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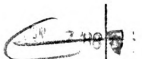

IMMUNOGLOBULIN SYNTHESIS IN P3 MYELOMA CELLS: DEFINING THE ROLE OF FREE POLYSOMES

By

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ABSTRACT

IMMUNOGLOBULIN SYNTHESIS IN P3 MYELOMA CELLS:
DEFINING THE ROLE OF FREE POLYSOMES

By

Paul Jeffrey Freidlin

Polyribosomes from P3 myeloma cells were fractionated into free, membrane-bound, and detached (formerly membrane-bound) populations. Free polysomes produced H chain and only poorly resolved precursor or authentic L chain. Membrane-bound polysomes produced H chain and authentic L chain. Detached polysomes synthesized H chain and precursor L chain with no detectable authentic L chain. Only polypeptides synthesized by membrane-bound polysomes were resistant to proteolysis. These results, including the production of Ig polypeptide by free polysomes, can be explained by the signal hypothesis for the synthesis of secretory proteins.

P3 myeloma cells were pulsed with [^{35}S]methionine in single or double label experiments. The molar ratio of intracellular L/H chain did not suggest an initial excess of L chains. The ratio of newly made to long-term labeled intracellular chains indicated that degradation did not greatly exceed synthesis for either chain. These results suggest that free polysomes did not produce Ig polypeptides that were degraded rapidly intracellularly. Additional experiments indicated that free polysomes did not produce Ig

polypeptides which accumulated intracellularly in lieu of their secretion or turnover on cell surface membrane. The metabolism of total protein (and probably Ig polypeptides) may vary greatly, depending on cell density.

Our data suggest that free polysomes do not produce Ig polypeptides that are accumulated or degraded in myeloma cells. Thus the possibility remains that free polysomes which contain Ig mRNA, if not contaminating detached polysomes, may be intermediates destined for RER, may provide cell surface Ig, or both.

We also investigated the effects of heparin on free and membrane-bound polysomes. Our observations may help resolve certain contradictory claims which concern the proposed binding of mRNA to membrane.

Free and membrane-bound polysome fractions were incubated with 1.0 mg/ml heparin, and the resulting polysome profiles were displayed on sucrose-RSB gradients. The major effects of heparin on free polysomes included a reduction in the size of large polysomes or aggregates, and enhanced resolution of ribosomal subunits, monosomes, and polysomes. Incubation of membrane-bound polysomes with heparin caused the release of material which migrated in the polysome, monosome, and subunit regions of the gradient. The released material corresponded to approximately one-half that which could be released in the presence of 1.0 mg/ml heparin plus a final concentration of 1.0% v/v Triton X-100. The action of heparin appeared to be related to its polyanionic nature.

DEDICATION

This dissertation is dedicated to my parents, Julius and Anna Freidlin, to my brothers, Mark and Aaron, to those others who cared, and to dedicated, responsible people everywhere.

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I would like to express my sincere gratitude to Dr. Ronald J. Patterson, my major advisor, for the time and effort which he expended to insure that I would participate in a rewarding, worthwhile doctoral program.

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INTRODUCTION

The current model of secretory protein synthesis and secretion by eukaryotic cells is documented by an extensive literature. A key point of the model is that secretory proteins enter the lumen of the rough (polysome studded) endoplasmic reticulum (RER) at one stage of their biogenesis. A logical corollary is that in order to facilitate their compartmentalization within the RER, secretory proteins are manufactured by membrane-bound polysomes. Indeed, this corollary receives overwhelming support from evidence accumulated since the 1950's. This same evidence, however, strongly suggests that a lesser, yet significant, portion of polysomes which are able to assemble secretory polypeptides are free in the cytoplasm, not membrane-bound. To be consistent with the model, one could hypothesize that these free polysomes are newly formed intermediates destined to become membrane-bound as soon as their nascent polypeptides have reached a certain size. Alternatively, if free polysomes complete secretory polypeptides, one could predict these polypeptides would not be secreted, but instead quickly degraded intracellularly.

The nature of such free polysomes, and the fate of their products, remain unresolved problems. In particular, the literature (with one exception: Scheele et al., 1978) has not been able to exclude the possibility of contaminating membrane-bound polysomes

being responsible for the secretory polypeptides synthesized by putative free polysomes. The contamination could arise in either of two fashions. The putative free fraction could be contaminated with detached polysomes (i.e., formerly membrane-bound polysomes which were separated from the membranes of the RER). Alternatively, the putative free fraction might be contaminated with intact microsomes (membrane-bound polysomes). Recent advances in our knowledge of the mechanism of secretory protein synthesis have made it possible in certain cases to distinguish between polypeptides produced by free and membrane-bound polysomes. Thus it is now possible to determine whether secretory polypeptides made by putative free polysomes originate from contaminating membrane-bound polysomes. This determination, and the possible fate of free polysomal secretory protein nascent chain, are the major topics of my dissertation.

This dissertation is organized into three divisions. The first division consists of a modest review (with emphasis on Ig synthesis) of a vast literature describing research on the molecular biology of the synthesis of proteins secreted by eukaryotic cells. The second division is a manuscript (to be submitted for publication) which describes the IgG-like polypeptides produced by MOPC 21 (P3) free, membrane-bound, and detached polysomes. The manuscript also considers the possible role of free polysomes in Ig synthesis *in vivo*. The final division is a manuscript (to be submitted for publication) describing the effects of heparin on MOPC 21 (P3) free and membrane-bound polysomes.

LITERATURE REVIEW

Isolation of Free and Membrane-Bound Polyribosomes

The isolation and description of free and membrane-bound polysomes have been reviewed extensively (Freidlin, 1976; McIntosh and O'Toole, 1976). Free and membrane-bound polysomes have been separated by exploiting differences in density, sedimentation rate, and surface properties. Each fractionation procedure has its drawbacks, and none can be guaranteed to yield free polysomes which are not contaminated with membrane-bound or detached polysomes. Currently there is no way to test for the presence of detached polysomes in a fraction of free polysomes. Membrane-bound polysomes formerly were detected by assaying for the presence of microsomes or lipid. Electron microscopy was used to detect microsomes morphologically. Chemical analysis of phospholipid and use of radioactive lipid precursors permitted the detection of lipid, although these methods did not allow one to conclude that only microsomal lipids were being detected. Finally, gross contamination of membrane-bound polysomes could be detected by observing the effect of detergent treatment on the polysome profile obtained by velocity centrifugation through a linear sucrose gradient. None of these methods for the detection of membrane-bound polysomes would enable an investigator to determine the origin of secretory polypeptides produced by a fraction of putative free polysomes. Even an extremely small degree of contamination

by membrane-bound polysomes could prevent the assignation of secretory polypeptide synthesis to free polysomes. Alternatively, the presence of an occasional microsome or barely detectable amount of lipid, which may or may not be microsomal, could unfairly prejudice one towards disbelief in the truly free polysomal origin of secretory polypeptide.

New advances in our knowledge of the processing of certain secretory proteins allow us to determine whether they were synthesized by membrane-bound polysomes. Many of these advances are encompassed in an experimentally derived model known as the "signal hypothesis." Unfortunately, there is no known method for distinguishing between the products of free and detached (formerly membrane-bound) polysomes, since detached polysomes may or may not produce a product which differs from that synthesized by free polysomes (differing observations have not yet been reconciled: Blobel and Dobberstein, 1975a,b; Scheele et al., 1978; vs. McIntosh et al., 1972; Harrison et al., 1974).

A Model for the Synthesis of Secretory Proteins: The Signal Hypothesis

The signal hypothesis was first proposed with accompanying evidence by Milstein et al. (1972) and later expanded and popularized by Blobel and Dobberstein (1975a,b) (although the origins of the Blobel and Dobberstein work appear to be a suggestion made by Blobel and Sabatini [1971]). Aspects of the signal hypothesis have also been reviewed by Kuehl (1977), Shore and Tata (1977), and McIntosh and O'Toole (1976). The model is constructed of a number of testable postulates. 1) Secretory protein synthesis begins on free polysomes. 2) The first codon in the mRNA specifies N-terminal

methionine. 3) The next 15-40 (the experimental average seems to be about 20) codons specify primarily (approximately 70%) hydrophobic amino acids. 4) The N-terminal stretch of hydrophobic amino acids acts as a "signal" on nascent polypeptide to direct the newly formed polysome to rough endoplasmic reticulum. 5) Following insertion of the signal sequence into membrane, two or more membrane proteins are recruited to the immediate vicinity of the nascent chain. These membrane proteins could bind the large ribosomal subunit to the RER, prevent the newly inserted nascent chain from slipping out of the membrane, provide a tunnel through the membrane for the less hydrophobic remainder of the polypeptide, or process (truncate by removing the signal sequence) the nascent chain. 6) The processed nascent chain is completed and then vectorially discharged into the cisternae of the RER for the start of post-translational modifications such as glycosylation (depending, of course, on the particular protein), and eventual secretion from the cell.

It should be noted that until this dissertation, only one work (Scheele et al., 1978) had been published containing direct evidence in support of the ability of free polysomes to synthesize secretory protein. Others had observed secretory protein synthesis by putative free polysomes, but attributed (without experimental proof) this synthesis to contaminating membrane-bound polysomes. As has been previously mentioned in this review, a simple observation of secretory protein synthesis is not satisfactory--one must establish that the protein did not come from contaminating membrane-bound polysomes.

The need to confirm the potential for secretory protein synthesis by free polysomes has taken on added importance with the

presentation of an alternative to that part of the signal hypothesis which predicts that all protein synthesis will begin on free polysomes. The alternate hypothesis (discussed by Kruppa and Sabatini, 1977) is that the 3'poly (A) end of mRNA is bound to RER. This affinity for membrane is not predicted by the signal hypothesis, and it does suggest another means by which polysomes may be directed to RER. Based on the detection of rapidly turning over newly made 40S subunits attached to newly made mRNA on RER, Mechler and Vassali (1975a,b) suggested that a polysome's initial contact with membrane was thorugh the 3' end of an mRNA-initiation complex. That is, free polysomes would not be expected to produce secretory polypeptide, and the signal region would lose a portion of its physiological significance. Using several procedures to disassemble polysomes *in vivo* (Shiokawa and Pogo, 1974; Adesnik et al., 1976; Van Venrooj et al., 1975) or *in vitro* (Milcarek and Penman, 1974; Lande et al., 1975; Cardelli et al., 1976), other investigators demonstrated the direct association of mRNA with RER. When tested, this association was found to involve a 3'poly (A) region-membrane connection (Milcarek and Penman, 1974; Lande et al., 1975; Cardelli et al., 1976). Heterogeneous nuclear RNA is reportedly bound through its poly (A) terminus to protein fibers in HeLa cell nuclei (Herman et al., 1978). Messenger RNA appears to be associated with a cytoplasmic cytoskeletal HeLa cell structure (Lenk et al., 1977). Thus all mRNA may be bound to some cellular structure, this having escaped notice due to the relatively harsh fractionation procedures used to obtain subcellular fractions.

Kruppa and Sabatini (1977) dispute the results of Cardelli et al. (1976) which indicate an mRNA-membrane attachment. Both groups

worked with rat liver microsomes, but Kruppa and Sabatini demonstrate in a number of ways using *in vivo* and *in vitro* approaches, that disassembly of polysomes causes extensive release of rat liver mRNA, i.e., rat liver mRNA is not bound to membrane. They feel, however, that the other investigators who worked with cultured cells may have truly observed an mRNA-membrane linkage. One problem is that total mRNA populations were being observed. In cases where specific mRNA could be assayed, there was no evidence for an mRNA-membrane linkage. Harrison et al. (1974) observed that membrane-bound polysome disassembly *in vitro* resulted in the release of immunoglobulin light chain mRNA. Virus probes have also been used to examine the question. Addition of puromycin or inhibitors of initiation *in vivo* caused an accumulation of the VSV transmembrane protein G mRNA in the cytosol, i.e., VSV G mRNA was no longer attached to the membrane (Grubman et al., 1977; Lodish and Froshauer, 1977).

Criteria for Demonstration of Ig mRNA in Free Polysomes

i. Presence of Ig mRNA

In order to claim that free polysomes are able to synthesize secretory polypeptides, it is necessary, but certainly not sufficient, to demonstrate that a subcellular fraction of putative free polysomes contains secretory protein mRNA. Studies which have attempted to establish the subcellular location of mRNA coding for secretory protein have been reviewed comprehensively by Rolleston (1974). These studies also have been reviewed by Freidlin (1976), McIntosh and O'Toole (1976), Shore and Tata (1977), and Kuehl (1977). The

general conclusion is that secretory protein mRNA is predominantly, but not exclusively, found in membrane-bound polysomes.

The presence of secretory protein mRNA has been demonstrated in a variety of ways, which include the use of cDNA probes, analysis of the products (by mobility in SDS-PAGE, immunoprecipitation, and mapping of tryptic peptides) of translation directed by mRNA in initiation or runoff (readout) systems for cell-free protein synthesis, the identification of nascent polypeptide chains, and electron microscopic localization (Kuehl, 1977). Only one group has used a cDNA probe to examine mRNA from free polysomes for the presence of Ig mRNA. Okuyama et al. (1977) found that free polysomes contained approximately one-third of the MOPC-315 light chain mRNA hybridizable to a cDNA probe. This result contrasts with the data gathered by Sonenshein et al. (1978), who could not detect Ig polypeptides synthesized by free polysomal mRNA in an initiation system.

Messenger RNA extracted from Ig or L chain secreting myeloma cell putative free polysomes has been translated in initiation systems for cell-free protein synthesis by other investigators, and Ig polypeptides have been made (Tonegawa and Baldi, 1973; Cowan et al., 1974). The total amount of Ig mRNA found in the free polysomes is still highly controversial. However, there is better agreement on the concentration of free polysomal Ig mRNA: no investigator, using any method, has found the μg Ig mRNA per mg free polysomal mRNA to be greater than about one-third the μg Ig mRNA per mg membrane-bound polysomal mRNA.

