Studies of Rat Liver Polyribosomes and RNA Metabolism

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Barry M.R.N.J. Woloski

A Thesis Submitted to
the Faculty of Graduate Studies and Research
of the University of Manitoba
in partial fulfillment
of the requirements of the degree
Master of Science

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Abstract

The significance of segregation of mammalian polyribosomes into bound and free classes is a topic of research capable of lending insight into many biochemical processes. This segregation seems to be important in consideration of evidence for preferred synthesis of secretory proteins on bound polyribosomes and intracellular proteins on free polyribosomes. The effect of induction of acute phase reactant synthesis was examined on rat hepatic RNA metabolism and the hepatic distribution of polyribosomes. Emphasis was placed on α_1-acid glycoprotein as a typical example of an acute phase reactant. The effects on polyribosome distribution of induction of ferritin synthesis by iron administration and metallothionein synthesis by zinc administration were also examined. Ferritin and metallothionein were considered to be examples of intracellular proteins.

Quantitation of bound and free polyribosome levels revealed that acute inflammation resulted in a shift of polyribosomes into the bound class as a result of increased levels of heavier aggregates of polyribosomes 18 h after inflammation. Induced synthesis of intracellular proteins did not induce a similar shift of polyribosomes into the free class.

Synthesis of α_1-acid glycoprotein and albumin were shown to occur preferentially on bound polyribosomes by immunochemical reactivity of nascent polypeptide chains on polyribosomes. These studies showed that inflammation elevated α_1-acid glycoprotein polyribosomal mRNA levels. Studies using an *in vitro* translation system suggested

preferential synthesis of α_1-acid glycoprotein on bound polyribosomes. Studies of orotate incorporation showed that rRNA levels increased as early as 4 h, while mRNA levels increased about 8 h after inflammation.

Changes in the levels of mRNA transcripts alone could not account for all the observed changes in polyribosome distribution. Assays of total alkaline ribonuclease levels showed that while this enzyme may be important to hepatic polyribosome distribution during acute inflammation, it does not account for the changes following iron and zinc administration.

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Finally, I am indebted to the National Research Council of Canada, the University of Manitoba and the Department of Chemistry, University of Manitoba for financial assistance.

List of abbreviations used

A	adenine
AA-tRNA	aminoacylated transfer RNA
ATP	adenosine triphosphate
DNA	deoxyribonucleic acid
dT	2'-deoxythymine
GTP	guanosine triphosphate
hnRNA	heteronuclear RNA
mRNA	messenger RNA
mRNP	messenger ribonucleoprotein particle
RNA	ribonucleic acid
rRNA	ribosomal RNA
tRNA	transfer RNA

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Introduction

Site of protein synthesis

The mammalian liver is an organ capable of modulating many biochemical and physiological changes that mammals are required to contend with. Biochemical mechanisms which accomplish these changes often involve biosynthesis of intracellular proteins and proteins which are secreted into the plasma. Control mechanisms for the biosynthesis of these proteins are not fully understood at this time.

Proteins are synthesized on polyribosomes formed by the aggregation of ribosomal subunits on strands of mRNA. There are two classes of mammalian polyribosomes, bound polyribosomes which are attached to the endoplasmic reticulum and free polyribosomes which are found in the cytosol. Examination of the site of synthesis of proteins (1-3), as is shown in Table 1, leads to the conclusion that secretory proteins are synthesized primarily on bound polyribosomes. Evidence for stating that intracellular proteins are synthesized on free polyribosomes is not as convincing.

The significance and consequences of the segregation of polyribosomes into two classes is a topic of research that is capable of lending insight into many other biochemical processes. Secretory proteins must pass through a membrane before they are secreted into the sinusoids of the liver. Modifications such as partial proteolysis, glycosylation and hydroxylation often occur before secretion. Both secretion and post-translational modification are processes associated with membrane structures and as such localization of secretory

Table 1

Synthesis of Proteins on Free and Bound Polyribosomes

Protein*	Synthesis of protein per mg RNA in bound:free polyribosomes
Secretory Proteins	
Serum proteins	7:1
Albumin	2.5:1
Albumin	1:5
Albumin**	24:1
α-globulins	4:1
α-globulins	77:1
β-lactoglobulin	20:1
α_1-acid glycoprotein***	6.6:1
Intracellular Proteins	
Ferritin	1:1.5
Ferritin	1:5
Arginase	1:7.7
Myosin	1:5
Serine dehydrogenase	1.2:1
Serine dehydrogenase	15:1
Catalase	1:1
Catalase	1:1
Catalase	1:1
Membrane Proteins	
NADP-cytochrome c-reductase	1:1
NADP-cytochrome c-reductase	0.14-1.5:1

