Membrane-bound Ribosomes of Myeloma Cells

VI. Initiation of Immunoglobulin mRNA Translation Occurs on Free Ribosomes

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ABSTRACT Immunoglobulin heavy (Ig H) and light (Ig L) chain mRNA molecules have been released from the endoplasmic reticulum (ER) membranes as free (F) mRNP particles when MOPC 21 (P3K) mouse myeloma cells are exposed to a hypertonic initiation block (HIB). The subsequent fate of these mRNA sequences has been examined when the cells are returned to normal growth medium. Upon return to isotonicity, all previously translated mRNA molecules reassociate with ribosomes and form functional polysomes. Ig H mRNA is found incorporated first into F polysomes and then into membrane-bound (MB) polysomes. Kinetic studies indicate that the time of passage of Ig H mRNA in F polysomes is ~30 s, during which a nascent polypeptide chain of ~80 amino acids would have been completed. When the rate of polypeptide elongation is depressed with emetine during the recovery from HIB, both Ig H and L mRNA molecules accumulate in small F polysomes. These results indicate that the formation of Ig-synthesizing polysomes proceeds in the sequence: mRNA → F polysomes → MB polysomes. With the additional observation that during HIB recovery puromycin completely prevents the reassociation of Ig mRNA with the ER, these findings support a model of MB polysome formation in which the specificity of membrane attachment is determined by the nature of the N-terminal amino acid sequence of the nascent polypeptide chain.

In eucaryotes, proteins intended for secretion or insertion into cellular membranes are synthesized on ribosomes attached to the endoplasmic reticulum (ER) membranes (27, 35, 25). Two mechanisms have been proposed to explain the specificity for mRNA translation on membrane-bound (MB) polysomes. The recognition mechanism might be determined either by the affinity of the N-terminal amino acid sequence of the nascent polypeptide chain for the ER (24, 3) or by a specific mRNA-membrane interaction (22, 11, 21). The major difference between these two mechanisms concerns the subcellular site where the initiation of the translation of an mRNA which will subsequently form an MB polysome takes place. The polypeptide signal hypothesis predicts that this initiation should occur in the free (F) ribosomal fraction, whereas the mRNA-membrane interaction hypothesis forecasts initiation on the ER membranes.

An attempt has been previously made to study the entry of new mRNA into polysomes of MOPC 21 (P3K) mouse mye-loma cells. This work demonstrated that normally the entry of new Ig mRNA into MB polysomes represents only a minute fraction of all the polypeptide initiation events that take place on Ig-synthesizing polysomes (16). Were conditions found in which the proportion of untranslated mRNAs available for association with ribosomes is drastically enhanced, the study of MB polysome formation would be facilitated. Such conditions are expected to occur when the initiation of protein synthesis is inhibited. Upon reversal of this inhibition, most of the previously translated mRNAs that have been freed from ribosomes should reenter almost synchronously into polysomes.

In this study, I show that hypertonicity in the growth medium, which results in a selective and reversible inhibition of the initiation of protein synthesis in cultured animal cells (29, 31, 36), can be used to study the relationship between mRNA and the ER membranes in P3K cells. After the arrest of protein synthesis by hypertonic initiation block (HIB), all F and MB

polysomal mRNA molecules are recovered free in the cytoplasm of P3K cells as mRNP particles. Upon return to isotonicity, immunoglobulin heavy chain (Ig H) mRNA is first found in F polysomes and subsequently becomes attached to the ER membranes when the nascent polypeptide chain is ~80 amino acids long. Furthermore, when initiation of protein synthesis is allowed to proceed during the recovery from the hypertonic initiation block in the presence of emetine, an inhibitor of ribosome movement along mRNA (8), both Ig H and L mRNAs accumulate in small F polysomes. Similar results have been obtained using puromycin. These experiments confirm that the N-terminal region of the nascent polypeptide chain specifies the attachment of polysomes to membranes.

MATERIALS AND METHODS

Preparation of Polysomes and mRNP Particles for Direct In Vitro Translation

For protein synthesis assays performed with ribosomal and mRNP particles, F and detergent-liberated MB ribosomal particles as well as mRNP particles were separated on a discontinuous sucrose density gradient as described previously (16, 19), diluted with TK$_{80}$M (0.08 M KCl, 0.005 M MgCl$_2$, 0.05 M Tris-HCl, pH 7.4), layered over 2 ml of 2.0 M sucrose TK$_{80}$M, and centrifuged for 40 h at 4°C in a Spinco SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) at 50,000 rpm. Pelleted ribosomal and mRNA particles were resuspended in SMISH (0.01 M NaCl, 0.025 M KCl, 0.001 M MgCl$_2$, 0.01 M Tris-HCl, pH 7.4) and then diluted in a messenger-dependent reticulocyte lysate protein synthesis system (28).

All other procedures for cell culture, cell fractionation, preparation of F and MB ribosomal fractions, as well as RNA extraction, in vitro protein synthesis, immunoprecipitation, polyacrylamide gel electrophoresis, and fluorography have been described previously (16).

Materials

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

RESULTS

Fate of mRNA Released upon Polysome Disruption Induced by Hypertonicity

When P3K cells are exposed for 10 min to an excess of 0.2 M NaCl in the growth medium, a condition which was found to achieve within 4 min a complete inhibition of protein synthesis (unpublished observation), all the polysomes are converted into 80S ribosomes (Fig. 1). Moreover, comparison of the F and MB ribosome sedimentation profiles of control cells (see Figs. 1 and 2 in preceding paper) and HIB-treated cells indicates that ~55% of the MB ribosomes were released into the F ribosomal fraction and recovered in the form of 80S ribosomes, as previously shown for the blockage of protein synthesis initiation in P3K cells by pactamycin (21).

