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Avian myeloblastosis virus RNA was fractionated into a high molecular weight RNA and a low molecular weight RNA fraction by sucrose density centrifugation. The larger RNA component had a sedimentation constant of 65S and the low molecular weight RNA about 4S. The low molecular RNA has two biological properties of interest. First, it behaves like tRNA in that it will attach amino acids in the presence of aminoacyl synthetases and second, it is a potent inhibitor of *in vitro* protein synthesis. The properties of this RNA fraction with regard to *in vitro* peptide synthesis were investigated in the *E. coli* and chick polysome cell-free systems. Peptide synthesis using a S30 preparation from *E. coli*, is inhibited about 50% with 0.5 μg of virus RNA when either Qβ phage RNA or *E. coli* RNA is used as a template. Peptide synthesis directed by the homopolymers poly U and poly A are not inhibited by the low molecular

weight virus RNA. When poly C is used as a template, proline polymerization is inhibited. Excess ribosomes added to the *in vitro* system did not reverse the inhibition by virus RNA. Increasing either mRNA or the ribosome-free supernatant reversed the inhibition. Peptide synthesis, using chick polysome cell-free systems was inhibited to a lesser extent. The results of these studies are consistent with a mechanism by which inhibition occurs prior to the formation of the messenger-ribosome complex.

The transfer activity of the low molecular weight virus RNA fraction was examined. Virus RNA charged with amino acid transferred amino acids to TCA-precipitable protein to a degree similar to that transferred by liver aminoacyl-tRNA. Low molecular weight virus RNA appears to contain an RNA fraction with properties identical to tRNA.

The development of systems for protein synthesis from chick cells was described. A ribosome preparation, capable of using poly U and Qβ RNA as templates for protein synthesis, contained low endogenous mRNA activity and incorporated phenylalanine linearly for 30 minutes in the presence of poly U. Results using a chick polysome cell-free system were described. The endogenous message-containing system required ATP and GTP for incorporation, was sensitive to the addition of puromycin and incorporated amino acids when added in the form of aminoacyl-tRNA.

Biological activity of high molecular weight virus RNA was examined in cell-free systems for protein synthesis. No inhibition of protein synthesis was seen using intact, heat-treated and alkaline-treated virus RNA. Added virus RNA stimulated amino acid incorporation in cell-free systems from *E. coli* and chick cells. This is used as an evidence that high molecular weight virus RNA may serve as a template for protein synthesis *in vivo*.

Biological Activity of Avian Myeloblastosis Virus RNA in Cell-Free Systems for Protein Synthesis

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To my wife

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Biological Activity of Avian Myeloblastosis Virus RNA in Cell-Free Systems for Protein Synthesis

INTRODUCTION

The Avian Myeloblastosis Virus

The current knowledge in biochemistry and virology is inadequate to explain the numerous cell transformations of viral etiology. Although the process of viral infection and multiplication has been examined in bacterial cells, little is known about viral infection in higher cell types. Many diseases of unknown origin may be of a viral origin.

A significant milestone in medicine was the discovery that viruses were capable of causing tumor growth. One of the first viruses detected with these properties, the Rous sarcoma virus, was discovered in 1911 by Peyton Rous. Another tumor virus, the myeloblastosis virus, was discovered in 1931 (39). This virus, formerly called the erythromyeloblastosis virus, has been identified in blood plasma of the infected chick (6, 40); and although it is difficult to observe in the intact myeloblast, it can be seen as an intracytoplasmic particle by the electron microscope in the spleen, bone marrow and liver of the infected host (87). Inoculation with plasma from infected birds, or transfer of the circulating, primitive cells results in the transmission of the disease (40). Infection results

in an accumulation of myeloblasts, a primitive ganulocyte, reaching a concentration of two million primitive cells/ml (34). This represents a volume equal to one-half that of the blood.