* Unless otherwise noted, examples were taken from (1) which cites the work of several authors. Different values cited represent differences in tissues, assay techniques or physiological states.
** Taken from (2) where albumin mRNA was directly assayed.
*** Taken from (3).

protein synthesis on membrane-bound polyribosomes has a special significance. Similarly, localization of the synthesis of intracellular proteins, which are neither secreted nor modified as secretory proteins are, would seem to have a significance. There are many factors which determine the distribution of ribosomal RNA between bound and free polyribosomes as well as between ribosomal subunits and polyribosome aggregates of various sizes. If, under a set of conditions, the distribution of ribosomal RNA is controlled primarily by the mRNA transcripts initiating protein synthesis, then analyses of polyribosomes could provide some insight into mRNA metabolism. Experiments with animals in other physiological states could provide insight into the capacity of the liver to meet requirements for protein synthesis and the mechanisms by which the translation process is controlled. The work presented in this thesis has attempted to more fully elucidate these aspects of the biochemistry of the mammalian liver.

Mammalian RNA metabolism

RNA metabolism in mammalian systems involves a complex interrelationship of all forms of nucleic acids as is shown in Figure 1. Species of RNA are transcribed as precursors which are later processed in the cell nucleus into forms that are active in the cytoplasm. Both the transcriptional and processing events offer opportunity for regulation of later cytoplasmic events. Transcription and processing of mRNA are of special relevance to the topic of differential expression of genes. Transcription of mRNA has been reviewed by Goldberger

Fig. 1. General Scheme of Mammalian RNA Metabolism

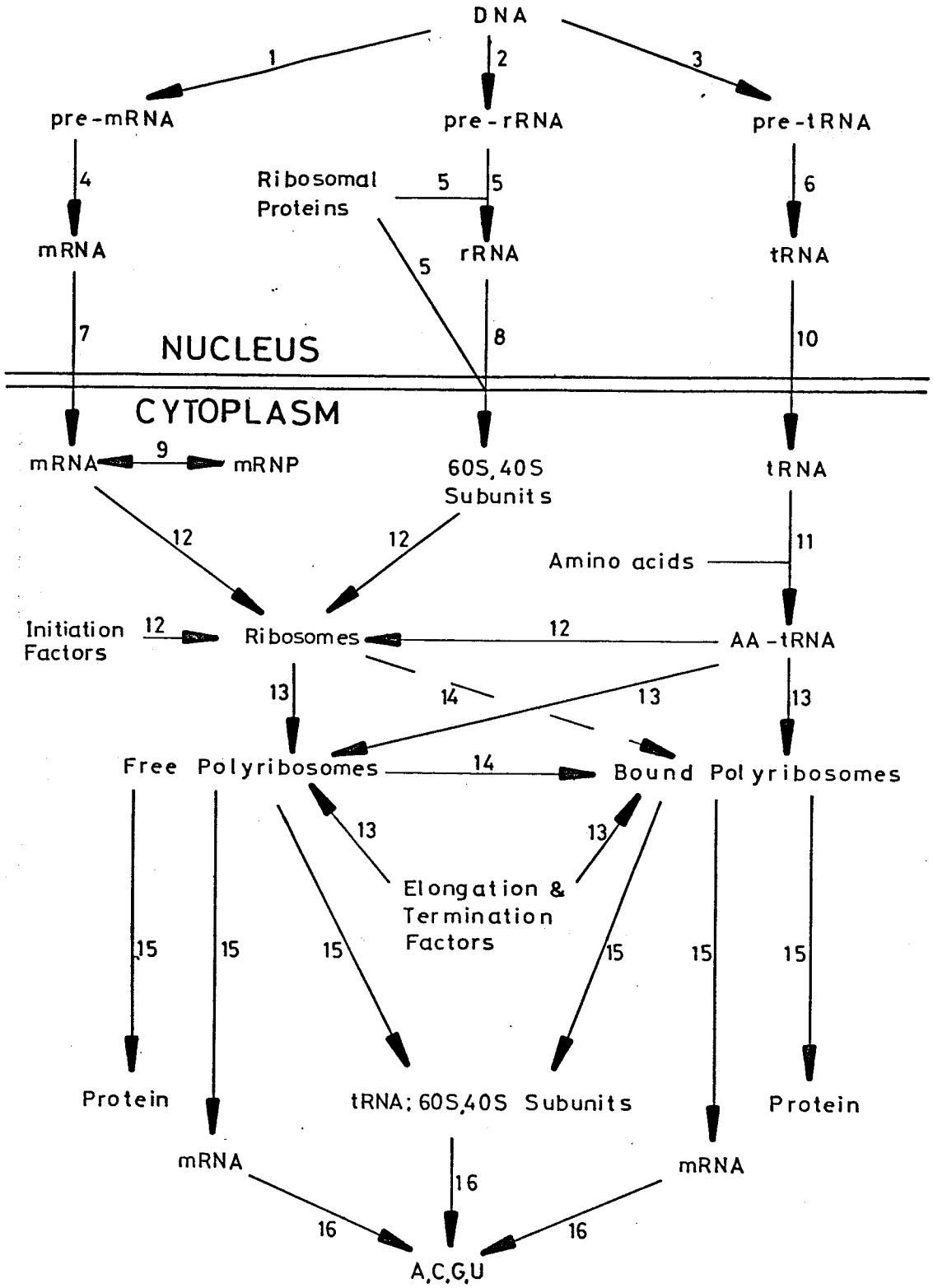
Adapted primarily from the text of (7) and (8)