The distribution of the mRNA template activity in HIB-treated cells reveals that almost all the polysomal mRNA molecules were converted into particles sedimenting in the range of 25–60S, presumably as mRNP particles (Fig. 1). Furthermore, 90% of the template activity of the phenol-extracted mRNA has disappeared from the membrane fraction. This observation suggests that MB polysomal mRNA has been detached from the membranes and, indeed, the fluorogram of the translation products directed by mRNA contained in the F ribosomal fraction (25S–80S particles) of HIB-treated cells confirms that Ig H and L mRNA sequences are among the most abundant sequences in the F mRNP particles.
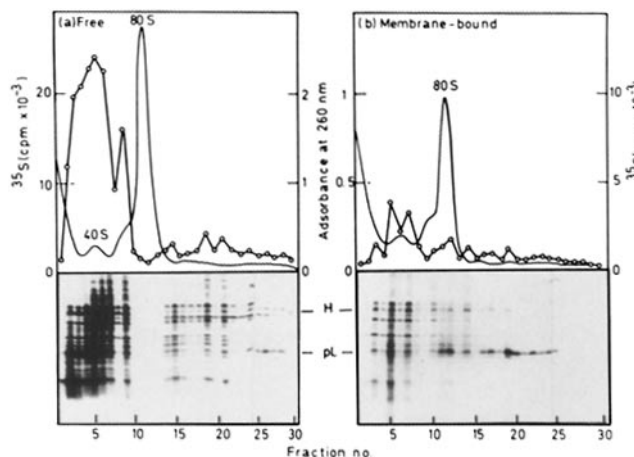

FIGURE 1 Effect of HIB on the mRNA distribution in the F Ribosomal Fraction and on the ER of P3K Cells. 5 × 10^7 P3K cells were exposed for 10 min to an excess of 0.2 M NaCl in the growth medium and fractionated. A quarter of the F ribosomal fraction and the totality of the membrane fraction (the latter treated with 0.5% sodium deoxycholate and 0.5% Brij 58) were layered on 15–55% linear sucrose density gradients and centrifuged for 8.5 h at 23,000 rpm and 4°C in a Spinco SW27 rotor. 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 of MDL containing [^{35}S]methionine. After incubation, the [^{35}S]methionine incorporation was measured in a 1-μl aliquot of each in vitro reaction and the remainder analyzed in a separate track of a 17.5% polyacrylamide slab gel containing SDS. Distribution of (a) F and (b) MB ribosomal particles and template activity. Sedimentation is from left to right. (—) optical density at 260 nm; (O——O) cpm (^{35}S). Analysis of the MDL-synthesized polypeptides by (a, bottom) F and (b, bottom) MB ribosomal and mRNP particles.

Some residual mRNA activity (10%) persists on the membranes of HIB-treated cells and is associated with small polysomes and particles sedimenting as mRNP particles. These polysomes contained genuine MB mRNA with Ig L mRNA as the major sequence, whereas the mRNA of the MB mRNP particles coded for numerous polypeptides that appeared to be similar to the polypeptides synthesized by mRNA sequences present in F mRNP particles. This similarity suggests that the apparent MB mRNP particles were in fact contaminating F particles. This assumption was further strengthened by the fact that purified F 40S particles which have been added to control cells and HIB-treated cells when they were homogenized were recovered in increased amount on the ER membranes of HIB-treated cells (unpublished results).

In Vitro Translation of Polysomes and mRNP Particles

To establish that HIB-released particles present in the F ribosomal fraction were not associated with ribosomes, I examined their translational properties in the messenger-dependent reticulocyte lysate (MDL). In such a system, polysomes and mRNP particles behave differently with respect to inhibition of the initiation of protein synthesis: the nascent chains carried by polysomes will be completed, but the initiation of new polypeptide chains on mRNA either incorporated into polysomes or present as mRNP particles will be prevented. Edeine, which served as the translation inhibitor, has been shown to block the joining of the native 60S ribosomal subunit to 40S/

mRNA/met-tRNA$_F$ complexes without interfering with elongation in the reticulocyte lysate (10, 32).

The analysis of the translation products (Fig. 2) demonstrates that edeine completely prevents the translation of the mRNA contained in HIB-released particles (slot *d*) and allows only the completion of the nascent chains carried by F (slot *c*) or MB (slot *g*) polysomes from untreated cells, because large polypeptides are labeled more than small polypeptides. Such a distribution is expected if the number and size of nascent polypeptide chains in relation to the polysome size are considered: the intensity of a labeled polypeptide should increase with its size. Completed Ig chains made on MB polysomes in the presence or absence of edeine appeared as three immunoprecipitable polypeptides (slot *m*): one intensively labeled H chain and two weakly labeled L chains, of which one has the mobility of the secreted L chain (L) and the other the mobility of the precursor of the L chain (pL) (24). Blobel and Dobberstein (3) have shown a precursor-product relationship between pL and L chains: the conversion of pL to L occurs during synthesis of the pL polypeptide on those polysomes present on rough microsomes. However, the conversion does not take place when the polysomes have been liberated from the membranes.

The fluorogram presented in Fig. 2 also shows that F polysomes (slot *a*) and HIB-released particles (slot *b*) directed the synthesis of the same basic set of polypeptides. In addition, the particles released by the HIB contained the mRNA sequences

that were originally associated with MB polysomes, as shown by the presence of two enhanced polypeptide bands corresponding to Ig H and pL chains. This experiment demonstrates that Ig mRNA molecules have almost completely shifted their location from MB polysomes to F mRNP particles during the HIB, suggesting that the nascent polypeptide chain is responsible for the attachment of Ig mRNA to the ER membranes.

It can nonetheless be argued that Ig mRNA binds directly to membranes, but through an interaction particularly sensitive to intracellular ionic strength change (like HIB). In such a case, treatment of P3K cells with puromycin alone should result in the retention of Ig mRNA on the ER membranes. On the contrary, if binding were instead mediated through the nascent polypeptide chain, puromycin treatment should lead to the detachment of Ig mRNA from the membranes. To distinguish these alternatives, P3K cells were incubated with 4.5×10^{-4} M puromycin for 1, 3, 5, and 10 min, and the F and detergent-liberated MB ribosomal particles were directly translated in an MDL. The results shown in Fig. 3 indicate that after puromycin treatment all the Ig mRNA was released from the membranes into the F ribosomal fraction of P3K cells. This release was faster for Ig L mRNA than for Ig H mRNA, consistent with an attachment dependent entirely on polypeptide anchorage in the membranes because, on the average, Ig H-synthesizing polysomes contained twice the number of nascent chains as Ig L-synthesizing polysomes (16).

Formation of Polysomes during the Recovery from the HIB

When HIB-treated P3K cells are returned to normal growth medium, protein synthesis resumes immediately (31), and correspondingly polysomes begin to form, as shown in Fig. 4 *a–i*. This analysis was performed by sedimenting total cytoplasmic extracts of control, HIB-treated, and HIB-recovering cells on sucrose density gradients made in high ionic strength buffer (i.e., 0.3 M KCl). It has been shown that under such conditions those ribosomes not engaged in protein synthesis are dissociated into ribosomal subunits, whereas 80S ribosomes and polysomes carrying nascent polypeptide chains remain intact (17). This analysis reveals that the formation of polysomes proceeded very rapidly with no apparent intermediate accumulation of mRNA-80S ribosome complexes during the 30 min necessary to achieve complete polysome recovery. The size of the polysomes gradually became larger with a progressive increase in the amount of the largest structures. After 1 min of recovery, dimers, trimers, and tetramers were already formed and the number of these small polysomes increased progressively during the first 10 min of recovery, and then remained constant for the next 20 min, during which there was essentially a loading of the larger polysomes. These results suggest that almost all mRNA molecules had entered polysomes in the first minutes of recovery.