These actively metabolizing, leukemic cells constitute an impediment to normal circulation in critical tissues and organs, and deplete the blood of glucose and other essential nutrients resulting in acute anemia (8).

Host susceptibility to the virus decreases 22 to 67 fold from three to 21 days as natural resistance increases. The natural resistance increases to a greater percent than the body weight during this period (33).

Chemical analysis has been performed on the purified virus (14). RNA[1] is present in the virus and represents 2.17% of the

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[1] The following abbreviations are used in this thesis: poly U, polyuridylic acid; poly A, polyadenylic acid; poly C, polycytidylic acid; poly rT, polyribothymidylic acid; ATP, adenosine-5'-triphosphate; GTP, guanosine-5'-triphosphate; TMV, tobacco mosaic virus; TYMV, turnip yellow mosaic virus; EMC, encephalomyocarditis virus; RSV, Rous sarcoma virus; Act. D, actinomycin D; RNA, ribonucleic acid; DNA, deoxyribonucleic acid; mRNA, messenger RNA; tRNA, transfer RNA; rRNA, ribosomal RNA; DOC, deoxycholate; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; RNase, ribonuclease; DNase, deoxyribonuclease; ATPase, adenosine triphosphatase; tris, tris (hydroxymethyl) aminomethane; E. coli, Escherichia coli; A, absorbance; EDTA, ethylene diaminetetraacetic acid; LMWt, low molecular weight; HMWt, high molecular weight; MAK, methylated albumin kieselguhr; C, centigrade; ml, milliliter(s); M, molar; cpm, counts per minute; μ, micro; g, gram; mg, milligram; +, present; -, absent; DEAE, diethylaminoethyl.

virus material by weight. No deoxypentose nucleic acid was detected indicating this is an RNA virus.

In Vitro Protein Synthesis

The knowledge of the mechanisms involved in protein synthesis can mainly be attributed to the in vitro system constructed from Escherichia coli by Nirenberg and Matthaei (82). The basic mechanisms of in vitro protein synthesis were summarized in a recent review (74, p. 419-420).

> Individual amino acids are activated in the presence of ATP, magnesium ions, and specific enzymes [aminoacyl soluble RNA (sRNA) synthetases], to form enzyme-bound aminoacyl adenylates and inorganic pyrophosphate. The carboxyl group of the amino acid is linked to the phosphate group of the adenylate moiety by an anhydride bond. The aminoacyl group of the enzyme-bound complex is transferred to amino acid-specific acceptor sRNA's where it is attached by an ester linkage to the ribose portion of the terminal adenosine residue. The amino acid-binding end of sRNA consists of a cytidylate-cytidylate-adenylate (CCA) sequence. Aminoacyl sRNA's react with template-bound ribosomes (polyribosomes, ergosomes), at specific positions determined by the nucleotide sequence of the template (or messenger) RNA. The aminoacyl sRNA's are specifically aligned in relation to the growing peptide chain, the C-terminal end of which is linked to sRNA. Nucleophilic attack by the α-amino group of the incoming aminoacyl sRNA on the carboxyl carbon atom of peptidyl sRNA results in the formation of a new peptide bond and release of the sRNA that was previously attached to the end of the growing chain. The polypeptide chain, now containing an additional amino acid residue, is linked through the new amino acid to its corresponding sRNA. Polyribosome-bound aminoacyl sRNA and peptidyl sRNA are therefore intermediates in protein synthesis. The incorporation of the aminoacyl moiety of

aminoacyl sRNA into the growing ribosome-bound peptide chain requires GTP, glutathione, and two enzymes. The messenger and the ribosome then move one coding unit over in relation to each other. A new nucleotide sequence in messenger RNA is thus placed in position to base-pair properly with the next aminoacyl sRNA. As this process is repeated, the polypeptide chain grows from its N-terminal to its C-terminal residue by the sequential addition of aminoacyl sRNA's. The sequence of amino acids is determined by the nucleotide sequence of messenger RNA; the latter is determined by the deoxynucleotide sequence in DNA, which is transcribed in the form of a complementary polyribonucleotide chain, the messenger. Subsequently, the ribosome moves far enough along the messenger molecule to allow a new ribosome to attach. A second identical peptide chain can now be initiated and synthesized in the path of the second ribosome in the same way. Thus, several identical peptide chains can be synthesized simultaneously on a polyribosome complex. Eventually, at the end of the messenger-RNA chain, the ribosome, the nascent completed polypeptide chain, and the terminal, esterified sRNA are released from the polyribosome.