Analysis of the products of runoff (readout) cell-free systems also consistently reveals the presence of Ig mRNA in putative free polysomes (Lisowska-Bernstein et al., 1970; Pyrme et al., 1973;

Baglioni and Liberti, 1974; Blobel and Dobberstein, 1975a). Blobel and Dobberstein (1975a) demonstrated that a significant portion of L chains produced in a runoff system probably came from contaminating membrane-bound polysomes. However, they could not rule out the presence of free polysome derived L chain.

Sherr and Uhr (1970) were able to immunoprecipitate nascent Ig polypeptide released from putative free polysomes. Choi et al. (1971) also were able to immunoprecipitate Ig nascent chains from free polysomes, although apparently they did not first release the chains. Pyrme et al. (1973), using ^{125}I-labeled antiserum, were able to detect nascent Ig H chain on free polysomes. Cioli and Lennox (1973) used ion-exchange chromatography to obtain peptidyl-tRNA. They were *not* able to immunoprecipitate nascent Ig chain from a very restricted population of free polysomes (i.e., probably not more than 20% of the total free polysomes).

Of the preceding, only Lisowski-Bernstein et al. (1970), Sherr and Uhr (1970), Baglioni and Liberti (1974), and Okuyama et al. (1977) interpret their results to mean that free polysomes contain Ig mRNA. One major problem is that some investigators, while they would undoubtedly agree that Ig mRNA is less concentrated in free polysomes, refuse to use a correspondingly larger amount of radioactive polypeptides made by free polysomes in order to detect Ig polypeptides. This is an important point, since many researchers are performing experiments at the limits of detectability where it is easy to bias the results.

Finally, it is pertinent to briefly consider the methods used to isolate free polysomes. While differential centrifugation has been used to isolate free polysomes, it is generally conceded that

the sedimentation coefficients of small microsomes overlap the S values of free polysomes too much for this procedure to be useful. Therefore, most investigators choose to exploit the density difference between free and membrane-bound polysomes. Perhaps the best separation procedure is the flotation method (Mechler and Vassalli, 1975a,b), in which total polysomes are mixed with dense sucrose and centrifuged. The membrane-bound polysomes float out of the dense sucrose, while the free polysomes remain or pellet. The major disadvantage of this procedure is that it cannot be used preparatively. The most commonly used density gradient procedure involves layering a postnuclear supernatant over a layer of 2M sucrose (Webb et al., 1964; Bloemendal et al., 1967). After appropriate centrifugation membrane-bound polysomes band at the 2M interface while the free polysomes, being denser, pellet. Unfortunately, some lipid detected chemically or by use of radioactive precursors, such as ^{3}H-choline or ^{3}H-oleic acid, also can pellet through at least 2M sucrose (Bloemendal et al., 1967; Murty and Hallinan, 1969; Caliguiri and Tamm, 1970; Cioli and Lennox, 1973). Blobel and Dobberstein (1975a) also show an electron micrograph of microsomes that pelleted through 2M sucrose. Other investigators, however, examined their free polysome fractions (pelleted through 2M sucrose) by electron microscopy and did not detect any (or many) contaminating microsomes (Bloemendal et al., 1967; Murty and Hallinan, 1969; Sherr and Uhr, 1970; Lisowska-Bernstein et al., 1970). Whether the lipid sedimenting with free polysomes is totally, predominantly, or not at all microsomal lipid is still an unresolved question. It is thus manifestly important to establish by other means that the secretory polypeptide being synthesized, or the secretory mRNA

detected by cDNA probe, is *not* due to the presence of contaminating membrane-bound polysomes.

ii. Protein composition and size: precursor vs. processed polypeptide

The signal hypothesis is supported by evidence gathered by translation of Ig mRNA in a number of initiation systems, e.g., reticulocyte lysate, Kreb's ascites lysate, and wheat germ lysate (reviewed by Kuehl, 1977). In every case examined, the products of translation have contained an additional six to fifty N-terminal predominantly hydrophobic amino acids that are not found in the secreted polypeptide. Methionine is the first amino-terminal residue, but does not occur as the last residue of the precursor sequence. Thus synthesis of the precursor protein is not an artifact of incorrect initiation, since the initiator amino acid in Ig protein biosynthesis is methionine (Milstein et al., 1972; Jones and Mach, 1973).

The signal hypothesis predicts that if protein synthesis is allowed to occur in the presence of rough endoplasmic reticulum membrane, the signal sequence will lead the nascent polypeptide (and polysome) to the membrane where the signal sequence will be removed by a membrane-bound enzymatic activity before completion of the nascent chain (Blobel and Dobberstein, 1975a,b). Key points of the prediction that are validated in initiation and runoff systems are 1) removal of the signal sequence can only be performed by RER derived membrane (though it need not originate from the same species as the mRNA, polysomes or ribosomes) or RER membrane extract, not smooth ER or any other cellular membrane or membrane extract (Jackson and Blobel, 1977; Warren and Dobberstein, 1978), and 2) RER membrane

must be present during protein synthesis (co-translational), that is, if membranes are added after termination of protein synthesis, the precursor polypeptide will not be processed into the smaller authentic polypeptide (Brownlee et al., 1972; Swan et al., 1972; Blobel and Dobberstein, 1975a,b; Boime et al., 1977; Birken et al., 1977; Lingappa et al., 1977; Shields and Blobel, 1977). The required presence of RER membrane during protein synthesis indicates that completed precursor chain cannot assume the proper configuration for insertion into membrane, and the enzymatic activity that removes the signal sequence is not located on the cytoplasmic surface of the membrane. Initial attempts to solubilize the processing activity failed (Blobel and Dobberstein, 1975b). Later attempts resulted in the successful isolation of a soluble sodium deoxycholate (0.5%) microsomal extract which was able to accurately remove the signal sequence from precursor proteins during, or *after*, protein synthesis (Jackson and Blobel, 1977). In possible contrast, the presence of low concentrations of Triton X-100 has been shown to inhibit the enzymatic cleavage activity (i.e., only precursor protein was synthesized) of microsomal membranes (Boime et al., 1977). The solubilization and inhibition observations have not yet been reconciled. Possible causes of the different results are 1) the different properties of Triton X-100, a nonionic detergent, and sodium deoxycholate, an anionic detergent, 2) the different concentrations of extract in each system, or 3) the different incubation temperatures--the microsomal extract cleavage activity is rapidly inactivated at 37°C, but not 25°C (Jackson and Blobel, 1977). Boime et al. (1977) incubated their cell free system at 30°C.

Another major expectation is that detached polysomes obtained from detergent-treated RER should synthesize both precursor and authentic polypeptides, the longer nascent chains already having been processed. This has been found in two cases (Blobel and Dobberstein, 1975a,b; Scheele et al., 1978), while other investigators have observed that detached polysomes only synthesize detectable precursor polypeptides (Milstein et al., 1972; Harrison et al., 1974).

In order to detect precursor polypeptides, most studies exploit the different mobilities of precursor and processed polypeptides on SDS-PAGE. The situation is therefore complicated when glycosylated polypeptides, such as Ig H chain, are examined. The loss of the signal sequence is more than offset by the glycosylation, i.e., precursor H chain migrates as if it were the same size or smaller than glycosylated authentic H polypeptide (Milstein et al., 1972; for review see Kuehl, 1977). The precursor H chain polypeptide has been shown to contain additional amino acids (Cowan and Milstein, 1973) which in at least one case precede the N-terminus of the secreted H chain (Jilka and Pestka, 1977). No one has yet compared the mobilities in SDS-PAGE of nonglycosylated H chain (e.g., Melchers, 1973) and precursor H chain.

Evidence obtained *in vivo* is consistent with processing of secretory nascent chain. No matter how short the pulse of radioactive amino acids, no precursor polypeptide can be detected in cells (Schmeckpeper et al., 1975; Kemper et al., 1976; Sussman et al., 1977). The addition of proteolytic inhibitors, however, allows certain cell lines to accumulate detectable precursor polypeptides (Schmeckpeper et al., 1975; Sussman et al., 1976).

The signal hypothesis (Blobel and Dobberstein, 1975a) also predicts that a point mutation might alter the amino acid sequence of the signal peptide in a manner which would prevent its cleavage by membrane enzymes. Nevertheless, the mutated signal peptide would still functionally interact with RER membrane. Ovalbumin is the only secretory protein found to date which may have such an altered signal peptide. The ovalbumin polypeptide manufactured in an initiation system for cell-free protein synthesis has the same N-terminus (after cleavage of the terminal methionine) as the authentic secreted product (Palmiter et al., 1978). The ovalbumin cell-free product also interacts with RER membrane in a manner functionally analogous to that of secretory precursors graced with cleavable signals (Lingappa et al., 1978).

From the preceding one would expect that if free polysomes contain secretory protein mRNA, their translation in a runoff (readout) system for cell-free protein synthesis should produce only precursor polypeptides. This was indeed the case for canine pancreas free polysomes which only synthesized pretrypsinogen, while detached polysomes appeared to synthesize both authentic trypsinogen and precursor trypsinogen (Scheele et al., 1978). No other study has demonstrated the synthesis of precursor polypeptides by free polysomes. Data showing the production of free polysomal authentic Ig L chain polypeptides (the size of processed chains) have been equivocal (due to lack of gel resolution), except in the case of Blobel and Dobberstein (1975a,b). However, they could not rule out the presence of precursor polypeptide, and they attributed the authentic chain to contamination by membrane-bound polysomes. This interpretation was partially confirmed by significant resistance

of the polypeptide to proteolytic digestion. The fact that at least some of the authentic chain might not have come from membrane-bound polysomes suggests another explanation for authentic-sized chains synthesized by free polysomes. Free polysomes may contain mRNA which lacks the template for the signal sequence. However, the results of Scheele et al. (1978), which demonstrate that canine pancreas free polysomes only synthesize precursor trypsinogen, seem to preclude this possibility. Alternatively, some authentic chain may originate from contaminating detached polysomes (Blobel and Dobberstein, 1975a; Scheele et al., 1978).

iii. Interaction of RER protein with ribosomes or nascent chain

The vectorial transport of secretory protein nascent chain, and the removal of the precursor's signal sequence, require the participation of RER proteins (reviewed by Rolleston, 1974; McIntosh and O'Toole, 1976; Freidlin, 1976; Shore and Tata, 1977). Studies mentioned in the preceding section (section ii) established that RER had to be present during protein synthesis in order for cleavage of the precursor form to occur. RER sodium deoxycholate extract could cleave nascent or completed precursors. Some progress has been made towards determination of which membrane proteins might be necessary. Olsnes (1971) isolated polysomes from rat liver RER by solubilization of the membrane with various detergents. Through experiments centering mainly on polyribosomal density in CsCl gradients, he established that polyribosomes isolated in the presence of Triton X-100 alone adsorbed many more RER proteins than polysomes isolated in the presence of either the anionic detergent sodium deoxycholate, or Triton X-100 plus 150 mM or greater KCl. Kreibich et al. (1978a,b)

utilized Olsnes' observation and previous work on the effects of various concentrations of sodium deoxycholate on the protein composition of rat liver microsomes (Kreibich et al., 1973; Kreibich and Sabatini, 1974) to identify two nonribosomal proteins unique to rat liver RER membrane. These ribophorins, of apparent molecular weight 65,000 and 63,000, did *not* bind to ribosomes released by puromycin-high salt or deoxycholate, but *did* bind to ribosomes released by nonionic detergent. Sedimentable puromycin-high salt treated membrane could be further extracted with another detergent, cholate, to yield a complex primarily composed of the ribophorin proteins. Proximity (a requisite for interaction) of the ribophorins to ribosomes was supported by isolating reversibly cross-linked ribophorin-ribosome complexes. Boulan et al. (1978) have shown that ribophorins are transmembrane glycoproteins that are core glycosylated with the sugar moiety exposed only on the luminal surface of the membrane.

The presence of ribophorins may provide an indication of large scale contamination of putative free polysomes by membrane-bound polysomes, but probably will not be useful for the more vexing problem of trace contamination. It is not yet known whether ribophorins are universally distributed, since all characterization studies to date have been done with rat liver.

Functional assays have been routinely performed with RER membrane and secretory mRNA from species and tissues other than rat and liver. This may *not* prevent an assessment of ribophorin function since systems composed of heterologous components (e.g., Blobel and Dobberstein, 1975b) appear to work well. One interesting question yet to be answered is, what is the relationship of the ribophorins

to the signal peptidase activity (Jackson and Blobel, 1977) which can be solubilized from canine pancreas microsomes by sodium deoxycholate? It would also be useful to know the degree of cooperativity ribophorins can exhibit with the microsomal salt extract which Warren and Dobberstein (1978) found was necessary, but not sufficient, for vectorial transfer and processing of nascent secretory polypeptide.

iv. Glycosylation of polypeptide

Various aspects of the glycosylation of Ig (e.g., see Kuehl, 1977; Shore and Tata, 1977) and other secretory proteins have been reviewed extensively. The oligosaccharide portion of Ig protein is attached (to H chain, and in some L-chain secreting myeloma variants, L chain) through an N-acetylglucosaminyl-asparagine linkage (Kornfeld and Kornfeld, 1976) to regions containing the sequence Asn-X-Ser (Thr) (e.g., see Ronin et al., 1978). Glycosyltransferases are apparently found on both the cytoplasmic and luminal side of rough and smooth endoplasmic reticulum, and of Golgi membranes (Nilsson et al., 1978). This presumably reflects the need to glycosylate diverse non-secretory proteins, or other molecules such as gangliosides which may even be glycosylated by plasma membrane glycosyltransferases (Fishman and Brady, 1976). The general mechanism of glycosyl transfer to secretory proteins containing Asn-linked oligosaccharides involves polyprenol-linked sugar intermediates concentrated in the RER (for review see Hemming, 1977). The core glycosylation of IgG heavy chain occurs by *en bloc* transfer of a preformed oligosaccharide chain from an oligosaccharide pyrophosphoryl-dolichol intermediate (intermediate: $(Man)_n\ GlcNAc \xrightarrow{\beta 1,4} GlcNAc\text{-}P\text{-}P\text{-}$ dolichol, $n \geq 5$; Tabas et al., 1978). This is followed by processing

which reduces the number of mannose residues, after which the sugars which comprise the outer branches of the oligosaccharide are added (Tabas et al., 1978). The addition of outer branch sugars takes place at subcellular locations other than the RER, consistent with a sequential addition of carbohydrate to Ig during the course of its transport and secretion (Zagury et al., 1970; Choi et al., 1971; Melchers, 1971, 1973).

