because the half-life of both Ig H and L mRNAs had been calculated to be ~14 h (5), which corresponds to the doubling time of the population of P3K cells (18), one can estimate that the translation of ~20 new Ig H or L mRNAs would be initiated per minute and per cell. Because each P3K cell contains on the average 9,000 Ig H or L mRNAs (17), these newly initiated Ig mRNAs should represent only 0.05% of the Ig mRNA engaged in protein synthesis, assuming that any message is initiated four times per minute (unpublished results). However, the possibility that at least some MB polysomes were dislodged from the membranes during the fractionation procedure cannot be ruled out. The presence of a relatively high proportion of Ig mRNA in F polysomes is in contradiction with the hypothesis of the polypeptide leader sequence. But, in the case of the myeloma, it is possible that the number of Ig mRNA molecules exceeds the translational capacity of the ER membranes. The excess of Ig mRNA would therefore be translated in the F polysomes. This view is strengthened by the previous observation that P3K cells contain a paucity of ER membranes, a reduced level of MB polysomes, and a limited number of ribosome-binding sites that prevents the direct attachment of all the ribosomes present in MB polysomes (20) as compared to differentiated tissues like liver cells (1, 28). However, it should be noted that in spite of these membrane limitations, the preferential translation of the Ig mRNA on MB polysomes is still favored by a 10/1 ratio.

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