The entry of mRNA into polysomes was further investigated by in vitro translation of the mRNA extracted from individual fractions of such sucrose gradients. In this case the gradients were made in low ionic strength to facilitate RNA extraction with SDS. As shown in Fig. 5, the mRNA template activity, which was originally present in polysomes, was almost entirely shifted to mRNP particles after HIB. However, within 3 min of recovery, most of the mRNA was again associated with polysomes. The mRNA activity was first confined to small polysomes (two and four ribosomes) and after 10 min became widely dispersed among larger polysomes. Those mRNA mol-
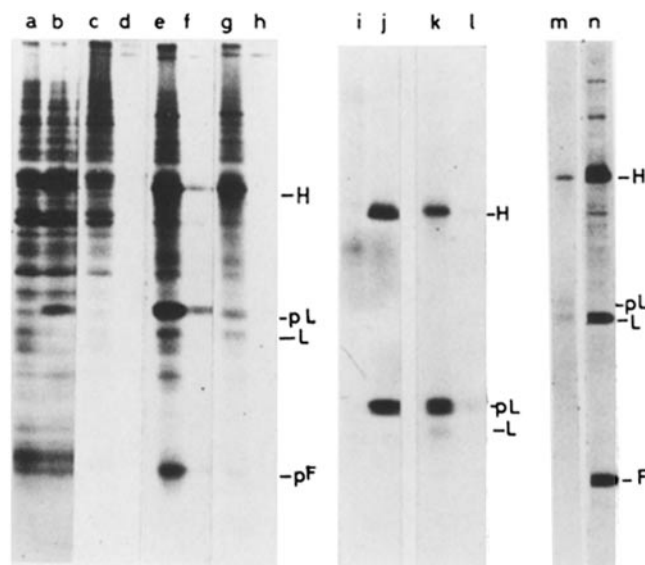

FIGURE 2 MDL-synthesized products F and MB ribosomes and mRNP particles of control and HIB-treated P3K cells. The [^{35}S]methionine-labeled polypeptides synthesized in an MDL by F and MB ribosomal and mRNP particles of control and HIB-treated cells were analyzed by SDS polyacrylamide gel electrophoresis. These fluorograms of three different electrophoretic runs show the products of the translation of F ribosomes and F mRNP particles of (a) control cells, (b) HIB-treated cells, (c) control cells in the presence of edeine, (d) HIB-treated cells in the presence of edeine; the products of the translation of MB ribosomes of (e) control cells, (f) HIB-treated cells, (g) control cells in the presence of edeine (h) HIB-treated cells in the presence of edeine; and the immunoprecipitated Ig chains synthesized by F ribosomes and F mRNP particles of (i) control cells, (j) HIB-treated cells and by MB ribosomes of (k) control cells, (l) HIB-treated cells, and (m) control cells in the presence of edeine. Slot n refers to [^{35}S]methionine-labeled polypeptides that have been synthesized and secreted by P3K cells.

FREE   MEMBRANE
(a)   (b)   (c)   (d)
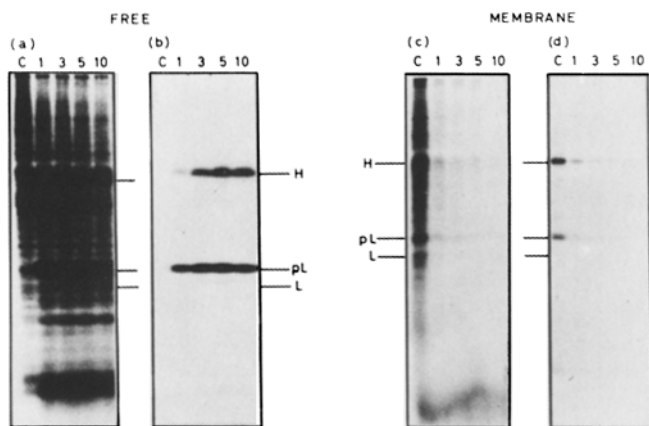C 1 3 5 10   C 1 3 5 10   C 1 3 5 10   C 1 3 5 10

FIGURE 3   Effect of puromycin on the subcellular distribution of Ig mRNA in P3K cells. Analysis by SDS polyacrylamide gel electrophoresis and fluorography of the MDL-synthesized products of (a) F and (c) MB ribosomes of control (C) and puromycin-treated cells for 1, 3, 5, and 10 min. Ig chains synthesized by (b) F and (d) MB ribosomes were immunoprecipitated and analyzed on 17.5% polyacrylamide slab gel containing SDS.

ecules associated with polysomes and those few species enriched in mRNP particles during recovery from HIB are distributed in the same fashion as in normal cells. Thus the same translational selectivity operates during the recovery from HIB as in untreated cells. Furthermore, the direct relationship observed in normal cells between the polysomal size and the size of the polypeptide products (16) applies also to the mRNA distribution in 10-min recovering polysomes, with the notable exception of Ig L mRNA which was essentially associated with relatively small polysomes (80S ribosomes and dimers). This last observation indicates that during recovery from HIB the rate of either initiation or polypeptide elongation is different for Ig L mRNA than for the average mRNA molecules of P3K cells.

Formation of MB Ig-synthesizing Polysomes

The formation of Ig-synthesizing polysomes was studied by analyzing the Ig synthesis in an MDL directed by F and detergent-liberated MB ribosomal particles of HIB-recovering cells. As indicated above, the use of edeine permits one to distinguish the mRNA sequences incorporated into polysomes (including 80S ribosomes actively engaged in protein synthesis) from those present in mRNP particles. In these experiments, the polypeptides were labeled with [^{35}S]cysteine because Ig H and Ig L contain more cysteine residues (13 and 5, respectively) more regularly spaced than methionine residues (9 and 4, respectively) (23, 33). The use of specific anti-Ig antiserum allows the identification and quantitation of the cell-free synthesized Ig polypeptide and thus permits the determination of the relative content of Ig mRNA in polysomes (18, 16). This strategy was used to follow the subcellular distribution of Ig mRNA when polysomes build up during the recovery from HIB.