Membrane-dependent glycosylation of nascent secretory protein chains has been demonstrated in initiation systems for protein synthesis (Lingappa et al., 1978; Bielinska and Boime, 1978) in which correct cleavage of the precursor (pre-α-lactalbumin) was also found (Lingappa et al., 1978).

Core glycosylation of nascent Ig H chain appears to occur *in vivo* except in the case of certain variants which may be able to glycosylate completed H chain (Bergman and Kuehl, 1978).

Secretory proteins which are normally *not* glycosylated are also processed correctly: for example, L chain from IgG producers (see section ii) or L chain from certain L chain secreting variants (e.g., MOPC 41, Blobel and Dobberstein, 1975a,b) have precursor forms truncated by signal peptidase. It is not yet known whether a normally glycosylated protein, which has somehow been prevented from being glycosylated, can be correctly processed by signal peptidase. In the case of Ig H chain, non-glycosylated forms can be studied with certain myeloma H chain variants (e.g., Weitzman and Scharff, 1976). Alternatively, non-glycosylated H chain can be produced by use of inhibitors of glycosylation such as excess glucosamine (Bergman and Kuehl, 1978), tunicamycin (Hickman and Kornfeld, 1978; Struck and Lennarz, 1977) or 2-deoxy-D-glucose

(Melchers, 1973; Schwarz et al., 1978). The question of whether cells are able to secrete non-glycosylated H chain will be covered in section vi.

The weight of evidence concerning the mechanism of glycosylation suggests that free polysomes which contain Ig mRNA should not be able to produce glycosylated Ig. Sherr and Uhr (1970) labeled Ig producing cells *in vivo* with radioactive sugars, fractionated polysomes and found carbohydrate associated with immunoprecipitable nascent chains from free polysomes. This may indicate contamination by membrane-bound polysomes, but the free polysomal product would have to be further characterized before one could be confident of the origins of the glycoprotein. No other studies have been made of the degree of glycosylation of Ig (or any other polypeptides) synthesized by free polysomes *in vivo* or *in vitro*. At least two other approaches could be taken to identify a glycosylated polypeptide. Treatment of the polypeptide with the appropriate glycosidase should cause a change in mobility in SDS-PAGE (Bielinska and Boime, 1978). One could try to isolate the glycoprotein on a Con-A sepharose affinity column. If the protein did not bind, one could tentatively assume that it was not glycosylated. Unfortunately, the affinity column route may be difficult to use in the characterization of H chain, since Weitzman and Scharff (1976) had myeloma variants which produced glycosylated and non-glycosylated chains (identified by incorporation of radioactive sugars), but were unable to separate these chains on a Con-A affinity column.

v. Susceptibility of polypeptide to proteolytic digestion

A well-accepted criterion of vectorial discharge of secretory protein nascent chain into the microsomal lumen is segregation of newly-made polypeptide into a proteolytic resistant space (Sabatini and Blobel, 1970; reviewed by Shore and Tata, 1977). Cell-free products synthesized by membrane-bound polysomes are protected from proteolytic digestion, while polypeptides made by detached polysomes (detergent-treated membrane-bound polysomes) are readily degraded by proteolytic enzymes such as trypsin and chymotrypsin (Blobel and Dobberstein, 1975a; Shore and Harris, 1977; Scheele et al., 1978).

Studies performed with initiation systems for protein synthesis indicate that in the presence of added membrane, the secretory polypeptides resistant to proteolysis have had their signal sequences removed (Blobel and Dobberstein, 1975a,b; Dobberstein and Blobel, 1977; Shields and Blobel, 1978). When glycoprotein mRNA is translated, only the core glycosylated form of the polypeptide is protected from proteolytic digestion (Lingappa et al., 1978a,b).

In order for vectorial transfer of secretory polypeptide to occur, RER derived membrane (Warren and Dobberstein, 1978) must be present during protein synthesis (Lingappa et al., 1978a). Certain proteins which can be washed from the membrane with 0.5 M KCl are required for vectorial transfer (Warren and Dobberstein, 1978). Smith and Boime (1977) have observed that 0.1-0.2 mM calcium chloride inhibits the vectorial transfer of secretory polypeptide (preplacental lactogen).

In general, treatments that inhibit the vectorial transfer of secretory precursor polypeptides make the polypeptides susceptible to proteolytic digestion (by definition) and prevent cleavage (but see Jackson and Blobel, 1977) or glycosylation of the polypeptide. Polypeptides that are normally non-glycosylated can be discharged into the microsomal lumen (e.g., Blobel and Dobberstein, 1975a,b). It is not known whether normally glycosylated polypeptides, made non-glycosylated by mutation or inhibition of glycosylation, can be vectorially transferred into the lumen. However, it may be inferred that vectorial transfer occurs since these "artificially" non-glycosylated proteins can be secreted (Kuehl, 1977). Other support comes from the finding that a transmembrane protein can be inserted into RER even when its glycosylation is prevented by an inhibitor (Garoff and Schwarz, 1978; Wirth et al., 1979). Truncation of the precursor does not seem to be a requisite for vectorial transfer, since ovalbumin does not have a cleavable amino terminal signal sequence but is nevertheless discharged into a proteolysis resistant space (Lingappa et al., 1978b).

There has been only one attempt to proteolytically degrade secretory polypeptide synthesized by free polysomes (Blobel and Dobberstein, 1975a). Since the gel system used was unable to resolve precursor polypeptide (due to crowding by other proteins of similar mobility), the results were equivocal--they could not support or preclude synthesis of Ig L chain by free polysomes. Much of the authentic L chain sized polypeptide produced by the putative free polysomes appeared to be resistant to proteolysis. However, further characterization of the apparently authentic L chain (e.g., proteolysis in the presence of detergent, which should completely

eliminate the protein) is needed before one can conclude that the authentic L chain sized polypeptide originated from contaminating membrane-bound polysomes.

vi. Fate of polypeptide *in vivo*

There is still some question about the route of transport and secretion followed by secretory protein synthesized by membrane-bound polysomes. However, in general the protein seems to proceed in stepwise fashion from RER to smooth membrane to Golgi apparatus and finally is somehow secreted from the cell (Kuehl, 1977). Both glycosylated and non-glycosylated protein can be secreted. Proteins whose precursors are substrates for signal peptidase, and at least one protein (ovalbumin) which cannot be cleaved, are secreted (see previous sections). The major requirement for participation in this movement towards the cell periphery is that protein synthesis must occur on RER membrane, i.e., the polypeptides must be synthesized by membrane-bound polysomes.

Free polysomes which contain secretory protein mRNA may merely be in transit to the membrane. When they reach the membrane they may become membrane-bound polysomes whose products follow the classical pathway for secretion. A more complicated situation would develop if free polysomes synthesized secretory protein *in vivo*. The free polysomal product would likely be the precursor form of the secretory polypeptide (Scheele et al., 1978). Although an uncleaved protein can be secreted (ovalbumin), cleavage is undoubtedly a physiologically important event because it is remarkably conserved among different species. Bacteria (Smith et al., 1978; Mandel and Wickner, 1978), algae (Dobberstein et al., 1978b), higher plants

(Burr et al., 1978; Cashmore et al., 1978) and amphibians (Jilka et al., 1979) possess signal peptidases. If mammalian mRNA is injected into frog oocytes, the secretory precursor polypeptide which is synthesized is properly and precisely cleaved (Jilka et al., 1979). It is not known whether signal peptidases found in bacteria and plants can accurately cleave mammalian precursor polypeptides. Also, as yet no yeast signal peptidase has been reported in the literature. Signal sequences are found both in phage (Mandel and Wickner, 1979) and mammalian (Irving et al., 1979) viral precursor proteins. The ubiquitous nature of signal peptidases and signal sequences leads this author to venture that if free polysomes do indeed synthesize secretory precursor polypeptide *in vivo*, that synthesis must have a significant physiological purpose or it must be a direct symptom and/or cause of cellular aberrancy such as uncontrolled proliferation.

Weitzman and Scharff (1976) studied mouse myeloma mutants blocked in the glycosylation of IgG. They observed that some cells were able to secrete significant amounts of non-glycosylated H chain (complexed with L or H chain). Using an inhibitor of glycosylation (the antibiotic tunicamycin), Hickman and Kornfeld (1978) were able to inhibit the secretion of IgM and IgA. However, IgG was still secreted by mouse plasmacytoma cells. They noted that IgG normally has less carbohydrate attached than IgM or IgA. The IgM which was not secreted was *not* rapidly degraded intracellularly. Melchers (1973) employed the inhibitor 2-deoxy-D-glucose (2dDG) to inhibit glycosylation of mouse IgG, and subsequently prevent secretion of Ig synthesized in the presence of 2dDG. The block in secretion may have been due to the effects of 2dDG on RER proteins,

rather than as a consequence of non-glycosylated Ig. In *most* cases core glycosylation appears to occur cotranslationally on nascent chains *in vivo*. Sometimes, however, completed Ig chains are able to be glycosylated *in vivo* (Bergman and Kuehl, 1978). When L chain mRNA is injected into frog oocytes, only L chain which is normally glycosylated by the donor cell is glycosylated by the oocyte (Jilka et al., 1977b). Since completed Ig chains can be glycosylated under special circumstances (Bergman and Kuehl, 1978), and since glycosyltransferases may be found on the cytoplasmic or other side of all cellular membranes (Fishman and Brady, 1976; Nilsson et al., 1978), there is a slight chance that Ig polypeptide produced by free polysomes could be glycosylated, albeit not by the classical pathway.

If free polysomes synthesize Ig polypeptide *in vivo*, what happens to that polypeptide? The normal mechanism for Ig secretion has been presented. Free polysome derived Ig polypeptide, non-glycosylated and in precursor form, probably cannot utilize this mechanism and is therefore probably not secreted. There is precedence for intracellular degradation of Ig (Baumal and Scharff, 1973, 1976; Weitzman and Scharff, 1976), and Ig polypeptides produced by free polysomes may indeed be destined for this important cellular process (Kay, 1978). Alternatively, precursor secretory polypeptide synthesized by free polysomes could serve a physiological function, such as being the Ig that ends up on the plasma membrane. Polypeptides apparently synthesized by free polysomes *in vivo* possibly may complex directly with chloroplast envelope membranes (Dobberstein et al., 1977) and plasma membranes (Atkinson, 1978). In addition, there are free polysomes associated with, but

not directly bound to, Golgi membranes (Elder and Morre, 1976) and some uncharacterized membranes (Choi et al., 1971). The Golgi-associated polysomes appear to synthesize plasma membrane proteins *in vitro* (Elder and Morré, 1976). Thus, given the well established existence of surface Ig on myeloma cells (Knopf, 1973; Kuehl, 1977), it is entirely possible that free polysomes synthesize Ig destined for the plasma membrane, whereas membrane-bound polysomes synthesize Ig destined for secretion.

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ARTICLE 1

IMMUNOGLOBULIN SYNTHESIS IN P3 MYELOMA CELLS: DEFINING THE ROLE OF FREE POLYSOMES

By

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IMMUNOGLOBULIN SYNTHESIS IN P3 MYELOMA CELLS: DEFINING THE ROLE OF FREE POLYSOMES

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Running Footnote: Ig synthesis in P3 myeloma cells

KEY TERMS

Immunoglobulin synthesis; Cell-free translation; Free and membrane-bound polyribosomes; Myeloma cells; Intracellular degradation.

ABSTRACT

Polyribosomes from P3 myeloma cells were fractionated into free, membrane bound, and detached (formerly membrane-bound) populations. Free polysomes produced H chain, and only poorly resolved precursor or authentic L chain. Membrane-bound polysomes produced H chain and authentic L chain. Detached polysomes synthesized H chain and precursor L chain with no detectable authentic L chain. Only polypeptides synthesized by membrane-bound polysomes were resistant to proteolysis. These results, including the production of Ig polypeptide by free polysomes, can be explained by the signal hypothesis for the synthesis of secretory proteins.

P3 myeloma cells were pulsed with [^{35}S]methionine in single or double label experiments. The molar ratio of intracellular L/H chain did not suggest an initial excess of L chains. The ratio of newly made to long-term labeled intracellular chains indicated that degradation did not greatly exceed synthesis for either chain. These results suggest that free polysomes did not produce Ig polypeptides that are degraded rapidly intracellularly. Additional experiments indicated that free polysomes did not produce Ig polypeptides which accumulated intracellularly in lieu of their secretion or turnover on cell surface membrane. The metabolism of total protein (and probably Ig polypeptides) may vary greatly, depending on cell density.

Our data suggest that free polysomes do not produce Ig polypeptides that are accumulated or degraded in myeloma cells. Thus the possibility remains that free polysomes which contain Ig mRNA, if not contaminating detached polysomes, may be intermediates destined for RER, may provide cell surface Ig, or both.

INTRODUCTION

Polysomes may be found free in the cytoplasm or bound to the membrane of the endoplasmic reticulum (ER) [cf. reference 40 for review]. Proteins destined for secretion are thought to be synthesized exclusively by membrane-bound polysomes [cf. reference 47 for review]. It has been proposed that such polysomes initially are directed to ER membrane by a nascent chain signal sequence [7,8,9,44] that consists of about twenty amino terminal amino acids enriched for hydrophobic residues [cf. reference 35 for review]. Thus one of the key testable postulates of the signal hypothesis delineated by Blobel and Dobberstein [7,8] is that the synthesis of proteins destined for secretion begins on free polysomes which serve as intermediates in the production of secreted protein. This concept receives support from numerous studies that have concluded that secretory protein mRNA is segregated predominantly in membrane-bound polysomes, with a lesser amount found in free polysomes [cf. reference 50 for review]. However, this support is not confirmatory since only one group [53] has applied rigorous criteria based on predictions of the signal hypothesis to exclude the possibility that secretory protein synthesis by free polysomes is due to contaminating membrane-bound or detached polysomes. The need to confirm the presence of secretory protein mRNA in free polysomes has taken on added importance with the report tnat initial polysomal contact with membrane may be mediated by the 3' terminus of mRNA in an initiation complex [42]. Studies using several procedures to disassemble polysomes *in vivo* [1,57,66] or *in vitro* [15,36,43] have demonstrated the direct association of mRNA with membrane [but see 34], which

supports, but does not confirm, the possible role of mRNA in initial polysomal contact with membrane.