Abbreviations used are:

pre	precursor form of RNA
mRNA	messenger RNA
rRNA	ribosomal RNA
tRNA	transfer RNA
A,C,G,U	nucleotides
mRNP	messenger ribonucleoprotein particle
60S & 40S	large and small ribosomal subunits
AA	amino acyl unit

Processes involved in this scheme are:

1,2,3	transcription
4,5,6	RNA processing
7,8,10	RNA transport across nuclear membrane
9	mRNA-protein association/dissociation
11	amino acylation of tRNA
12	initiation of polypeptide synthesis
13	elongation of polypeptides
14	segregation of bound polyribosomes
15	termination of polypeptide synthesis
16	cytoplasmic RNA degradation


DNA
1
2
3
pre-mRNA
pre-rRNA
pre-tRNA
4
Ribosomal Proteins
5
5
6
mRNA
rRNA
tRNA
5
7
NUCLEUS
8
10
CYTOPLASM
mRNA
9
mRNP
60S, 40S Subunits
tRNA
12
12
Amino acids
11
Initiation Factors
12
Ribosomes
12
AA-tRNA
13
14
13
13
Free Polyribosomes
14
Bound Polyribosomes
13
13
Elongation & Termination Factors
15
15
15
15
15
15
Protein
tRNA; 60S,40S Subunits
Protein
mRNA
16
mRNA
16
16
A,C,G,U

(4), Stein et al. (5) and Potter (6). The topic of processing of mRNA has been reviewed by Greenberg (7) and Revel and Groner (8).

Primary transcripts of mRNA or pre-mRNA are generally accepted (7) to be heteronuclear RNA (hnRNA). Molecules of hnRNA may be larger than 10^7 daltons while mRNA molecules are generally smaller than 2×10^6 daltons. The majority of hnRNA does not enter the cytoplasm but rather is degraded in the nucleus. Those molecules of mRNA that are transported into the cytoplasm are not necessarily translated as a matter of consequence. Control can be exerted over the processes of initiation, elongation and termination of polypeptide synthesis, as well as degradation of mRNA which will prevent entry or re-entry into the pool of translatable mRNA.

It is known that mRNA may bind specific classes of protein. There have been a number of functions suggested for these messenger ribonucleoprotein particles (mRNP) which include involvement in messenger storage, transport and initiation (9). These mRNP have sedimentation properties similar to ribosomal subunits (7) and thus mRNP and ribosome subunits are indistinguishable under some conditions for sedimentation analyses of polyribosome populations. It has been suggested that binding of specific proteins may result in an untranslatable mRNP, whereas dissociation of binding proteins results in translation of the free mRNA transcript (7). Translation of rat liver ferritin may be controlled post-transcriptionally in this manner (10). There also is evidence to suggest that proteins associated with mRNA transcripts may be involved in other ways in the translation process (9).

One of the differences between eukaryotes and prokaryotes is that eukaryotes have polyribosomes which are bound to the endoplasmic reticulum while prokaryotes do not have this membrane structure. However, it should not be forgotten that there is evidence that the synthesis of some bacterial proteins occurs with the nascent chain attached to the cell membrane (11). The experimental data presented in Table 1 suggests that segregation of polyribosomes into free and bound classes is a process which may be capable of determining the fate of proteins.