Fig. 6 a and b show the results of two such experiments in which HIB-treated cells were returned to isotonic medium for various periods of time (0.5–10 min). These experiments show that, during recovery from HIB, Ig mRNA molecules rapidly disappeared from the F ribosomal fraction to become gradually bound to the ER, as indicated by the decreasing amounts of Ig polypeptides synthesized without edeine by F ribosomal par-

ticles and the correspondingly increasing amounts of Ig made by detergent-liberated MB ribosomal particles. The complete attachment of all Ig mRNA molecules to the ER requires ~10 min. Furthermore, during the very first minutes of recovery, some Ig H mRNA molecules were incorporated into F polysomes because Ig H polypeptides were completed in the edeine-treated MDL by F ribosomes. These Ig H-synthesizing F polysomes disappeared very rapidly and were only clearly observed during the first 1.5 min of recovery, after which they were present in an amount comparable to that found in control cells, as indicated by a faint band of labeled Ig H polypeptides on the fluorograms. During this latter period, increasing amounts of Ig H-synthesizing polysomes were found on the ER. The case of the Ig L mRNA deserves mention because there was almost no detectable Ig pL polypeptides completed in the MDL by the F polysomes at the beginning of the recovery. However, the less intense labeling the Ig pL or L
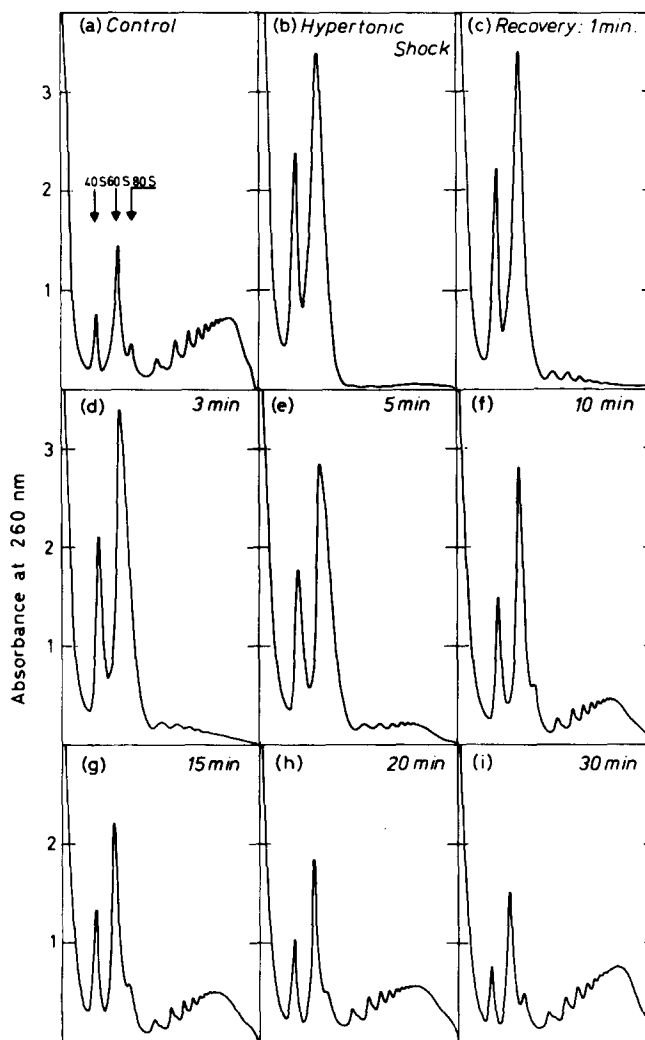

FIGURE 4   Formation of polysomes during the recovery from HIB. Cytoplasmic extracts of 5 × 10^7 control cells (a), HIB-treated cells (b), and cells recovering from HIB for various periods of time: 1 min (c), 3 min (d), 5 min (e), 10 min (f), 15 min (g), 20 min (h), and 30 min (i). Extracts were prepared by detergent lysis of cells, followed by a centrifugation to remove nuclei, and were sedimented on 15–55% linear sucrose density gradients made in TK$_{300}$M (50 mM Tris-HCl, pH 7.5, 300 mM KCl, and 5 mM MgCl$_2$) (12) in a Spinco rotor SW 27 for 8.5 h at 23,000 rpm at 4°C. Sedimentation is from left to right. (—) optical density at 260 nm.
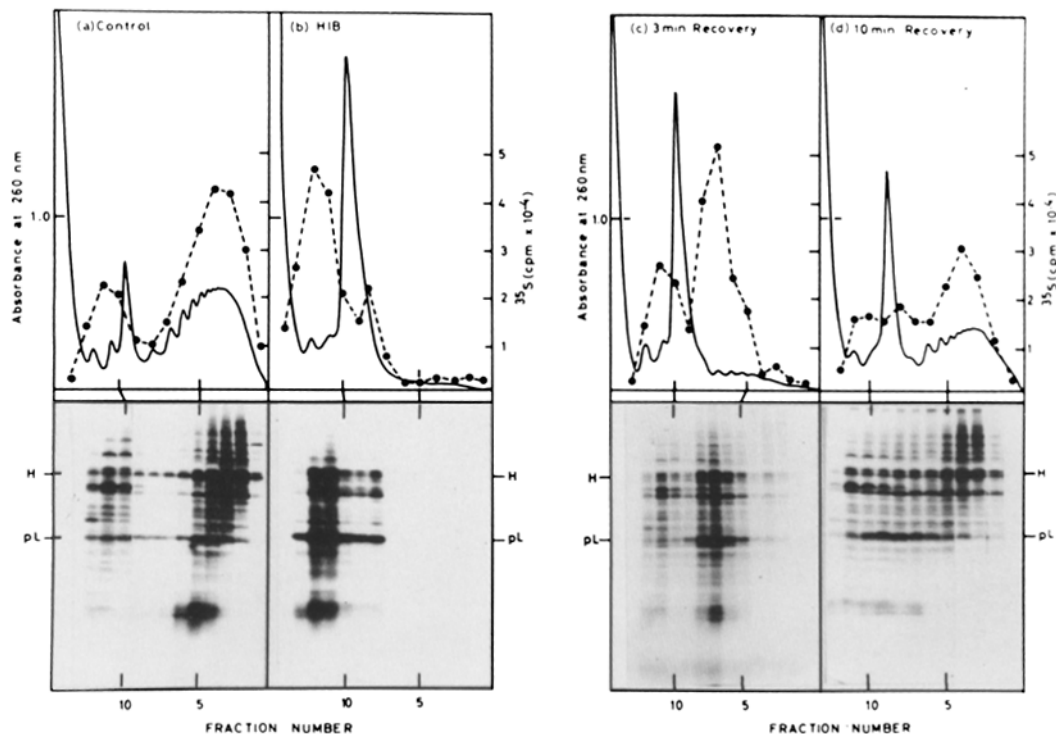
FIGURE 5 mRNA distribution in cytoplasmic extracts of control, HIB-treated, and HIB-recovering cells. Cytoplasmic extracts of 2 × 10^7 control cells (a), HIB-treated cells (b), and cells recovering from HIB for 3 min (c) and 10 min (d) were sedimented on 15–55% linear sucrose density gradients made up in TK80M in a Spinco rotor SW 27 for 8.5 h at 23,000 rpm at 4°C. The RNA from each individual fraction of the gradients was extracted with phenol-chloroform and 50% of the RNA of each fraction was used to direct protein synthesis in 10 μl MDL containing [35S]methionine. After incubation the [35S]methionine incorporation was measured in a 1-μl aliquot of each in vitro reaction and the remainder analyzed in a separate track of a 17.5% polyacrylamide slab gel containing SDS. Sedimentation is from left to right. (—) optical density at 260 nm; (●- - -●) cpm (35S).