In this paper we present a study of immunoglobulin synthesis in P3 myeloma cells which secrete an IgG1-like molecule. We found that cell-free translation of free, membrane-bound, and detached polysomes yielded Ig polypeptides, but only Ig polypeptides synthesized by membrane-bound polysomes were resistant to proteolysis. Analysis of pulse-labeled and relatively long term labeled intracellular Ig polypeptides revealed that no detectable rapid intracellular degradation of Ig polypeptides took place, nor did Ig polypeptides accumulate in the cells. A preliminary communication of this work has been published [24].

MATERIALS AND METHODS

Cell Maintenance

IgG1-secreting P3 murine myeloma tissue culture cells (kindly provided by Dr. Matthew D. Scharff, Albert Einstein College of Medicine) were maintained in flasks and roller bottles in Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS), 74 μg/ml streptomycin, 100 units/ml penicillin, and 40 units/ml mycostatin. The composition of the medium used when cells were labeled with radioactive precursors is given in the figure legends. For cell fractionation, cells grown to a density of 5-8 x 10^5/ml were diluted with approximately one volume of fresh medium three to five hours before use. Periodic biochemical assays for mycoplasma contamination were negative.

Cell Fractionation

Cultures were poured over crushed frozen saline and all subsequent procedures were performed at 0-4°C. The cells were pelleted by centrifugation for 8 min at 500 x g max, washed once with RSB (20 mM HEPES, pH 7.5, 10 mM Na^+-NaCl plus NaOH used to pH the HEPES, 3 mM $MgCl_2$), resuspended in RSB and allowed to swell for 7 min. The cells were pelleted, resuspended in 15% w/w sucrose (ribonuclease-free)-RSB and immediately homogenized in a Dounce homogenizer (Kontes Co., Vineland, NJ) with ten strokes of the B (loose) pestle. Nuclei were pelleted from the homogenate by centrifugation for 5 min at 900 x g max and washed once with 15% w/w sucrose-RSB. The wash was added to the first postnuclear supernatant which was then used as the source of polysomes. The postnuclear supernatant was layered over a 2 ml 15 x 32% w/w sucrose-RSB linear gradient [51] which was then centrifuged in the SW 50.1 rotor (Beckman) for 45 min at 27,000 x g max (15,000 rpm). The pellet was resuspended in 15% w/w sucrose-RSB and centrifuged (45 min, 27,000 x g max, SW 50.1) through a 2 ml 15 x 32% w/w sucrose-RSB linear gradient. The twice pelleted material was resuspended (Dounce homogenized) in 15% w/w sucrose-RSB and stored at -80°C for use as a source of membrane-bound polysomes. Detached polysomes were prepared from membrane-bound polysomes by addition of Triton X-100 (TX-100) to a final concentration of 1.0% v/v. The detached polysomes were then sedimented through 2 ml of 32% w/w sucrose-RSB by centrifugation for 90 min at 100,000 x g max (29,000 rpm) in the SW 50.1 rotor. The pellet of detached polysomes was resuspended in 15% w/w sucrose-RSB and stored at -80°C.

The postmicrosomal supernatant obtained from the first centrifugation of the postnuclear supernatant was layered over a

discontinuous sucrose-RSB gradient composed of 62% w/w (2 ml), 56% w/w (1 ml) and 40% w/w (1 ml) sucrose-RSB. The gradient was centrifuged for 16 hours at 195,000 x g max (34,000 rpm) in an SW 41 Ti rotor (Beckman). Fractions were collected by volume as shown in Figure 1. The 62% layer was collected with a 5 ml syringe and 18 g needle in order to avoid inclusion of the 56%/62% interface. The pellet was washed with RSB, resuspended in 15% w/w sucrose-RSB and added to the 62% layer which was then stored at -80°C for use as a source of free polysomes.

System for Cell-free Protein Synthesis and Assays for Cell-free Translation Products

COMPOSITION OF SYSTEM: The final concentrations of reactants were: 1) 100 mM KCl, 5 mM $MgCl_2$, 20 mM HEPES, pH 7.5; 2) 6 mM 2-mercaptoethanol; 3) 1 mM ATP, 0.02 mM GTP, 8 mM creatine phosphate (dipotassium or di-Tris salts); 4) 25 µM amino acids minus leucine or methionine or both; 5) L-[4,5-^{3}H] leucine (Amersham, 105 Ci/mmol, final concentration of 100 µCi/ml) or [^{35}S]methionine (Amersham, 1040 Ci/mmol, final concentration of 100 µCi/ml) or both; 6) deionized, glass-distilled, autoclaved water which was also used for solubilizing reagents; 7) 1.55 units/ml creatine phosphokinase; 8) high speed supernatant (HSS) at a final concentration of about 0.1 mg protein/ml; and 9) 3-5 A_{260} units/ml free or detached polysomes, or 5-8 A_{260} units/ml membrane-bound polysomes.

Incubation was at 37°C for 40 min. Incorporation of radioactivity was accessed by hot TCA precipitation of aliquots spotted on Whatman 3 MM filter paper disks. Radioactivity was determined in

toluene-Omnifluor (New England Nuclear Corp., Boston, MA) in a Searle Delta 300 liquid scintillation counter.

HSS was prepared from P3 cells by centrifugation of a post-nuclear supernatant for 120 min at 305,000 x g max (50,000 rpm) in the SW 50.1 rotor. The HSS was dialyzed against 10 mM HEPES, pH 7.5, 10 mM KCl, 2 mM $MgCl_2$, and stored at -80°C for up to 3 months for use in the cell-free system.

IMMUNOPRECIPITATION OF CELL-FREE PRODUCTS: Antisera were prepared as previously described [22]. MOPC-21 myeloma protein was isolated and purified as previously described [22]. Antisera against the heavy (H) and light (L) chains of MOPC-21 protein were raised in rabbits by subcutaneous injection of H and L chains separated by SDS-PAGE. The H and L chain-containing polyacrylamide gel fractions were emulsified in Freund's complete adjuvant. The animals periodically were given booster injections and bled through the marginal ear vein 7 to 10 days after each injection. The gamma globulin fraction was purified as described for the myeloma protein [22]. The rabbit anti-myeloma protein was further purified by affinity chromatography as previously described [22].

Immunoprecipitation was performed with antisera and bacteria-bound staphylococcal protein A essentially as described by Kessler [31]. To prepare the bacteria, the Cowen I strain of *S. aureus* (kindly provided by Dr. J. N. Behnke, USUHS) was streaked on an agar plate and incubated at 37°C. Tubes containing 5 ml of broth were inoculated from a colony and incubated overnight at 37°C. One liter Erlenmeyer flasks containing 250 ml of broth/flask were inoculated with 1 ml of the overnight culture/flask. The flasks were incubated

for 24 hours at 37°C on a shaker at 240 rpm. Both agar and broth were made with Trypticase soy broth soybean-casein digest medium, USP (BBL. Div. Becton, Dickinson and Company, Cockeysville, MD). The bacteria were harvested by centrifugation for 10 min at 8,000 x g (7,000 rpm) in the Sorval GSA rotor and washed once with 10 mM HEPES, pH 7.5, 150 mM NaCl (HN). They were then resuspended to approximately 10% v/v in HN plus 1.5% final concentration formaldehyde, and stirred for 1.5 hours. The bacteria were then pelleted, washed once with HN, resuspended in HN to approximately 10% v/v, and swirled continuously for 5 min in an Erlenmeyer flask in an 80°C waterbath, then immediately cooled on ice. The bacteria were washed once with HN and adjusted to 10% v/v in HN. This preparation of bacteria was divided into aliquots and stored at -80°C until use.

Within 24 hours of use, bacteria were thawed, washed twice with 1.0% NP40, 10 mM HEPES, pH 7.5, 150 mM NaCl (1% NHN) by centrifugation for 15 min at 900 x g max, and resuspended in 0.5% NHN to approximately 10% v/v.

Cell-free translation was stopped by placing tubes on ice and adding a final concentration of 16 mM unlabeled leucine (leu) or 4 mM unlabeled methionine (met) to the reaction mixture. For immunoprecipitation, all samples were treated with 0.5% v/v NP40, and sometimes also 0.5% v/v sodium deoxycholate. After 10 min, the samples were centrifuged for 15 min at 900 x g max to pellet insoluble material. The samples were transferred to Eppendorff microtubes (maximum capacity 1.5 ml) and centrifuged for 2 min in a Brinkman centrifuge to remove nonadsorbed material that would nonspecifically sediment with bacteria. The cleared samples were incubated at 0-4°C for one hour with anti-H and anti-L antisera,

followed by addition of 0.2 ml of the 10% v/v Cowen I *S. aureus* preparation. After 10 min incubation on ice, the bacteria were pelleted by centrifugation for 1.5 min in the Brinkmann centrifuge. The bacterial pellet was resuspended in 0.05 ml of solubilization buffer composed of 0.4 ml protein solution (10 mM Tris, pH 8.0, 1 mM EDTA, 1% w/v SDS), 0.1 ml 20% SDS, and 0.01 ml 14.3N 2-ME. The bacteria were incubated in solubilization buffer for 15 min at 37°C, 10 sec at 100°C, and pelleted for 2 min in the Brinkmann centrifuge. The supernatant containing antibody (and homologous antigen) which had specifically adsorbed to the bacteria was removed with a Hamilton syringe and processed as described in the section on analysis of translation products by SDS-PAGE.

PROTEOLYSIS OF TRANSLATION PRODUCTS: Cell-free translation was stopped by cooling the mix to 0-4°C and adding unlabeled leu or met as described in the preceding section. Direct TCA precipitation of the translation products was accomplished by the addition of 0.1 ml reaction mix to 0.9 ml 10% w/v TCA. For proteolytic digestion, 0.012 ml of a 1 mg/ml solution of trypsin (185 U/mg, Worthington Biochemical Corp., Freehold, NJ) was added to 0.1 ml reaction mix (7). In some instances 0.012 ml of a 10% v/v solution of TX-100 (for a final concentration of 1% TX-100) was added before the proteolytic enzyme. Digestion was allowed to proceed for 3 hours at 0-4°C, after which the reaction was stopped and the remaining products were precipitated by the addition of 0.9 ml 10% w/v TCA. The TCA precipitates were pelleted for 10 min at 900 x g max, washed once with -20°C freezer cold acetone, and resolubilized as

described in the section on analysis of translation products by SDS-PAGE.

ANALYSIS OF TRANSLATION PRODUCTS BY SDS-PAGE: TCA precipitates were resolubilized in 0.05 ml of solubilization buffer (see section on immunoprecipitation of cell-free products). Subsequently, the TCA precipitates and immunoprecipitates were treated identically. Samples (0.04 ml) were removed and, after addition of 0.008 ml of dye-sucrose solution (bromphenol blue in sucrose), incubated for 30 min at 37°C, then for 10 sec at 100°C. To each sample 0.012 ml of 1 M iodoacetamide was then added. After the bromphenol blue had turned yellow (acidic), usually within 10 min, each sample was neutralized with 0.003 ml of 2.5N NaOH. The samples were added directly to the gel (0.02 ml/slot) for SDS-PAGE.

The slab gel (0.75 mm thick) consisted of a 7.5-15% acrylamide gradient serving as a resolving gel and a 5% acrylamide stacking gel, both in SDS, and buffers as described by Fairbanks et al. [23]. Electrophoresis was for 15 hours at constant voltage.

After electrophoresis, the slab gel was either fixed and stained with Coomassie blue by conventional procedures, or processed by fluorography as described by Bonner and Laskey [11].

RESULTS

Fractionation of Free and Membrane-Bound Polysomes

Figure 1 shows a diagram of the fractionation procedure used to obtain free and membrane-bound polysomes. Centrifugation of the postnuclear supernatant was performed so as to pellet all particles of an apparent S value greater than or equal to 1000S [51].

Resuspension and recentrifugation of the pellet yielded the fraction of membrane-bound polysomes. Free polysomes were obtained by centrifugation of the polysomes in the initial postmicrosomal supernatant through a discontinuous sucrose gradient as shown. Detached polysomes were obtained from detergent treated (1% TX-100) membrane-bound polysomes which then were pelleted through 32% w/w sucrose-RSB. Routinely 10% to 15% of the A_{260} material of the postnuclear supernatant was recovered with the membrane-bound polysomes, and 40% to 50% of the A_{260} material with the free polysomes. Thus, by this method approximately 20% of the polysomes in the postnuclear supernatant were membrane-bound. A similar percentage of membrane-bound polysomes in P3 myeloma cell postnuclear supernatant has been reported [6,41]. About 50% of the [^{14}C]choline labeled material co-sedimented with the membrane-bound polysomes, while less than 3% was found with the free polysomes.

To assess further the purity of the free and membrane-bound polysome fractions, we performed the [^{125}I]labeled Concanavalin A ([^{125}I]ConA) binding experiments shown in Table I. We sought to detect the presence of core glycosylated proteins (putative H polypeptides) which should be present in the lumen of rough microsomes. Free, membrane-bound, and detached polysome fractions were either directly TCA precipitated, or detergent-treated and immunoprecipitated, and the precipitates were analyzed by SDS-PAGE. The H chain regions were cut from the gel, overlaid with [^{125}I]ConA ([^{125}I-ConA], specifically inhibitable by glucose, was graciously supplied by Dr. Walter Esselman) and thoroughly washed to remove unbound [^{125}I]ConA. In immunoprecipitated samples, more [^{125}I]ConA was bound to microsomal glycoproteins migrating in the H chain region than to polypeptides

of corresponding mobility obtained from free or detached polysome fractions. Since significant amounts of [^{125}I]ConA bound to the H chain of the antisera used for immunoprecipitation (antisera control), we directly TCA precipitated portions of the polysome fractions. In a comparison of binding to H chain regions, ConA bound almost exclusively to glycoproteins derived from the membrane-bound polysome fraction, with little or no binding to TCA precipitated proteins of similar mobility obtained from free or detached polysomes. The [^{125}I]ConA did not bind to nonglycosylated protein (L chain control).