Many of the features of cytoplasmic mRNA are acquired during the maturation process (8). The enzymic activities involved in the processing events are presented in Figure 2. The primary transcripts of mRNA contain sequences which will appear in the processed mRNA separated by intervening sequences, which will be cleaved from the molecule. A stretch of fifty to two hundred adenine residues is added to the 3'-terminus of the primary transcript as a post-transcriptional event. This may occur as a nuclear event prior to the processing of hnRNA, or as a cytoplasmic event (8). It is currently thought that at an early step of the maturation process, some internal adenine residues are methylated at the 6 position of the nitrogenous base. A guanine residue is also added at the 5'-terminus and then methylated at the 7 position of the nitrogenous base. Two nucleotides adjacent to the 5'-terminal guanine are methylated at the 2' position of ribose to form a "cap" structure with the 5'-terminus methylated guanine residue. The 5' cap structure has at least two functions in

Fig. 2. Maturation of messenger RNA

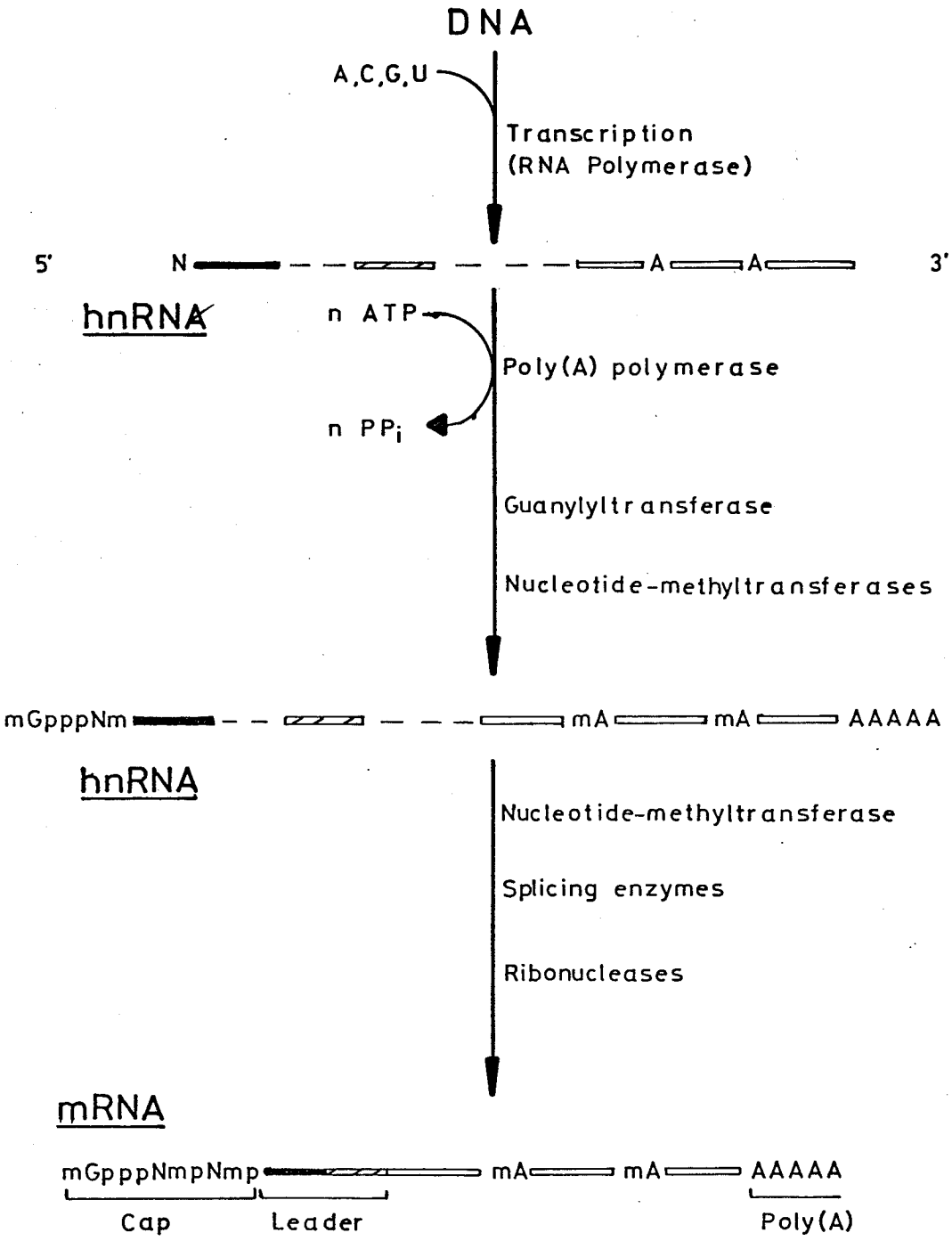
Adapted from (8)