polypeptides (which contain five cysteine residues (33) whereas Ig H chain possesses 13 cysteine residues [23]) and the relatively low ribosome density of Ig L mRNA during the recovery from HIB (see Fig. 5) are such that the amount of Ig pL polypeptides completed by F polysomes could be too small to be detected under the present experimental conditions. Therefore, the kinetic analysis (vide infra) will deal only with Ig H mRNA.

The subcellular distribution of Ig H mRNA was further quantitated by measuring the Ig H polypeptides that have been synthesized by the various F and MB ribosomal particles. As shown in Fig. 6c, 0.5 min after return to isotonicity, 15–20% of Ig H mRNA was incorporated into F polysomes and almost none into MB polysomes. However, after 1 min of recovery, the proportion of Ig H mRNA was already greater in MB than in F polysomes and, as the amount of Ig H mRNA increased in MB polysomes, it decreased in F polysomes to reach, after 2 min, a level comparable to the amount of F polysomal Ig H mRNA present in control cells. This suggests that the return to isotonicity triggered the entry of a large fraction of Ig H mRNA into F polysomes which subsequently reattached to the ER.

To confirm that the translation of all Ig H mRNA molecules occurred first in the soluble compartment, the kinetics of entry of Ig H mRNA into MB polysomes were compared to the kinetics of entry into total cytoplasmic polysomes (which were obtained by summation of the contents of Ig H polypeptides completed by F and MB polysomes). This comparison (Fig. 6d) shows that the time of passage of Ig H mRNA into F polysomes took ~30 s.

If the growth of the Ig H polypeptide is linear with time, then the approximate length of the nascent chain at the time of

its association with the ER can be determined. The rate of polypeptide elongation was measured in control and HIB-recovering P3K cells by the method of Fan and Penman (7). As indicated in Table I, elongation proceeded at similar rates in control and HIB-recovering cells: 160 amino acids polymerized per minute. Thus, during the 30 s necessary for the passage of the Ig H mRNA into the F polysomes, a nascent chain of ~80 amino acids would have been completed.

Effects of Inhibitors of Protein Synthesis on the Entry of Ig mRNA into Polysomes

The above kinetic analysis indicates that Ig H mRNA was incorporated into F polysomes before its association with the ER. However, no such transitory intermediates of MB polysomes could be found in the case of Ig H mRNA. As mentioned above, Ig L-synthesizing F polysomes might not be detected under the present experimental conditions. If the mechanism of MB polysome formation were similar for all mRNA species that are translated on the ER, a block of the elongation of protein synthesis during HIB recovery should increase the proportion of such F transitory intermediates for both Ig H and L mRNAs.

To test this prediction, HIB-treated cells were exposed to 10^-3 M emetine (8), 5 min before their return to isotonicity, and were further incubated for various recovery periods of time (1–10 min) in the presence of the drug. Emetine inhibition was found to be reversible in the sense that purified polysomes from emetine-treated P3K cells directed protein synthesis in the MDL with the same efficiency as polysomes from untreated cells (unpublished results). It is thus possible to analyze the Ig
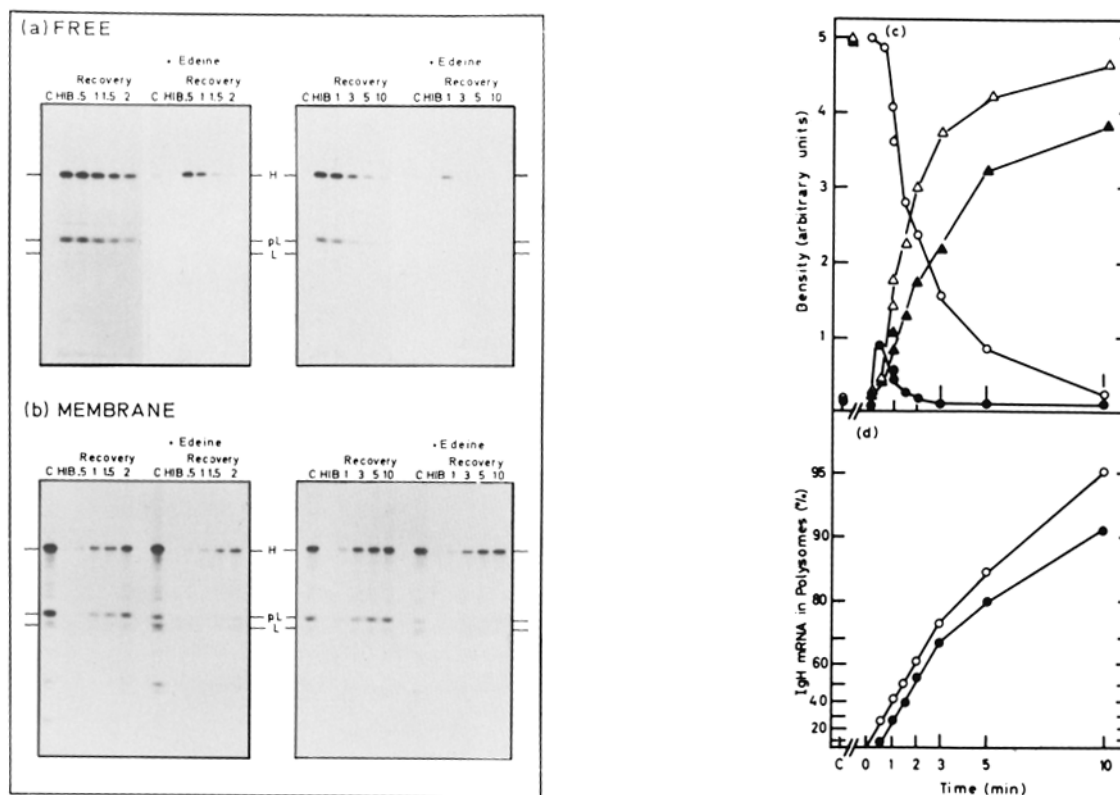
FIGURE 6   Entry of Ig mRNA in F and MB polysomes. (a and b) Subcellular distribution of Ig mRNAs in F and MB polysomes and mRNPs during the recovery from HIB. Analysis by SDS polyacrylamide gel electrophoresis and fluorography of the immunoprecipitated Ig H and L (pL + L) chains synthesized in MDL and labeled with [35S]cysteine by: F polysomes and F mRNPs (a, no edeine), detergent-liberated MB polysomes (b, no edeine), or completed by: F polysomes (a, + edeine), detergent-liberated MB (b, + edeine) of control cells (C), HIB-treated cells (HIB), and cells recovering from HIB for 0.5, 1, 1.5, 2, 3, 5, and 10 min. (c) Time-course of the intracellular redistribution of Ig H mRNA during the recovery from HIB. Fluorograms shown in a and b were scanned using a scanning densitometer (Joyce, Loebl & Co., Gateshead-on-Tyne, England). Ig H chain peaks from the densitometer traces were excised and weighed to allow estimation of the different amounts of MDL-synthesized Ig H products. Ig H chains synthesized in MDL without edeine by F polysomes and F mRNPs. (O) or MB polysomes (△). Ig H chains completed in MDL in presence of edeine by F polysomes (●) or MB polysomes (▲). (d) Kinetics of entry of Ig H mRNA into total cellular polysomes (O), obtained by summation of the curves of Ig H chains completed by F and MB polysomes as indicated in (c), and into MB polysomes (●).