Immunoglobulin Polypeptides Synthesized by Free, Membrane-Bound and Detached Polysomes

Figure 2, slot A, shows a fluorograph of immunoprecipitable polypeptides synthesized by detached polysomes. H and precursor L chains were visible. No authentic L chain could be detected. The precursor L chain appeared as a single band. Immunoprecipitated H and L chains from secreted myeloma protein are shown in slot B for comparison.

Figure 3 shows a fluorograph of immunoprecipitable polypeptides synthesized by free and membrane-bound polysomes. For comparison, Ig polypeptides synthesized by detached polysomes (slot D) and secreted by myeloma cells (slot S) are shown. The gel was overexposed to improve the visibility of the polypeptides produced by membrane-bound polysomes. Free polysomes synthesized H chain, but little or poorly resolved precursor L or authentic L chain (slot F). Membrane-bound polysomes synthesized authentic L chain and a doublet band of H chain (slot M). H chain produced by free and detached polysomes appeared to migrate with the faster moving doublet band. The H chain

doublet synthesized by membrane-bound polysomes was seen in total TCA precipitable material (Fig. 4M-) and was resistant to proteolysis by trypsin (Fig. 4M+). In contrast, H chain produced by free polysomes (Fig. 4, slots F2- and F-) or detached polysomes (Fig. 4D-) could be digested proteolytically (Fig. 4, slots F+, D+). When detergent was added to the system with membrane-bound polysomes prior to proteolysis, the H chain doublet (as well as other polypeptides greater than 25,000 daltons) became susceptible to proteolytic digestion (Fig. 4, slot M+d). In comparison to free and detached polysomal cell-free TCA precipitable counts, approximately one-third more material synthesized by membrane-bound polysomes was resistant to proteolysis (data not shown). It should be noted that in some experiments we were not able to resolve the H chain produced by membrane-bound polysomes into a doublet. The appearance of an artifact of proteolysis (e.g., Fig. 4, slots M+, F+, D+) made it impossible for us to interpret the effect of proteolysis on L chain. The artifact was observed even in the presence of detergent (Fig. 4, slots F+d, M+d, D+d) and an additional proteolytic enzyme (chymotrypsin, data not shown).

Fate of Immunoglobulin Polypeptides Synthesized in vivo

Figure 5 shows the time course of [^{3}H]leu incorporation into total intracellular and secreted protein made by P3 cells at two different initial cell densities. The lower density, 4.7×10^5 cells/ml is characteristic of exponentially growing cells, while the higher density (achieved by concentrating the cells), 1.3×10^6 cells/ml may be characteristic of cells in stationary phase. Production of intracellular protein plateaus within 4 h for the cells at the highest

density. In contrast, no plateau is observed even after 6 h for cells at the lower density. Depletion of [^{3}H]leu was probably not the cause of the plateau exhibited by the higher density cells since the pattern of secretion was not similarly affected. We chose to investigate the fate of Ig synthesized by cells at the lower density, as we felt the lower density cells were most likely to represent cells with optimal metabolic activity.

The time course of incorporation of [^{3}H]leu into intracellular and secreted IgG1 myeloma protein at the initial cell density of 4.7×10^{5} cells/ml is shown in Figure 6A. The rate of secretion became linear at about 90 to 120 minutes. The rate of production of intracellular Ig lessened during this interval (compare slopes before and after) but did not plateau. Figure 6B shows the percentage of total intracellular protein immunoprecipitable as Ig during the course of this experiment. In this experiment approximately 5% of the newly synthesized intracellular material was immunoprecipitable. As more intracellular proteins were labeled, the percent immunoprecipitated reached a lower limit of about 2.5%. The predominance of newly synthesized Ig compared to other intracellular proteins could be seen in fluorographs of total TCA precipitable proteins (data not shown).

P3 myeloma cells at a density of $5\text{-}7 \times 10^{5}$ cells/ml were grown for 3 h (in Dulbecco's high glucose medium, 10% FCS, 1/40 normal amount met) in the presence of [^{3}H]leu. They were pulsed with [^{35}S]met for 5 min, then chased with 20 times the normal amount of cold met ([^{3}H]leu at the original concentration was included with the cold met). The chase was effective, as there was no increase in TCA precipitable [^{35}S]labeled intracellular protein after

addition of the cold met (data not shown). Aliquots were periodically withdrawn beginning at 1.5 minutes after the chase (6.5 min postlabeling) and the secreted and intracellular Ig polypeptides were immunoprecipitated and analyzed by SDS-PAGE. Intracellular precursor L chain was not observed at any time interval during the chase (Fig. 7, slots 6.5-35). We could only detect intracellular H and L chains which migrated with the same mobility as the secreted (Fig. 7, slot S) Ig polypeptides.

For further analysis the gels were stained and the H and L regions were cut out. H and L chains were eluted from the gel portions with NCS tissue solubilizer, and the amount of radioactivity in each chain was determined for each time point. Table II presents the L/H molar ratio of newly synthesized Ig in P3 myeloma cells. The experimental design was similar to that of Baumal and Scharff [4]. The L/H molar ratio was calculated by dividing the L/H ratio at each time point by the L/H ratio of secreted protein obtained after 3 hours of labeling, with the assumption that secreted protein is maximally labeled and its ratio of L/H radioactivity represents a 1/1 molar ratio of L/H chains [4]. Table II shows that the L/H molar ratio of intracellular Ig was slightly depressed at the start of the experiment, but increased rapidly to reach a value of about 1.0 or slightly more. This suggests that these P3 cells were not producing L chain in 2- to 3-fold initial excess as previously reported for P3 cells [4]. Table III (using data obtained from two experiments, each replicated twice) shows that the slight depression and rapid increase in the L/H molar ratio was not due to the degradation of a slight excess of H chains. The [^{35}S]met incorporated into newly synthesized H and L chains was compared to the

relatively stable amount of Ig provided by the long term (3 h) [^{3}H]leu labeled Ig. The [^{3}H]leu labeled Ig provided an internal control for variable immunoprecipitation. The [^{35}S]met counts were normalized to the proportional amounts of [^{3}H]leu labeled H or L chains seen in secreted Ig. The amount of newly synthesized ([^{35}S]met labeled) to long term ([^{3}H]leu labeled) H chain remained relatively constant during the first 20 minutes of postlabeling (15 min chase). The amount of newly synthesized to long-term labeled L chain actually increased slightly during the first 20 minutes of postlabeling. These results suggest that neither H nor L chain were subject to rapid intracellular degradation.

DISCUSSION

We have detected cell-free synthesis of Ig polypeptide by a well-characterized fraction of free polysomes, and have provided some data on the possible *in vivo* fate of free polysomal Ig nascent chain or completed product. The results were not able to preclude contamination of the putative free polysomes by detached polysomes, but suggested the complete absence of microsomal contamination. We exploited density differences between free and membrane-bound polysomes to further purify free polysomes found in the postmicrosomal supernatant after differential centrifugation. Perhaps the best separation procedure that utilizes density differences is the flotation method [41] in which total polysomes are mixed with dense sucrose and centrifuged. The membrane-bound polysomes float out of the dense sucrose, while the free polysomes remain in the dense sucrose or pellet. The major disadvantage of this procedure is that it cannot be used preparatively. The most commonly used density

gradient procedure involves layering a postnuclear supernatant over a layer of 2M sucrose [10,67]. After appropriate centrifugation membrane-bound polysomes band at the 2M interface while the free polysomes pellet. However, varying amounts of lipid, detected chemically or by use of radioactive precursors such as [^{3}H]choline or [^{3}H]oleic acid, also can pellet thorugh 2M, or even greater than 2M, sucrose [10,14,18,45]. Blobel and Dobberstein [7] showed an electron micrograph of microsomes that pelleted through 2M sucrose. Other investigators, however, examined their free polysome fractions (pelleted through 2M sucrose) by electron microscopy and did not detect contaminating microsomes [10,38,45,56]. Whether the lipid sedimenting with free polysomes is totally, predominantly, or not at all microsomal lipid is still an unresolved question.

We assessed the microsomal contamination of our free polysome fraction by two procedures. First, less than 3% of membrane lipid (quantitated by long-term labeling with [^{14}C]choline) co-purified with the free polysome fraction. Thus the [^{14}C]choline/A_{260} ratio was much lower for free than membrane-bound polysomes. Second, we reasoned that a detectable quantity of core glycosylated myeloma H chain [64] should be contained within the cisternae of rough microsomes whereas free polysomes would have no core glycosylated H chains. Binding of [^{125}I]COnA [cf. reference 55 for review on lectin affinities] to polysomal fraction glycoproteins with H chain mobility confirmed this suggestion. Glycoproteins migrating with the mobility of P3 myeloma protein H chains on SDS-PAGE were isolated from free and membrane-bound polysomes either by direct TCA precipitation or immunoprecipitation. The glycoproteins derived from the membrane-bound polysome fraction showed significant [^{125}I]ConA

binding whereas similar proteins isolated from the free polysome fraction exhibited negligible binding. H chain sized material from detached polysomes bound low, but greater than background, levels of [^{125}I]ConA. Boulan et al. [12] used a similar procedure to identify rat liver microsomal glycoproteins.

Our data showing minimal [^{14}C]choline and negligible glycoprotein contamination of the free polysome fraction strongly suggest that the free polysome fraction was not contaminated with membrane-bound polysomes. The detection of secretory protein (e.g., Ig) mRNA in free polysomes, assessed either by analysis of cell-free translation products or molecular hybridization, has been reported [3,38,46,56] while others have been unable to confirm these observations [7,18,19,49,62,65]. Some who were not able to confirm the observations also could not exclude completely the possibility of Ig mRNA in free polysomes [7,19,49,65], while another group performed experiments using only a small, selected proportion of the total free polysomes [18]. One major problem in these studies was that some investigators, while they undoubtedly agree that Ig mRNA is less concentrated in free polysomes, did not use a correspondingly larger amount of radioactive polypeptides made by free polysomes in order to detect Ig polypeptides. This is an important point, since most experiments have been performed at the limits of detectability where it is easy to bias the results.

It is still manifestly important to establish by other means that the secretory polypeptide being synthesized, or the secretory mRNA detected by cDNA hybridization, is not due to the presence of contaminating membrane-bound polysomes. Accordingly, functional assays based on predictions of the signal hypothesis [7,8] were

performed to evaluate whether immunoprecipitable Ig polypeptides synthesized by free polysomes originated from contaminating membrane-bound or detached polysomes. One such study has been reported [7], but the results were inconclusive. The signal hypothesis predicts that if protein synthesis occurs in the presence of RER membrane, the signal sequence will lead the nascent polypeptide (and polysome) to the membrane. The signal sequence will be removed by a membrane-bound enzymic activity before completion of the nascent chain [7,8]. A major expectation is that detached polysomes obtained from detergent-treated RER should synthesize both precursor and authentic polypeptides, the longer nascent chains already having been processed. This has been found in two cases [8,53], while others (using the same cell lines as ours) have observed that detached polysomes only synthesize precursor polypeptides [25,44]. We, too, detected only the synthesis of precursor polypeptide (precursor L chain) by detached polysomes, although our precursor was not resolved into a doublet as reported by others [25,44]. The fact that we could not detect authentic L chain after translation of detached polysomes may be related to the physiology of the P3 cell line, since the ribosome-membrane attachment is largely disrupted by mild RNase treatment [42]. That is, only one or two ribosomes of an Ig H or L chain producing polysome may be bound to the membrane and too few nascent chains would be cleaved to result in detectable authentic L chain after completion in a cell-free system for protein synthesis.

One would expect that free-polysomes would produce only precursor L chains [53], but we were unable to resolve either precursor or authentic L chain synthesized by free polysome. Even if we had detected precursor L chain, we would have been unable to differentiate

between polypeptides synthesized by free and detached polysomes, since we observed that P3 detached polysomes only synthesized precursor L chain. Membrane-bound polysomes synthesized the expected authentic L chain. We are unsure of the nature of the doublet H band which was synthesized by membrane-bound polysomes, but it is possible that we have resolved core glycosylated and nonglycosylated H chain, especially since the H chain produced by free and detached polysomes appears to migrate with the same mobility as the lower band of the doublet. Another possible explanation is that one band represents an altered H chain produced by a mutant population of P3 cells, but this is unlikely since free and detached polysomes never synthesized a doublet.

Another prediction of the signal hypothesis [7,8] is that synthesis of secretory protein by microsomal polysomes should be accompanied by vectorial discharge of the nascent chain into the microsomal lumen. A well-accepted criterion of vectorial discharge is segregation of newly-made polypeptide into a proteolytic resistant space [52, cf. reference 59 for review]. Cell-free products synthesized by membrane-bound polysomes are protected from proteolytic digestion, while polypeptides made by detached polysomes are readily degraded by proteolytic enzymes such as trypsin and chymotrypsin [7,53,58]. There has been only one previous attempt to degrade proteolytically secretory polypeptide synthesized by free polysomes [7]. Since the gel system used was unable to resolve precursor polypeptide (due to crowding by other proteins of similar mobility), the results were equivocal and did not support or preclude synthesis of Ig L chain by free polysomes from the L chain secreting cells. We also had a problem, though of a different nature, in resolving L

chain after proteolysis. Fortunately, P3 cells secrete a whole IgG molecule and thus we were able to examine the effect(s) of proteolysis on H chain synthesis by the various fractions of polysomes. H chains produced by free and detached polysomes were digested by the proteolytic enzyme, trypsin, while H chains produced by membrane-bound polysomes were resistant to proteolysis. We conclude that H chains synthesized by the free polysomes are not produced by contaminating membrane-bound polysomes. Data from proteolytic digestion experiments cannot be used to differentiate between Ig polypeptides produced by free polysomes or detached polysomes.