Abbreviations used are:

DNA	deoxyribonucleic acid
hnRNA	heteronuclear ribonucleic acid
mRNA	messenger RNA
A	adenine
C	cytosine
G	guanine
U	uracil
N	ribonucleotide
ATP	adenosine triphosphate
PP_i	pyrophosphate
AAAAA	poly(A) tract
P	phosphate
m	methyl group, nitrogenous base is methylated if written on left side of the base, sugar is methylated if written on right

Symbols used are:

▭	translatable transcript
▬▨	leader sequence
— — —	sequences which are cleaved from hnRNA during processing


DNA
A,C,G,U
Transcription
(RNA Polymerase)
5'
N
A
A
3'
hnRNA
n ATP
Poly(A) polymerase
n PPi
Guanylyltransferase
Nucleotide-methyltransferases
mGpppNm
mA
mA
AAAAA
hnRNA
Nucleotide-methyltransferase
Splicing enzymes
Ribonucleases
mRNA
mGpppNmpNmp
mA
mA
AAAAA
Cap
Leader
Poly(A)

mRNA translation. The 5'-terminal methylated guanine is recognized during initiation and the cap itself protects the mRNA from 5'-exonucleolytic degradation (8). At a later stage of the maturation process, intervening sequences are cleaved out of the transcript to leave a "leader sequence" on the 5' side of the initiation codon. The leader sequence has been implicated in such processes as initiation and the binding of protein to form mRNP. The processed mRNA that results from the maturation process is the form of mRNA which will be transported into the cytoplasm to be translated.

Translation of messenger RNA

Initiation of protein synthesis is essentially a two step process as is shown in Figure 3. First, the initiation region of a mRNA transcript binds to a complex of 40S ribosomal subunit, initiation factor 2 and GTP. This process requires the initiation factors usually referred to as 1, 2, 3, 4A, 4B and 4C as well as the expenditure of energy as ATP. Binding of the 60S ribosomal subunit is the second step of initiation and requires initiation factor 5 which also has a GTPase activity. The 80S monoribosome formed by the aggregation of ribosomal subunits is incapable of protein synthesis without an adequate supply of AA-tRNAs and transfer factors.

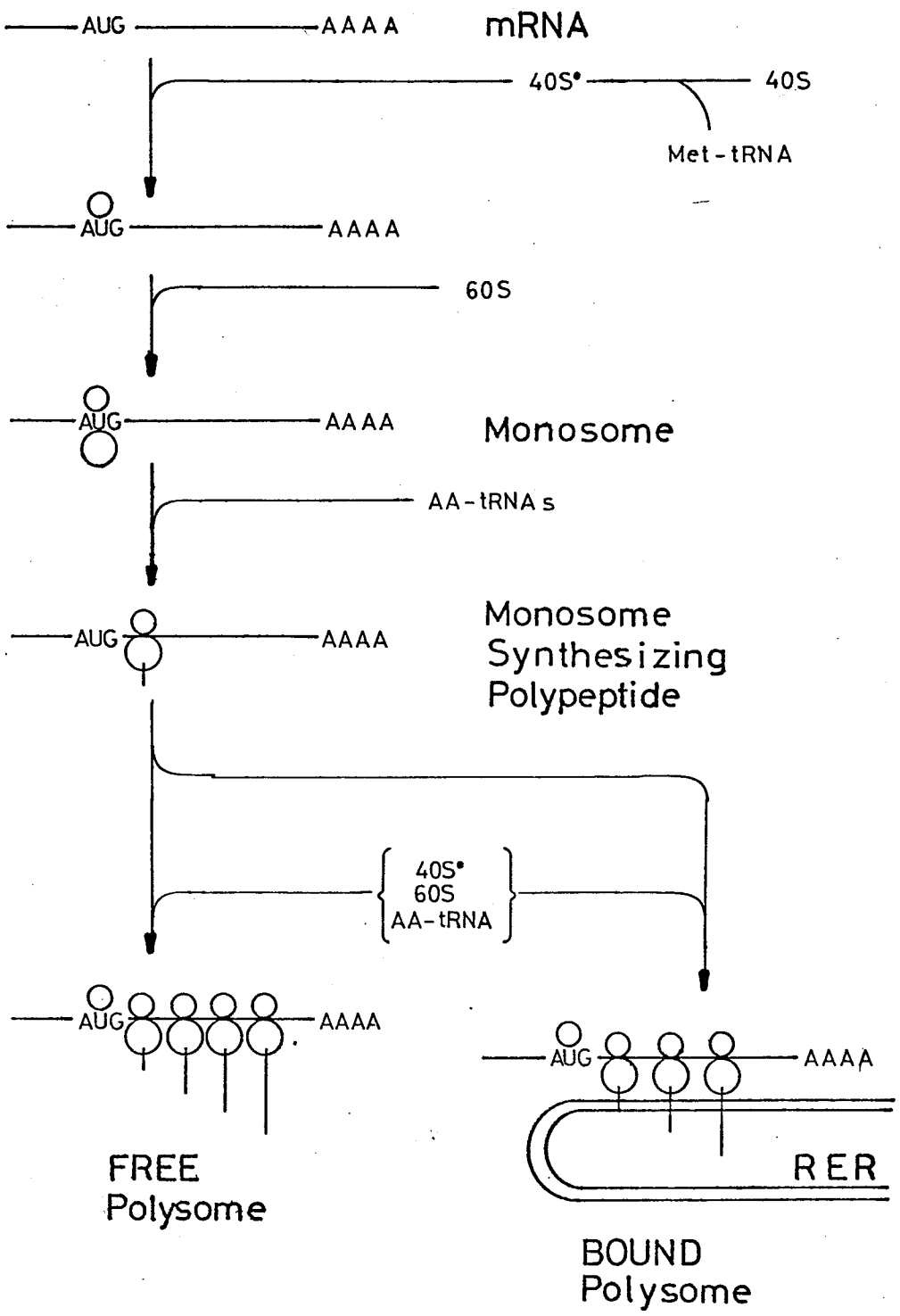
There is a growing amount of evidence that the initiation step of protein synthesis allows for a selection and discrimination of mRNA transcripts as is discussed by Revel and Groner (8). In a reticulocyte lysate translation system, increasing levels of hemoglobin mRNA decrease the ratio of synthesis of α- to β-hemoglobin. An