TABLE I

Rate of Polypeptide Elongation

Average mol wt of P3K polypeptides*	Assembly time	Elongation rate
	min‡	amino acids/min§
53,000	3	160

* Estimated from the densitometric scans of fluorograms of proteins extracted from [35S]methionine pulse-labeled P3K cells and separated by SDS polyacrylamide gel electrophoresis. Comparison with reference polypeptides allowed a calculation of the average molecular weight of P3K polypeptides assuming that during the course of the experiment newly made proteins were not degraded nor exported out of the cells.

‡ Determined according to Fan and Penman (7). The assembly time was estimated in control and HIB-recovering cells by measuring the horizontal displacement between the curves of total isotope incorporation (nascent polypeptides plus released proteins) and incorporation into released proteins. At steady state, the time difference between the two curves equals one-half the assembly time. In this measure, the assembly time includes the time required for termination which appears virtually negligible (14). Determination for HIB-recovering cells was made after sufficient time to allow full recovery in the establishment of steady state.

§ Assuming that the average molecular weight of amino acids in animal cells is 110.

mRNA entry into polysomes by the same experimental approach used previously.

The results shown in Fig. 7 indicate that emetine did not completely prevent the entry of Ig mRNA into MB polysomes

but considerably increased the proportion of Ig mRNA in F polysomes. Indeed, in the presence of emetine, polysomal Ig L mRNA could be detected in the F ribosomal fraction after 5 and 10 min of recovery, although the completed pL polypeptides were weakly labeled. In comparison to the normal recovery (without emetine), the uptake of Ig H and L mRNAs into F and MB polysomes was slower in the presence of emetine; this indicates that the drug had affected some step in the initiation of protein synthesis, probably as a secondary consequence of the inhibition of elongation.

To determine the extent of mRNA entry into polysomes, cytoplasmic extracts of 3- and 10-min emetine-treated HIB-recovering cells were sedimented on sucrose density gradients, and the RNA extracted from each individual fraction of such gradients was translated in MDL. Analysis of the mRNA template activity across the gradients and examination of the translation products (Fig. 8) reveal that, indeed, mRNA entered polysomes at a slower rate in presence of emetine (compare Fig. 8a and Fig. 5c) and accumulated into small polysomes (one to three ribosomes).

The results from the experiment using emetine further support the contention that the events leading to the formation of Ig-synthesizing MB polysomes involve the obligatory passage of Ig mRNA via the F polysomes. To further demonstrate that