Fate of Polypeptide in vivo

There is still some question concerning the route of transport and secretion of secretory protein synthesized by membrane-bound polysomes. However, in general, the protein proceeds in stepwise fashion from RER to smooth membrane to Golgi apparatus and finally is secreted from the cell [cf. reference 35 for review]. Both glycosylated and nonglycosylated protein can be secreted. Proteins whose precursors are substrates for signal peptidase, and at least one protein (ovalbumin) which is not cleaved [37,48], are secreted. The major requirement for participation in this movement to the cell periphery is that protein synthesis must occur on RER membrane (i.e., the polypeptides must be synthesized by membrane-bound polysomes).

Free polysomes which contain secretory protein mRNA merely may be in transit to the membrane. When they reach the membrane they may become membrane-bound polysomes whose products follow the classical pathway for secretion. A more complicated situation would

develop if free polysomes synthesized secretory protein *in vivo*. The free polysomal product would likely be the precursor form of the secretory polypeptide [53]. Although an uncleaved protein can be secreted (ovalbumin), cleavage is undoubtedly a physiologically important event because it is remarkably conserved among different species. Bacteria [39,61], algae [20], higher plants [13,16] and amphibians [28] possess signal peptidase(s). If mammalian mRNA is injected into frog oocytes, the secretory precursor peptide which is synthesized is properly and precisely cleaved [28].

It is not known whether signal peptidases found in bacteria and plants can accurately cleave mammalian precursor polypeptides. Also, as yet, no yeast signal peptidase has been reported in the literature. Signal sequences are found both in phage [39] and mammalian [27] viral precursor proteins. The ubiquitous nature of signal peptidases and signal sequences leads us to venture that if free polysomes do indeed synthesize secretory precursor polypeptide *in vivo*, that synthesis must have a significant physiological purpose or it may be a direct symptom and/or cause of cellular aberrancy such as uncontrolled proliferation.

If free polysomes synthesize Ig polypeptide *in vivo*, what happens to that polypeptide? Free polysome-derived Ig polypeptide, nonglycosylated and in precursor form, probably cannot utilize the normal mechanism for Ig secretion and therefore probably would not be secreted. We have never detected precursor H or L chains in polypeptides secreted from the cells, although our analytic procedures might not allow us to differentiate between glycosylated and precursor H chains.

The Ig could accumulate intracellularly, but our evidence suggests this does not happen. No precursor polypeptide could be detected in P3 cells regardless of time of labeling. This agrees with the results of other investigators [30,54,63]. The addition of proteolytic inhibitors allows certain cell lines to accumulate detectable precursor polypeptides [30,54,63]. Another indication that Ig does not accumulate intracellularly in P3 cells is that when cells are labeled in a time course experiment, the percent immunoprecipitable radioactivity decreases to a relatively constant value. That is, although the predominant newly synthesized polypeptides are H and L chains, we do not observe with time a disproportionate increase in Ig polypeptides relative to the amounts of other labeled proteins.

If the Ig polypeptides produced by free polysomes *in vivo* do not accumulate intracellularly, what then is their fate? There is precedent for intracellular degradation of Ig polypeptides [4,5,68] and Ig polypeptides produced by free polysomes may indeed be destined for this important cellular process [cf. reference 29 for review]. However, Ig which is not secreted does not necessarily have to be rapidly degraded [26]. Our data suggest that neither H nor L chains are rapidly degraded intracellularly. Our results contrast with those of Baumal and Scharff [4], who reported that P3 myeloma cells initially synthesize excess L chains which are rapidly degraded. We observed differences in intracellular incorporation of [^{3}H]leu by cells at 5 x 10^5 cells/ml, and cells at a higher density (1.3 x 10^6 cells/ml) approaching the density of 10 x 10^6 cells/ml used by Baumal and Scharff in their studies of protein turnover. Density differences definitely affect intracellular

metabolism in myeloma cells [32,60], and we suggest this may be the cause of the contrasting results. Currently experiments are being performed to investigate this possibility. On the other hand, Hickman and Kornfeld [26] used cells at a density greater than 10^6 cells/ml and did not observe rapid degradation of Ig which had been prevented from leaving the cell by tunicamycin. Hickman and Kornfeld's results may differ from ours due to unknown effects of tunicamycin, or because they used an IgM secretor cell line while we and Baumal and Scharff [4] were working with IgG-secreting P3 cells. At any rate, density differences have been reported to cause marked effects in the intracellular metabolism of myeloma [32] and transformed lymphoid cells [60].

Since Ig is apparently neither rapidly degraded nor accumulated in P3 cells, there are three reasonable possibilities to explain the ultimate fate of free polysomal Ig nascent chain. The first possibility is that we are detecting Ig mRNA in detached, not free polysomes. The second possibility, which we have already mentioned, is that free polysomes serve as intermediates in the synthesis of Ig as predicted by the signal hypothesis. That is, free polysomes containing Ig mRNA ultimately end up bound to membrane. Finally, precursor secretory polypeptide synthesized by free polysomes could serve a physiological function, such as surface Ig. Polypeptides apparently synthesized by free polysomes *in vivo* may complex directly with chloroplast envelope membranes and plasma membranes [20]. In addition, there are free polysomes associated with, but not directly bound to, Golgi membranes [21] and some uncharacterized membranes [17]. The Golgi-associated polysomes appear to synthesize plasma membrane proteins *in vitro* [21]. Thus, given the

well-established existence of surface Ig on myeloma cells [33,35], it is entirely possible that free polysomes synthesize Ig destined for the plasma membrane, whereas membrane-bound polysomes synthesize Ig destined for secretion.

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TABLE I

[^{125}I]ConA binding to free, membrane-bound, and detached polysome fraction glycoproteins

Procedure for glycoprotein isolation	Polysome fraction	Bound [^{125}I]ConA (CPM) Expt. 1	Expt. 2
Immunoprecipitation	Free	728	557
	Membrane-bound	1720	1051
	Detached	*	672
TCA precipitation	Free	56	-4
	Membrane-bound	1073	232
	Detached	*	56
Controls			
Gel region containing L chain		-40	4
Gel region containing H chain from antisera		940	454

Polysome fractions were isolated as described in Materials and Methods. In expt. 1, 1.2 A_{260} membrane-bound fraction and 0.9 A_{260} free fraction were TCA precipitated. In expt. 2, 0.4 A_{260} membrane-bound fraction, 0.6 A_{260} free fraction, and 0.5 A_{260} detached fraction were TCA precipitated. For each expt., immunoprecipitation was performed with 5 times the A_{260} used in TCA precipitation. Gels were stained with Coomassie blue and the region corresponding to H chain (or L chain for control) was cut out and washed with 10 mM HEPES, pH 7.5, 150 mM NaCl till the pH rose to about 7. The gel was then overlaid for 1 hour at room temperature with [^{125}I]ConA solution containing Mn^{++} and Ca^{++} ions. The [^{125}I]ConA was then aspirated from the gel. The gel was washed thoroughly with buffer and cut into regions of interest which were counted in a Packard gamma scintillation spectrometer, Model 3001.
*not done

TABLE II

L/H molar ratio of newly synthesized Ig in P3 myeloma cells

	Time Post-Labeling (min)						
	6.5	8	11	15.5	20	35	(180 continuous label)
Molar L/H	0.77	0.72	0.76	0.89	0.96	1.11	(1.17)
± s.d.	0.05	0.03	0.03	0.07	0.02	0.09	(0.14)

Logarithmically growing cells at 4 x 10^5 cells/ml were pelleted and resuspended to 5-7 x 10^5 cells/ml in prewarmed Dulbecco's medium minus leu and containing 1/40 the normal concentration of met plus 10% FCS and antibiotics. The cells were labeled for 3 h with L-[4,5-^{3}H]leu (10 µCi/ml, 105 Ci/mmol, Amersham) and then pulsed with [^{35}S]met (10 µCi/ml, 1040 Ci/mmol, Amersham) for 5 min. One ml of cells was incubated for 3 h with [^{3}H]leu for secreted Ig. Incorporation of [^{35}S]met was stopped by diluting the cells with one volume of prewarmed medium containing 40 times the normal concentration of met plus 10 µCi/ml [^{3}H]leu. Beginning 1.5 min after the chase (6.5 min post-labeling) and at the indicated intervals, samples containing 1.5 x 10^6 cells were added to an equal volume of ice cold medium containing 40 times the normal concentration of met. The cells were pelleted, washed twice with RSB and lysed with 0.5% NP40-RSB. The salt concentration was raised to 150 mM NaCl and nuclei and some ribosomes pelleted by centrifugation for 30 min at 100,000 x g max (29,000 rpm) in an SW 50.1 rotor. The supernatant was immunoprecipitated and analyzed by SDS-PAGE as described in Materials and Methods. After 3 h, secreted P3 myeloma protein was immunoprecipitated and analyzed by SDS-PAGE. H and L chain bands were cut from the gel and radioactivity determined. The L/H radioactivity of secreted Ig was considered to represent equimolar amounts of H and L chains and was used in all subsequent calculations of molar L/H ratios [4].

TABLE III

Analysis of newly synthesized H and L chains for evidence of intracellular degradation

	Normalized CPM in H and L Chains* Time Post-Labeling (min)							
	6.5	8	11	15.5	20	35	180[II]	secreted (180)[II]
H	7.97	8.53	8.95	8.99	8.80	7.20	158	45.6
± s.d.	0.56	0.33	0.30	0.18	0.06	0.02	17.0	3.1
L	3.71	3.79	4.11	4.89	5.27	5.28	123	32.9
± s.d.	0.25	0.25	0.16	0.56	0.55	0.66	11.0	1.2

Conditions for pulse-chase labeling of long-term labeled cells and analysis of the radioactivity in H and L chains were identical to those described in the legend to Table II.

To compare newly labeled [^{35}S]met to long-term labeled [^{3}H]leu H and L chains, the [^{35}S]met counts were normalized to [^{3}H]leu counts (arbitrarily chosen to be in the same proportion as the radioactivity found in secreted Ig). H chain radioactivity is expressed as [^{35}S]met-labeled H chain radioactivity per 10^5 CPM [^{3}H]leu-labeled H chains. L chain radioactivity is expressed as [^{35}S]met-labeled L chain radioactivity per 3.52×10^4 [^{3}H]leu-labeled L chains.

[II]No chase.

Figure 1. Fractionation procedure for the isolation of free and membrane-bound polysomes. Percent A_{260} and [^{14}C]choline in each fraction.

FRACTIONATION PROCEDURE

PERCENT A_{260} AND ^{14}C-CHOLINE IN EACH FRACTION

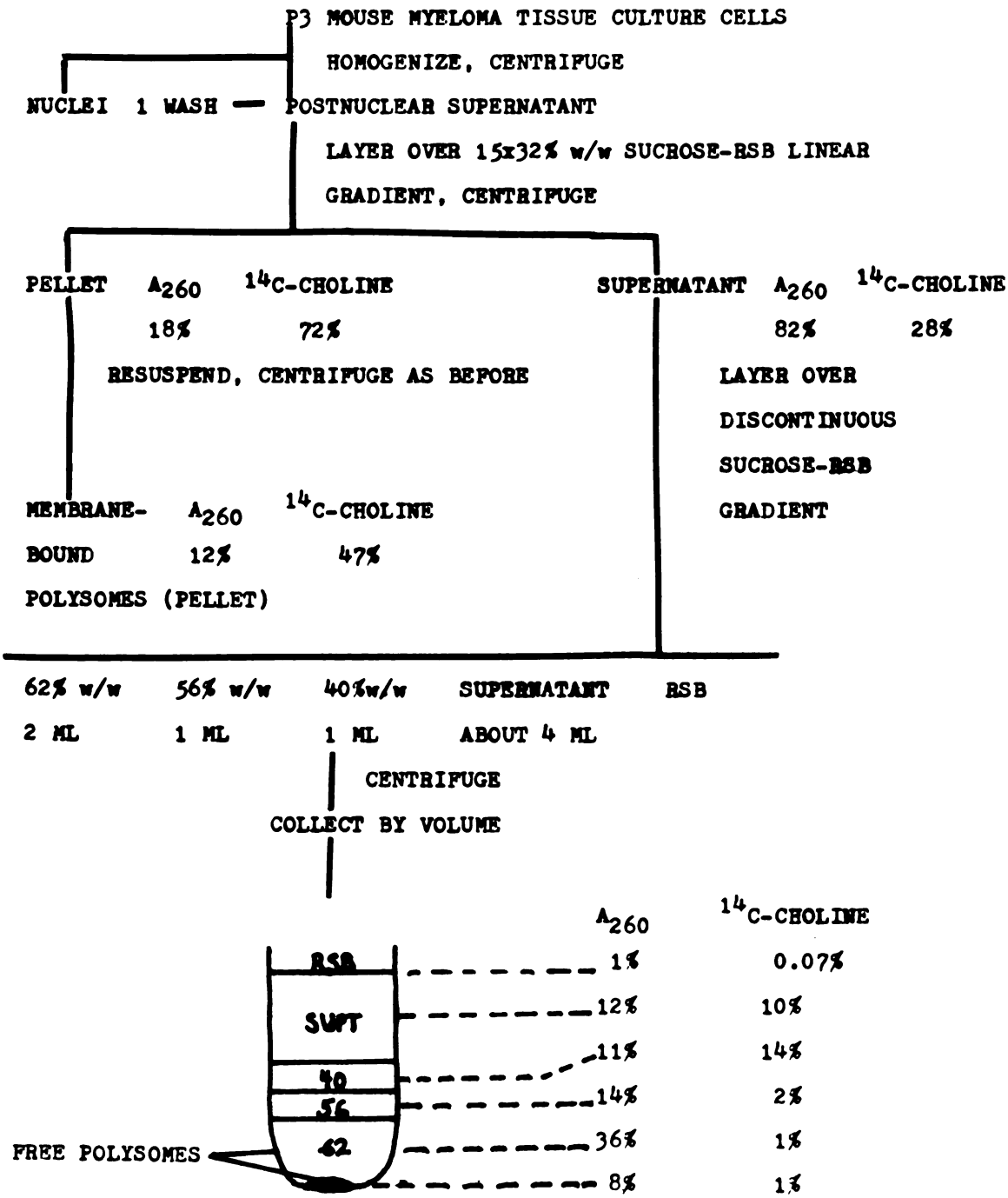

Figure 1

Figure 2. Immunoprecipitable polypeptides synthesized by detached polysomes. Immunoprecipitable polypeptides synthesized by detached polysomes are shown in slot A. For comparison the secreted H and L chains are shown in slot B. pL: precursor L chain.