Fig. 3. Initiation and Translation of messenger RNA

Adapted from (8,12 & 15)

Abbreviations used are:

AUG	initiation codon
AAAA	poly(A) tract
40S	small ribosomal subunit
Met-tRNA	methionyl-tRNA
40S'	complex of 40S, Met-tRNA, GTP and initiation factor 2
60S	large ribosomal subunit
AA-tRNA	amino acyl-tRNA
RER	rough endoplasmic reticulum


AUG
AAAA
mRNA
40S*
40S
Met-tRNA
AUG
AAAA
60S
AUG
AAAA
Monosome
AA-tRNAs
AUG
AAAA
Monosome
Synthesizing
Polypeptide
40S*
60S
AA-tRNA
AUG
AAAA
FREE
Polysome
AUG
AAAA
RER
BOUND
Polysome

explanation invoked to explain this observation relies on the argument that α-globin mRNA depends on cap recognition while β-globin mRNA depends on initiation factor 4B binding during the initiation event. Other examples of apparent selection and discrimination support models of initiation control.

Elongation of polypeptides requires amino acyl tRNAs, elongation or transfer factors and the expenditure of energy as GTP. Elongation factor 1, which previously had been referred to as transferase 1, is responsible for the transfer of AA-tRNA into the acceptor site on the ribosome. A component of the large ribosomal subunit catalyzes the formation of the peptide bond and elongation factor 2 translocates the peptidyl-tRNA into the donor site of the ribosomes. A more detailed discussion is presented by Wainwright (12).

As previously mentioned, polyribosomes synthesizing polypeptides can exist in one of two states, free in the cytosol or bound to a membrane such as the endoplasmic reticulum. Discussions on the nature of the interaction between ribosomes and membrane are presented by Rolleston (1), Shore and Tata (13) and McIntosh and O'Toole (14). These authors discuss evidence to support the existence of interactions between components in the membrane and the leader sequence or poly(A) tract of mRNA, specific sites on ribosomal subunits or the nascent chain synthesized by the polyribosomes.