the membrane attachment of Ig-synthesizing polysomes requires the presence of a nascent polypeptide chain, HIB-recovering cells were exposed to 4.5×10^{-4} M puromycin, which causes premature termination of the growing polypeptide chain (2, 26). As shown in Fig. 9, puromycin allowed the entry of some Ig H and L mRNAs into the F polysomes, as indicated by the completion of small amounts of Ig H and pL polypeptides by F ribosomal particles extracted from 5- and 10-min
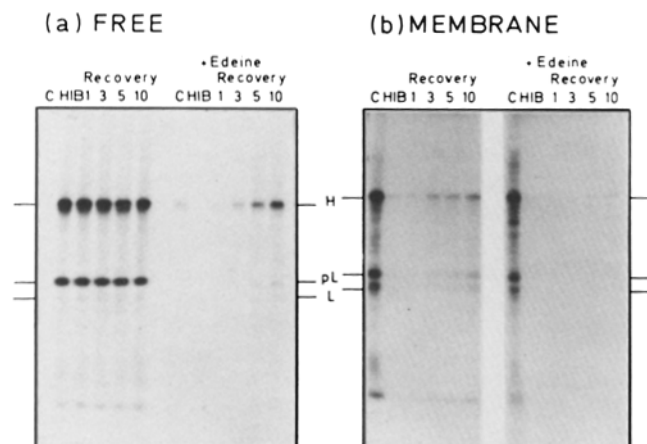
FIGURE 7   Effect of emetine on the subcellular distribution of Ig mRNAs during recovery from HIB. Analysis by SDS polyacrylamide gel electrophoresis and fluorography of the immunoprecipitated Ig chains synthesized in MDL and labeled with [^{35}S]cysteine by: F ribosomal and mRNP particles (a) or detergent-liberated MB ribosomal particles (b) of control cells (C), HIB-treated cells (HIB), and cells recovering from HIB in presence of 10^{-3} M emetine for 1, 3, 5, and 10 min.
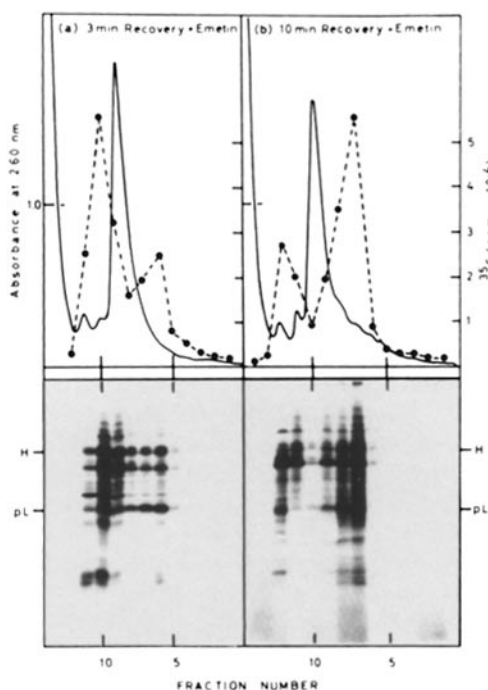
FIGURE 8   Effect of emetine on the mRNA distribution in cytoplasmic extracts of HIB-recovering cells. The mRNA distributions in cytoplasmic extracts of HIB-recovering cells in presence of 10^{-3} M emetine for 3 and 10 min were analyzed as indicated in Fig. 2. Sedimentation is from left to right. (—) optical density at 260 nm; (●- - -●) cpm (^{35}S).
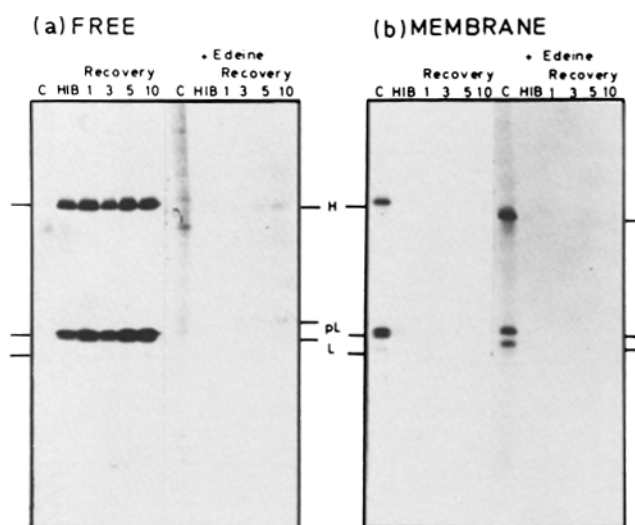
FIGURE 9   Effect of puromycin on the subcellular distribution of Ig mRNAs during recovery from HIB. Analysis by SDS polyacrylamide gel electrophoresis and fluorography of the immunoprecipitated Ig chains synthesized in MDL and labeled with [^{35}S]cysteine by: F ribosomal mRNP particles (a) or detergent-liberated MB-ribosomal particles (b) of control cells (C), HIB-treated cells (HIB), and cells recovering from HIB in presence of 4.5×10^{-4} M puromycin for 1, 3, 5, and 10 min.

HIB-recovering cells, but completely prevented their attachment to the ER. Because of the complex relationship of ribosomes and mRNA in presence of puromycin—mRNA might be loaded by several ribosomes that do not necessarily carry a nascent polypeptide chain—the amount of Ig H and pL polypeptides completed in the MDL certainly does not reflect the actual content of Ig mRNA associated with the F polysomes. However, the results of this experiment confirm the essential role of the nascent polypeptide chain in the attachment of Ig-synthesizing F polysomes to the ER.

DISCUSSION

This report describes the fate of mRNA after in vivo polysome disruption and reconstitution, and examines in particular the subcellular location of the mRNA coding for Ig. The mRNA subcellular distribution was determined by analyzing the in vitro translation products directed either by polysomes and mRNP particles or by the mRNA extracted from these structures. On the one hand, the analysis of the products synthesized by mRNA extracted from ribosomal and mRNP particles that have been sedimented in sucrose density gradients allows the size determination of the mRNA-containing structures. On the other hand, the different translation properties of polysomes and mRNP particles with respect to the inhibition of initiation of protein synthesis in the MDL provides a useful tool to discriminate readily those mRNA sequence engaged in polysomes from all other mRNA sequences. These two approaches were used to characterize the mRNA-containing structures of HIB-treated cells. The sedimentation analysis suggested that the HIB-released mRNA structures were mRNP particles, and, indeed, the translation properties of the intact particles confirmed that most of the cytoplasmic mRNA molecules were in ribosome-free structures.

These results also show that the mRNPs are not an untranslatable form of mRNA, as has been suggested in the case of duck reticulocyte mRNP particles (5), as these mRNP

particles prove to be translatable in vitro to the same extent as mRNAs incorporated into polysomes. Furthermore, the template activity of the HIB-treated mRNPs is comparable to that of deproteinized polysomal mRNA isolated from cells grown under normal conditions (results not shown).

Unlike other inhibitors of protein synthesis, HIB induces the release of polysomal mRNA in the form of mRNP particles. Work of other investigators using pactamycin and muconomycin A to examine the fate of vesicular stomatitis viral mRNAs during inhibition of initiation of protein synthesis indicates that these messages remain associated with ribosomes (9, 13).

When P3K cells are exposed to an excess of 0.2 M NaCl in the growth medium, initiation of the synthesis of new chains is blocked, but elongation and termination of nascent polypeptides proceed normally. This leads to a rapid disappearance of polysomes which is accompanied by the release of all F and MB polysomal mRNAs as F mRNP particles. The results presented in this paper indicate that the previously translated mRNA is retained in a form that allows a rapid resumption of its activity when the cells return to normal growth medium. Thus, the recovery from HIB provides an adequate experimental system (*a*) for the study of mRNA entry into polysomes and (*b*) for the analysis of the events leading to the formation of MB polysomes.