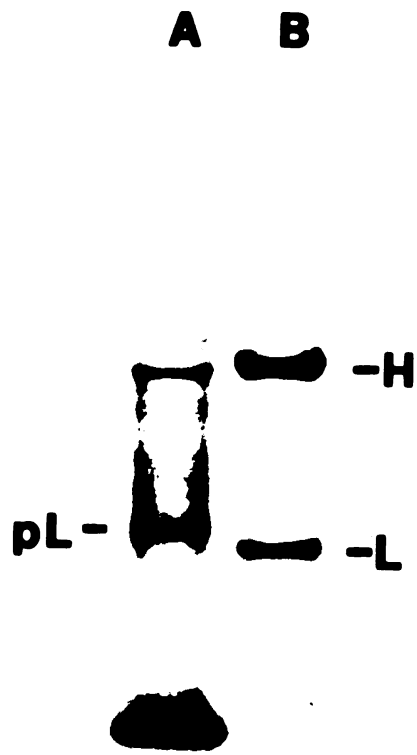

Figure 2

Figure 3. Immunoprecipitable polypeptides synthesized by free and membrane-bound polysomes. Shown are the immunoprecipitable labeled cell-free products synthesized by free polysomes (slots F) and membrane-bound polysomes (slots M). For comparison labeled secreted H and L chains are shown in slots S, and immunoprecipitable products synthesized by detached polysomes are shown in slots D. The film was overexposed to increase the visibility of the cell-free product synthesized by membrane-bound polysomes.

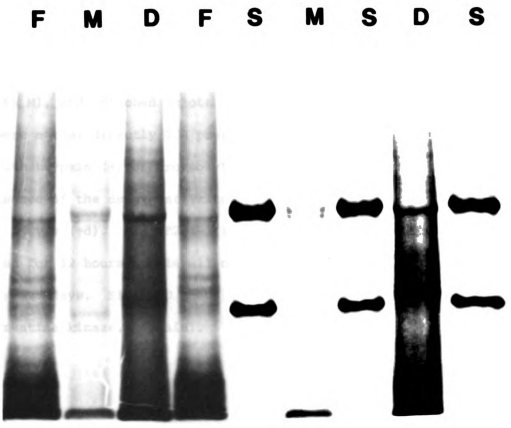

Figure 3

Figure 4. Proteolytic digestion of polypeptides synthesized by free, membrane-bound, and detached polysomes. Shown are the labeled products synthesized by free (slots F and F2), membrane-bound (slots M), and detached (slots D) polysomes. The cell-free products were either directly TCA precipitated (-), proteolytically digested with trypsin (+) or proteolytically digested with trypsin in the presence of the detergent Triton X-100 at a final concentration of 1% v/v (+d). Slot F2 (-) is slot F (-1), but from a film exposed for 12 hours, while all other slots were from a film exposed for 6.5 days. Slot STD shows [^{14}C]labeled standards (BSA, H chain, creatine kinase, L chain).

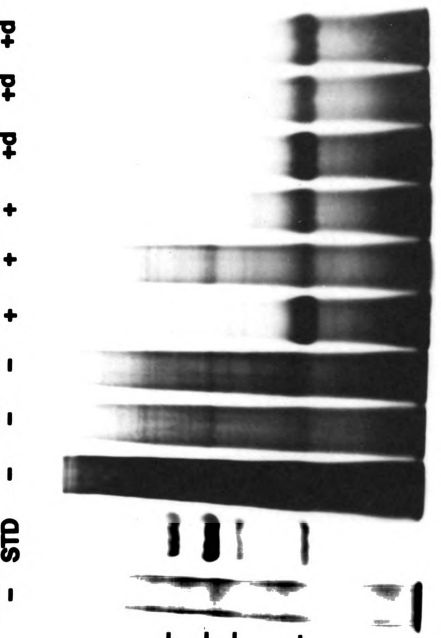

Figure 4

Figure 5. Time course of incorporation of L-[4,5-^{3}H]leu into total intracellular (———) and secreted (· · ·) protein at two different initial cell densities: 4.7 x 10^{5} cells/ml (●) and 1.3 x 10^{6} cells/ml (○). Logarithmically growing cells were pelleted and resuspended at the indicated cell densities in Dulbecco's medium plus 10% FCS (4.7 x 10^{5} cells/ml) or 10% dialyzed FCS (1.3 x 10^{6} cells/ml) plus streptomycin, penicillin, mycostatin, but minus leucine. The cells were labeled with [^{3}H]leu (4.7 x 10^{5} cells/ml:10 µCi/ml, L-[4,5-^{3}H]leu, 105 Ci/mmol; 1.3 x 10^{6} cells/ml:5 µCi/ml, L-[4,5-^{3}H]leu, 50 Ci/mmol), and samples were withdrawn periodically for analysis of total TCA precipitable intracellular and secreted protein.

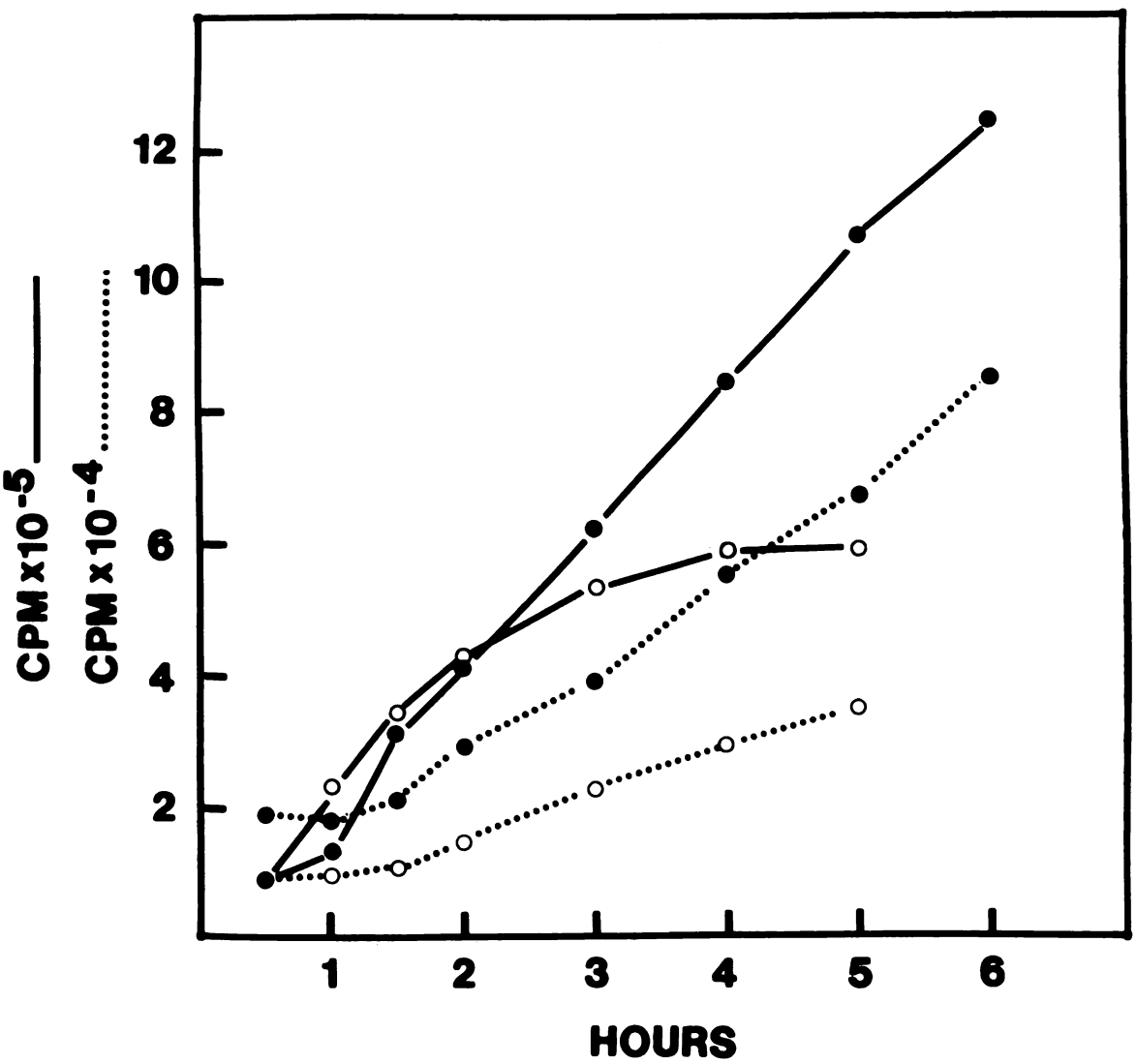

Figure 5

Figure 6. A) Time course of incorporation of L-[4,5-^{3}H]leu into intracellular (———) and secreted (· · ·) IgG1 P3 myeloma protein at an initial cell density of 4.7 x 10^{5} cells/ml. B) Percentage of intracellular protein immunoprecipitated during incorporation experiment shown in A. Cells were labeled as described in the legend to Figure 5. Immunoprecipitation was performed as described in Materials and Methods and in the legend to Table II, except that nuclei were pelleted for 5 min at 900 x g max.

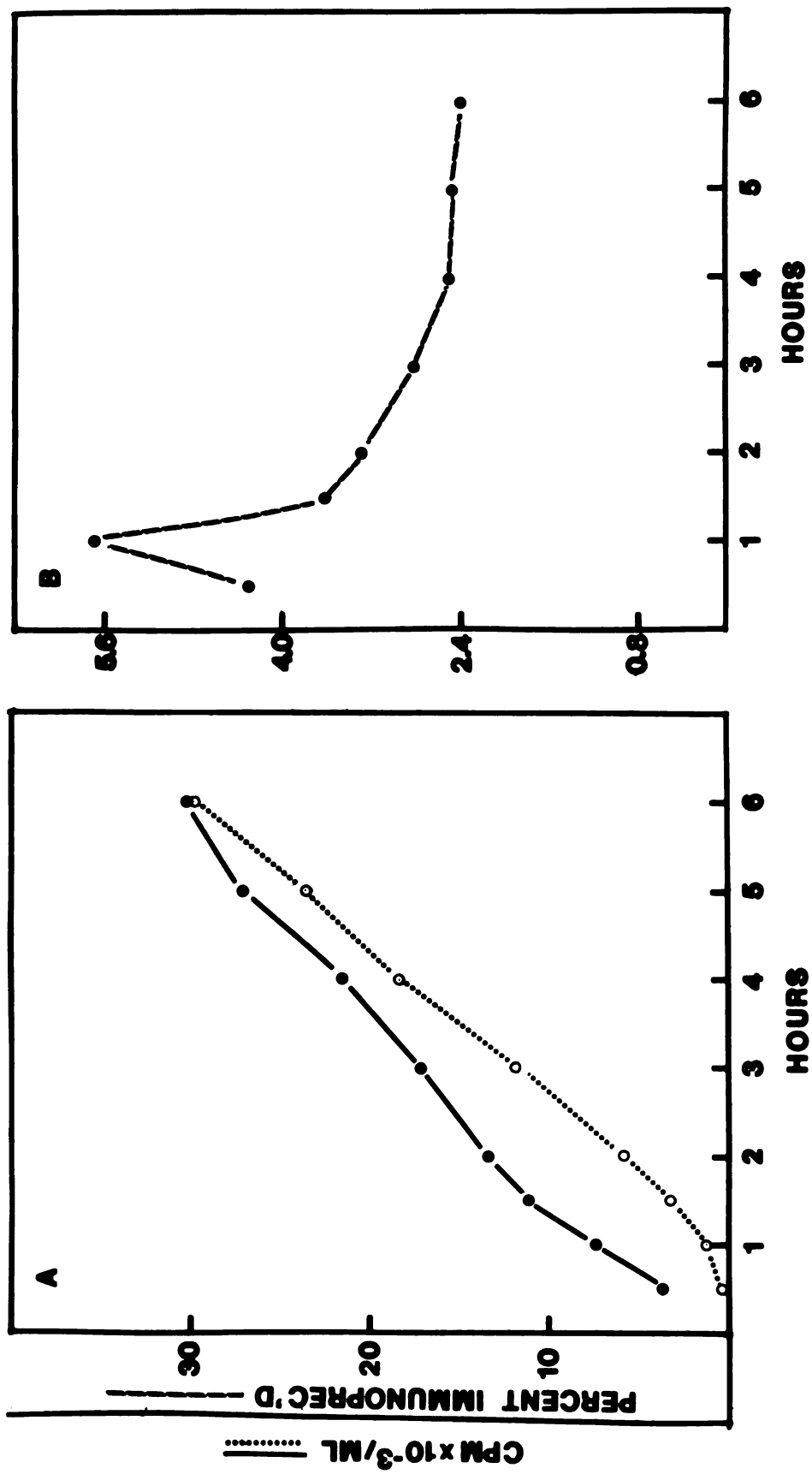
A
B
HOURS
HOURS
PERCENT IMMUNOPREC'D
CPM x 10⁻³/ML
30
20
10
5.0
4.0
2.4
0.8
1
2
3
4
5
6

Figure 6

Figure 7. Immunoprecipitable polypeptides synthesized *in vivo*. Shown are polypeptides immunoprecipitated in a double-label pulse-chase experiment as described in the legend to Table II. Slot numbers refer to time post-labeling (min) as in Table II. Slot S contains the immunoprecipitable IgG H and L chains secreted during 180 min of double labeling (no chase).