A model that offers an explanation for the synthesis of secretory proteins on bound polyribosomes (Table 1) has been proposed by Blobel and Dobberstein (15) and elaborated upon by Blobel (16). These workers

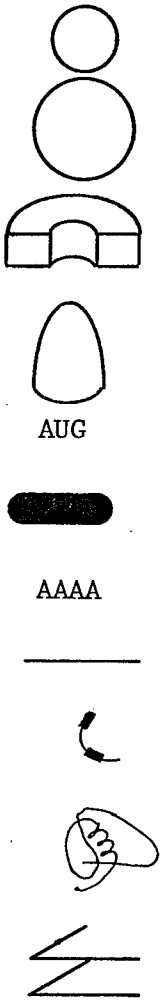
and others (15,17) have found that primary translation products of mRNA coding for secretory proteins are larger than the products secreted *in vivo*. The "signal" hypothesis proposed by Blobel and Dobberstein, and shown in Figure 4, proposes that there exist "signal" codons 3' to the initiation codon for a "signal" sequence which will interact with membrane proteins prior to completion of the nascent polypeptide chain. In this model, the "signal" peptide-membrane protein interaction brings about the aggregation of some membrane proteins to form a tunnel through which the nascent chain can pass into the lumen of the endoplasmic reticulum. It is also proposed that the interaction between the nascent chain and membrane proteins is further stabilized by a ribosome-membrane protein interaction. Blobel and Dobberstein found that during immunoglobulin synthesis, proteolysis of the "signal" peptide occurred prior to completion and release of the polypeptide from the ribosome and suggest that this may be an universal occurrence. This model has been extended by Blobel beyond the insertion of secretory proteins through the endoplasmic reticulum to include the insertion of membrane proteins into the endoplasmic reticulum and mitochondrial membrane, and the process by which the conformation of these proteins in these membranes is assumed (16).

A number of amino terminals of secretory proteins have been identified from the products of *in vitro* translation systems utilizing purified exogenous mRNA (15,17). These sequences are generally hydrophobic in nature, and are apparently cleaved before completion and release of the nascent chain from the ribosomes. In addition to these

Fig. 4. The "Signal" Hypothesis of Blobel and Dobberstein

Taken from (15)

Symbols used are:



small ribosomal subunit

large ribosomal subunit

membrane receptor protein for signal sequence

general membrane protein

initiation codon

signal codons

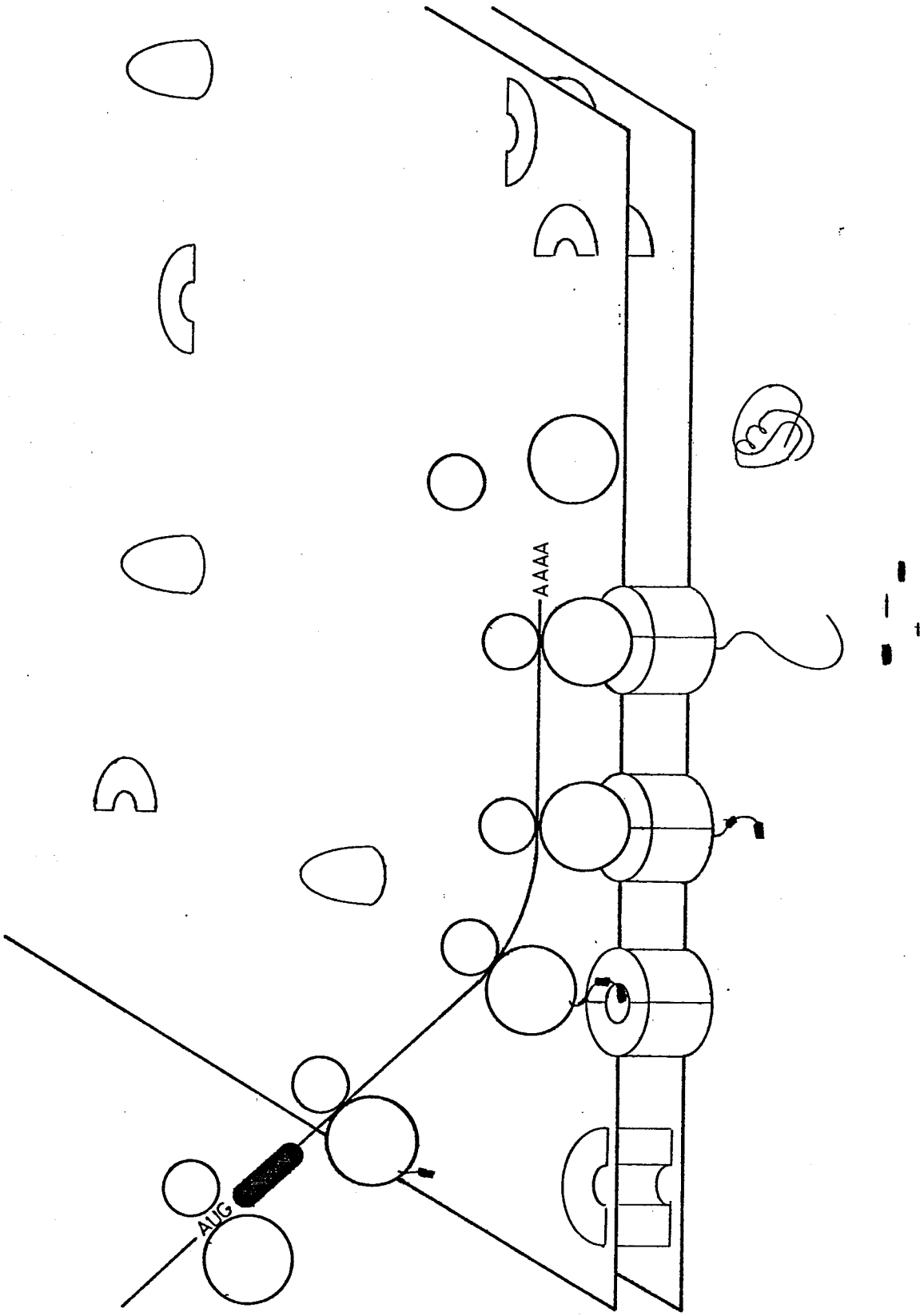
Poly(A) tract

mRNA

signal peptide

polypeptide

rough endoplasmic reticulum


AAAA
AUG

sequences, proteins such as albumin, parathyroid hormone, trypsin, insulin and glucagon are known to contain extra sequences which are cleaved from the translation products (17,19). Although no generally accepted nomenclature exists for precursor proteins, it has been suggested that the designation "pre" should be adapted for precursor proteins with a "signal region" cleaved by microsomal peptidases, whereas "pro" should refer to protein segments cleaved by either intracellular (localized in Golgi and condensing vacuole membranes) or extracellular peptidases (17). It has also been proposed that precursor proteins be grouped into four structural classes (17). Type 1 contains only a presegment, type 2 contains a presegment and an amino terminal prosegment, type 3 contains a presegment and an internal prosegment and type 4 contains a presegment and a carboxyl terminal prosegment. Examples of these sequences are shown in Table 2. Although the existence of presegments at amino terminals of translation products is explained by the signal hypothesis, not all prosegments share a common function. Prosegments seem to be intricately involved in controlling the biological activity of proteins such as insulin, collagen, trypsin and chymotrypsin. Functions also suggested for the prosegments of albumin include mediation of membrane binding, masking of binding sites, regulation of degradation or synthesis, and facilitation of tertiary structure formation.

The secretion of secretory proteins involves passage of the proteins from the rough endoplasmic reticulum to the smooth endoplasmic reticulum to the Golgi apparatus to secretory vesicles to the plasma

Table 2

Presegments

Serum Albumin (rat)	Met Lys Trp Val Thr Phe Leu Leu Leu Leu Phe Ile Ser Gly Ser Ala Phe Ser
Trypsin (dog)	Ala Lys Leu Phe Leu Phe Leu Ala Leu Leu Leu Ala Tyr Val Ala Phe
Insulin (rat)	X Leu Lys Met X Phe Leu Phe Leu Leu Lys Leu Leu X Leu X X X X X X X X
Ovomucoid (chicken)	Ala Met Ala Gly Val Phe Val Leu Phe Ser Phe Val Leu X Gly Phe Leu Pro Asp Ala Ala Phe Gly

Prosegments (type 2)

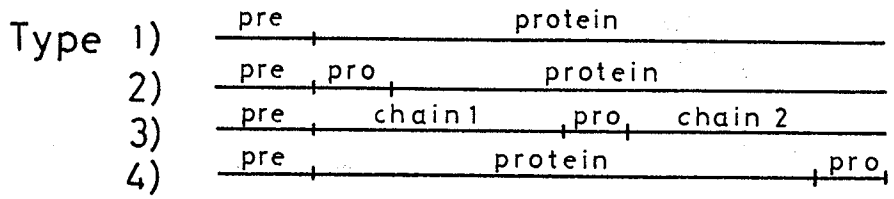
Albumin	Arg Gly Val Phe Arg Arg
Parathyroid Hormone	Lys Ser Val Lys Lys Arg

Prosegment (type 3)

Glycophorin	Arg Arg Leu Ile Lys Lys

Prosegment (type 4)

Glucagon	Thr Lys Arg Asn Lys Asn Asn Ile Ala



The amino end of these segments is on the left side of the Table while the carboxyl end of the segments is on the right side. The precursors are classified by the scheme shown immediately above. The code used to indicate the amino acids is: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartate; Gly, glycine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine; X, unidentified amino acid.

These sequences were taken from (17) and (19). See text for further explanation.