Release of MB Polysomal mRNA

The release of the MB Ig induced by an HIB indicates that the binding of Ig mRNA molecules to ER membranes is only mediated by the nascent polypeptide chains of the Ig-synthesizing polysomes. In no case was it possible to detect direct mRNA binding to the ER. Whether protein synthesis proceeds normally (16), or is inhibited at the level of initiation, nonfunctional Ig mRNA molecules were only found free in the cytoplasm. No significant amount of Ig mRNP particles can be detected on the ER membranes, either in normal cells (16) or in HIB-treated cells. The presence of a small amount of mRNP particles on the membranes of HIB-treated cells reflects simply a contamination of the membrane fraction by F mRNP particles, by far the most abundant mRNA-containing structures in the cytoplasm of these cells. Thus, the absence of any membrane accumulation of Ig mRNP particles after HIB indicates that Ig mRNA does not interact directly with the ER.

The hypothesis that the nascent polypeptide chain mediates the membrane attachment of Ig-synthesizing polysomes is further strengthened by the fact that in vivo puromycin treatment induced the release of Ig mRNA from the ER. A similar result has previously been observed in the case of vesicular stomatitis virus G protein mRNA by Lodish and Froshauer (13), as well as by Grubman et al. (9) using pactamycin as an inhibitor of protein synthesis initiation. In addition, the observation that Ig L mRNA is released by puromycin from the ER membranes faster than Ig H mRNA indicates that the membrane binding of Ig-synthesizing polysomes depends uniquely on the number of nascent chains simultaneously synthesized from Ig mRNA template: on the average, Ig L mRNA containing polysomes carry six ribosomes, while Ig H polysomes carry 12 ribosomes (16).

The finding of Lenk et al. (12) which shows that most cytoplasmic mRNA in HeLa cells is associated with a skeletal-like structure may explain previous results suggesting a direct linkage of mRNA to the ER membranes (1, 22, 11, 20, 21) which are contradictory to those presented here. In particular,

during cell fractionation, fragments of the cytoskeleton can be recovered in the membrane fraction, accounting for the presence of mRNA sequences encoding soluble proteins on microsomes, as shown in the case of tubulin mRNA (6). The finding of MB mRNP particles not containing genuine MB mRNA sequences in HIB-treated cells may well be an example of adventitious binding of mRNA molecules to microsomes through such cytoskeleton remnants.

mRNA Entry into Polysomes

HIB-released mRNAs are found as mRNP particles that do not seem to be bound to native 40S ribosomal subunits. These structures closely resemble those present in untreated P3K cells, although their content in mRNA sequences appears to be different (16). mRNA of HIB-released mRNPs contains the entire set of previously translated and untranslated sequences, whereas mRNA present in normally occurring mRNPs seems to be enriched in specific sequences. In recovery from HIB, this class of mRNAs resists incorporation into polysomes. This translational selectivity suggests that at least a large fraction of the extrapolysomal mRNA in cells actively engaged in protein synthesis is not precursor to polysomal mRNA. However, the demonstration that HIB-released mRNPs are readily incorporated into polysomes upon return to isotonicity indicates that the recovery process might mimic the normal uptake of newly synthesized mRNA into polysomes.

Protein synthesis and polysome formation resume at once during recovery. There is no visible accumulation of mRNA-80S ribosomes as had been observed by Schochetman and Perry (34) when heat-shocked cells were returned to normal temperature. It is possible that the hypertonic treatment might affect aspects of initiation different than those modified by heat shock.

All previously translated mRNA molecules become rapidly incorporated into polysomes, while the entry of ribosomes is more gradual. mRNA uptake into polysomes is almost achieved in 10 min, whereas complete ribosome incorporation requires ~30 min. The analysis of the polysomal mRNA distribution during recovery from HIB suggests that the entry of most of the previously translated mRNAs proceeds at a similar rate, with the exception of Ig L mRNA. After 10 min of recovery, most of the mRNA is distributed in polysomes according to size, although these polysomes are still incompletely loaded with ribosomes. By contrast, Ig L mRNA contains many fewer ribosomes compared with steady-state conditions during which it has the same ribosome density as other messages (16). This lower ribosome density on Ig L mRNA might result from either a slower rate of initiation or a greater rate of elongation. I have not distinguished between these two possibilities.

Formation of MB Polysomes

During the recovery from the HIB, the formation of Ig-synthesizing polysomes evidently proceeds in the sequence: F mRNP → F polysomes → MB polysomes. This conclusion is supported by (*a*) the observation of a sudden burst of Ig H-synthesizing F polysomes; (*b*) the comparison of the kinetics of Ig H mRNA entry into total cellular polysomes and into MB polysomes; and (*c*) the depletion of F mRNPs and the accumulation of small F polysomes containing both Ig H and L mRNAs when the rate of elongation is depressed by emetine. A similar sequence of events has been deduced for the entry of

vesicular stomatitis virus G protein into MB polysomes by Grubman et al. (9).

These findings support a general model of the formation of MB polysomes in which the specificity of membrane attachment is determined by the nascent polypeptide chain (24, 3). According to this model, translation of mRNA coding for proteins intended for secretion begins in the F ribosomal fraction by the joining of this mRNA to ribosomes. These F polysomes (formed of one or more ribosomes) then synthesize nascent polypeptide chains whose N-terminal amino acid sequence serves as the signal for recognition by the membrane. Polysomes containing a suitable protruding "signal sequence" become attached to the ER and the growing polypeptide chains are transferred into and across the membrane, presumably through a protein channel. Therefore, the formation of MB polysomes is determined by the affinity of the nascent polypeptide chain for the ER rather than by a specific primary sequence of mRNA interacting directly with the membranes. Once on the ER, polysomes remain attached to the membrane by the successive reinsertion of nascent chains synthesized by the numerous ribosomes that translate simultaneously each mRNA molecule.

The length of the nascent polypeptide chain at the time of membrane attachment of polysomes was estimated by indirect measurements of the rate of polypeptide elongation in normal and HIB-recovering cells. Although the error of this determination may be large, I calculate that the membrane attachment occurs when the nascent chain is ~80 amino acids long. This result is consistent with that obtained by Rothman and Lodish (30) on in vitro membrane insertion of vesicular stomatitis virus G protein. Among these 80 amino acids, 40 are shielded by the large ribosomal subunit (15, 4), so that ~40 amino acids would be available to interact with the membranes. On the basis of the average ribosome density on mRNA molecules in normal cells (16), approximately two ribosomes should be loaded on newly formed polysomes at a time when the longest nascent chain is 80 amino acids long. Thus one can estimate that during HIB-recovery, Ig H synthesizing polysomes should contain about two ribosomes when they become attached to the membranes. Although this number may be an overestimation, it is consistent with the number of ribosomes found in polysomes after 1 min of recovery.

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