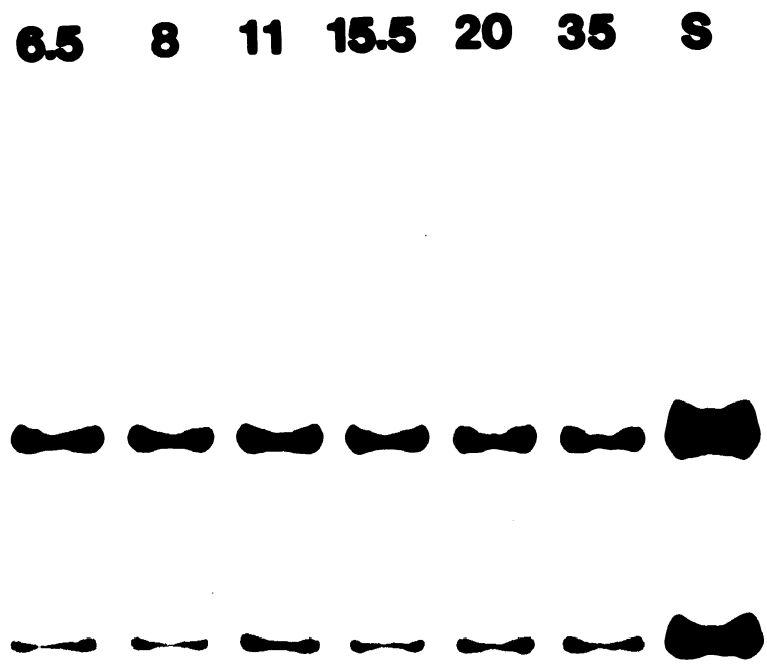

Figure 7

ARTICLE 2

EFFECTS OF HEPARIN ON FREE AND MEMBRANE-BOUND POLYRIBOSOMES

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SUMMARY

Free and membrane-bound polysome fractions were incubated with 1.0 mg/ml heparin, and the resulting polysome profiles were displayed on sucrose-RSB gradients. The major effects of heparin on free polysomes included a reduction in the size of large polysomes or aggregates, and enhanced resolution of ribosomal subunits, monosomes, and polysomes. Incubation of membrane-bound polysomes with heparin caused the release of material which migrated in the polysome, monosome, and subunit regions of the gradient. The released material corresponded to approximately one half that which could be released in the presence of 1.0 mg/ml heparin plus a final concentration of 1.0% v/v Triton X-100. The action of heparin appeared to be related to its polyanionic nature.

INTRODUCTION

Using several procedures to disassemble polysomes *in vivo* (1,15,16) or *in vitro* (4,8,11) a direct association of eukaryotic mRNA with rough endoplasmic reticulum has been demonstrated. When tested, this association was found to involve a 3' poly(A) region-membrane connection (4,8,11). Kruppa and Sabatini (7) dispute the results of Cardelli et al. (4) which indicated a rat liver mRNA-membrane attachment. As an initial step in the isolation of rough microsomes, Kruppa and Sabatini (7) added heparin to a final concentration of 0.5-1.0 mg/ml. Using similar concentrations of heparin, we show that heparin affects the polysome profiles of both free and membrane-bound polysomes. In particular, our data suggest that

heparin causes the release of some P3 polysomes from membrane. We comment on the relevance of these data to the findings of Kruppa and Sabatini (7).

MATERIALS AND METHODS

Cell Maintenance and Isotopic Labeling: The IgG1 secreting mouse myeloma tissue culture line P3 (kindly provided by Dr. Matthew D. Scharff, Albert Einstein College of Medicine) was maintained in Dulbecco's modified medium (Grand Island Biological Co.) supplemented with 10% fetal calf serum, and 74.0 µg streptomycin, 100 units penicillin, 40 units mycostatin per ml. P3 cells at 7-8.5 x 10^5 cells/ml were diluted with one volume of fresh medium and incubated for 4 hours with 1 µCi/ml [5,6-^{3}H]uridine (Amersham, 49 Ci/mmol) immediately before isolation of polysomes. Identically treated unlabeled cells (2.4 x 10^8) were added to 8 x 10^7 labeled cells before polysome isolation in order to obtain enough polysomes for absorbance profiles.

Isolation of Polysome Fractions: The cells were rapidly cooled by pouring over crushed, frozen saline. All subsequent procedures were performed at 0-4°C. The cells were pelleted by centrifugation for 8 min at 500 x g max, washed once with RSB (20 mM HEPES, pH 7.5, 10 mM Na^+-NaCl plus NaOH used to pH the HEPES, 3 mM $MgCl_2$), resuspended in RSB and allowed to swell for 7 min. The cells were then pelleted, resuspended in 15% w/w sucrose (ribonuclease-free, Schwarz/Mann)-RSB and immediately homogenized in a Dounce homogenizer (Kontes Co.) with ten strokes of the B (loose) pestle. Nuclei were pelleted from the homogenate by centrifugation for 5 min at 900 x g max and washed once with 15% w/w sucrose-RSB. The wash was added to the first postnuclear supernatant which was then used as the source of polysomes. The postnuclear supernatant was layered over 2 ml of a 15% x 32% w/w sucrose-RSB linear gradient (13) which was centrifuged in an SW 50.1 rotor (Beckman) for 45 min at 27,000 x g max (15,000 rpm). The supernatant was stored at -80°C and used as a source of postmicrosomal polysomes. The pellet was resuspended in 15% w/w sucrose-RBS and centrifuged as before. The pellet was resuspended in 15% w/w sucrose-RSB and stored at -80°C for use as a source of membrane-bound polysomes.

Sucrose Gradients: Polysomes were analyzed on 15% x 40% w/w sucrose-RSB linear gradients consisting of 4.4 ml of gradient formed over a 0.5 ml cusion of 62% w/w sucrose-RSB. The gradients were centrifuged at 4°C for 40 min at 243,000 x g max (45,000 rpm) in the SW 50.1 rotor (Beckman). Fractions of 0.4 ml were collected from the top using an ISCO Model 640 density gradient fractionator. Absorbance at 254 nm was monitored continuously. Fractions were collected into scintillation vials and counted in 5 ml of toluene, Triton X-100, water (6:3:1) plus Omnifluor (New England Nuclear).

RESULTS

Membrane-bound polysomes were obtained from P3 myeloma cells by differential centrifugation of a postnuclear supernatant. Polysome profiles were displayed on linear sucrose-RSB gradients. The membrane-bound polysomes did not exhibit a polysome profile in the polysome region of the gradient (Fig. 1). Membrane-bound polysomes that had been incubated with 1.0 mg/ml heparin released material which migrated in the polysome region of the gradient (Fig. 1) and corresponded to approximately one-half of the material which could be released in the presence of 1.0 mg/ml heparin plus a final concentration of 1.0% v/v Triton X-100 (Table I).

Membrane-bound polysomes incubated with 1.0 mg/ml of the polyanion dextran sulfate released approximately the same quantity and type of material as that released by 1.0 mg/ml heparin (data not shown). Incubation with 1.0 mg/ml of the polycation spermine did not result in the release of any material from the membrane-bound polysomes (data not shown).

The polysome profile of polysomes which did not sediment with the membrane-bound polysomes (i.e., polysomes in the postmicrosomal supernatant) also was affected by incubation with 1.0 mg/ml heparin (Fig. 2). Three major effects were observed: 1) the apparent size of the large polysomes or aggregates was reduced, 2) a shoulder merging with the monosome peak on the polysome side of the profile was noticeably absent, and 3) a shoulder merging with the monosome peak of the subunit side of the gradient was noticeably absent. Similar effects were observed on the profiles of postmicrosomal supernatant polysomes that had been further purified by sedimentation into or through 62% w/w sucrose-RSB (data not shown). Triton X-100

alone (final concentration, 1%) had no noticeable effect on the polysome profile of any fraction of postmicrosomal polysomes (data not shown).

DISCUSSION

We have shown that incubation with 1.0 mg/ml of heparin affects the polysome profile of both free and membrane-bound polysome fractions. Heparin appears to allow better resolution of the subunit and ribosome composition of the free polysome fraction. We speculate that heparin may be releasing polysome components that associate with a cytoskeletal structure similar to that observed by Lenk et al. (9).

At a concentration of 1.0 mg/ml, heparin releases approximately one-half of the membrane-bound polysomes that are detached by heparin plus Triton X-100 (final concentration, 1%). Dextran sulfate (a polyanion) has approximately the same effect as heparin, while the polycation spermine causes no measurable release of polysomes. Thus the action of heparin presumably is due to its polyanionic nature (e.g., see reference 3).

We speculate that the polysomes released from membrane were bound to membrane through fewer than two ribosomes, and possibly through the 3' end of the mRNA. This would be consistent with the reported RNase catalyzed release of a large portion of P3 membrane-bound polysomes (10). In any case, if undegraded polysomes were released from membrane by heparin and if those polysomes were bound to membrane in part through mRNA-membrane interaction (10), then this release implies the disruption of the mRNA-membrane linkage. Other evidence suggests, however, that heparin does not remove all membrane-bound ribosomes susceptible to RNase catalyzed release

(perhaps it creates new susceptible ribosomes), nor does it cause an increase in the percentage of large polysomes bound to membrane (Freidlin and Patterson, unpublished data). Another observation consistent with membrane attachment being mediated by only one or two ribosomes of a P3 polysome, and perhaps the mRNA, is that detached P3 polysomes do not produce authentic L chain in addition to the expected precursor L chain (6,12,Freidlin and Patterson, manuscript submitted for publication). This contrasts with the situation in other cell lines in which more ribosomes per polysome appear to be bound to membrane so that a detached polysome contains nascent chains (or a larger proportion of each chain) which have already been processed by signal peptidase (2,14).

Our data also suggest a way to reconcile contrasting results on the binding of rat liver mRNA to rough endoplasmic reticulum (4,7). Cardelli et al. (4) detected a direct association of mRNA with membrane while Kruppa and Sabatini (7) presented evidence that the mRNA was not directly attached. As an initial step in the isolation of rough microsomes, Kruppa and Sabatini (7) added heparin to a final concentration of 0.5-1.0 mg/ml. Our results suggest that this concentration of heparin may have been sufficient to disrupt possible mRNA-membrane linkages. This would have allowed Kruppa and Sabatini (7) to release mRNA from membrane under conditions which did not result in release for Cardelli et al. (4). Cardelli et al. (5) also reported release of mRNA from rat liver RER that had been washed with 1 mg/ml heparin. They found more release than they previously reported (4) but apparently not as much as reported by Kruppa and Sabatini (7).

Heparin may merely release contaminating free polysomes from the microsomal fraction, but this is unlikely since centrifugation of the membrane-bound polysome fraction through a linear sucrose gradient does not produce the polysome profile which would be characteristic of significant contamination by free polysomes.

Our results suggest that at least for P3 myeloma cell homogenates, the presence of polyanion nuclease inhibitors such as heparin or dextran sulfate could cause artifactual accumulation of detached polysomes in the free polysome fraction.

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TABLE I

RELATIVE DISTRIBUTION OF ^{3}H-URIDINE LABELED MATERIAL RELEASED FROM P3 ROUGH MICROSOMES

Fractions	Gradient Region	Relative Distribution (% CPM) Following Treatment with: Nothing (Control)	Heparin	Heparin + TX-100
1-3	Subunits-Monosomes	19.5	39.1	60.8
4-9	Polysomes	6.8	20.0	34.4
10-14	Membrane-bound Polysomes	73.7	40.9	4.8

Samples of the membrane-bound polysome preparation were incubated (at final concentrations of 22.5 A_{260}/ml) for 10 min at 4°C with RSB (control), 1.0 mg/ml heparin (sodium salt), or 1.0 mg/ml heparin plus 1% v/v Triton X-100. The treatment and control samples of the membrane-bound polysome preparation were analyzed on linear sucrose gradients (3.1 A_{260}/gradient). The gradient regions were determined by correlating the radioisotope distribution with the absorbance profiles shown in Fig. 1.

FIGURE 1. Profiles of P3 membrane-bound polysome fractions incubated for 10 min at 4°C with RSB (control, ———), 1.0 mg/ml heparin (-----), or 1.0 mg/ml heparin plus 1% v/v Triton X-100 (— — — —).

FIGURE 2. Profiles of P3 postmicrosomal polysome fraction incubated for 10 min at 4°C with RSB (control, ———) or 1.0 mg/ml heparin (-----). The concentration of the postmicrosomal fraction during incubation was 11.4 A_{260}/ml. Treated and control samples were analyzed on linear sucrose gradients (1.6 A_{260} control sample, 1.1 A_{260} heparin-treated sample).

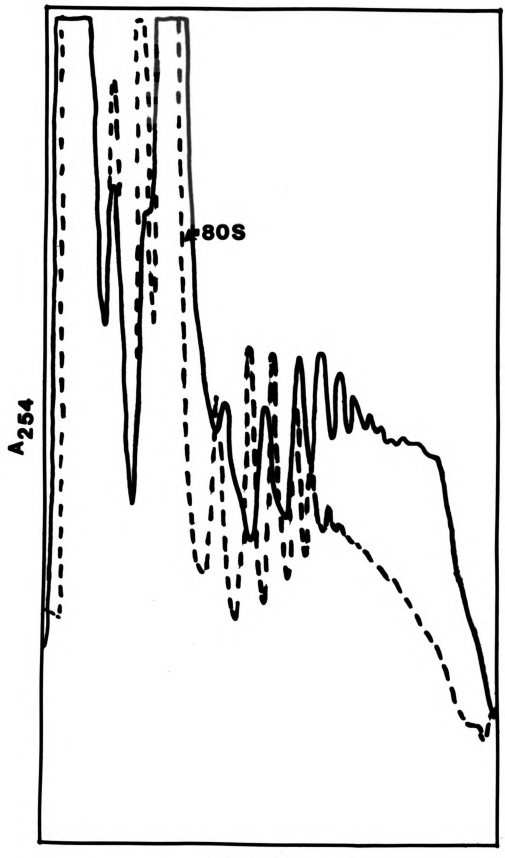

Figure 2