Two different in vitro systems can be utilized in the study of the mechanisms of protein synthesis, one which is dependent upon the addition of messenger RNA, and one which utilizes intact messenger RNA in the form of template-bound ribosomes. In each case the incorporation of amino acids into trichloroacetic acid-precipitable material using radioactive amino acids can be used as a measure of protein synthesis.

Template RNA

It was hypothesized in 1961 that enzyme synthesis is dependent upon rapidly-induced, short-lived RNA molecules (57). A number of

in vitro studies have demonstrated the role of a messenger RNA in protein synthesis. RNA isolated from the nucleus of Walker tumors (101) and rat liver (4, 30, 94) stimulates amino acid incorporation in the *E. coli* cell-free system. The stimulatory RNA fraction from rat liver corresponded to the RNA fraction that was labeled early by pulse-labeling techniques, one criterion of mRNA. RNA extracted from liver nuclei is much more active than liver cytoplasmic RNA in stimulating amino acid incorporation in the *E. coli* cell-free system (18). Nuclear RNA contains a larger ratio of messenger RNA to ribosomal RNA. RNA isolated from sheep thyroid is also stimulatory in the *E. coli* system (23). Only a slight stimulation of amino acid incorporation is obtained from RNA extracted from higher cell types when compared to that obtained from the addition of RNA from a bacterial virus. Likewise, a slight stimulation is seen when RNA fractions are added to cell-free systems for protein synthesis derived from higher organisms (53). Reasons for the slight stimulation will be presented in the text of this thesis.

Synthetic polynucleotides are composed of an orderly, linear sequence of ribonucleotides. Because the synthesis of polyribonucleotides can be controlled, polynucleotides of known base sequences can be obtained. As a result of their similarity to single-stranded RNA, these polyribonucleotides can function in a manner similar to mRNA. The genetic code owes much of its development to the use

of synthetic polyribonucleotides. Added to the E. coli cell-free system, polyuridylic acid (poly U) stimulates only phenylalanine incorporation (18, 46, 82). Using sucrose density gradient techniques, ribosomal aggregation on polyuridylic acid is observed (5, 48). Stimulation of amino acid incorporation by poly U is also seen in the yeast cell-free system (29, 72) and in the reticulocyte cell-free system (130). Polyadenylic acid and polycytidylic acid are less able to serve as messenger molecules (87).

The reticulocyte system has several advantages in the study of protein synthesis. Reticulocytes are not capable of synthesizing messenger RNA and are dependent upon preformed RNA (73). The reticulocyte also synthesizes mainly one protein, hemoglobin (65), thus yielding a homogenous messenger RNA fraction. Reticulocyte RNA will stimulate amino acid incorporation in the E. coli cell-free system (16, 31, 45, 107). Chromatography of the peptides produced on DEAE Sephadex indicates only five to ten percent of the peptides produced resemble those of hemoglobin (45). RNA isolated from reticulocytes will bind to ribosomes in a reticulocyte cell-free system and stimulate amino acid incorporation (3). These peptides thus produced appear to be similar to those of globin (67).

Viral RNA serves as a template for the synthesis of viral-specific enzymes and viral coat proteins. For RNA-containing virus, the viral genome is believed to carry this function. DNA-

containing virus, such as the vaccinia virus, are required to synthesize viral-specific template RNA for viral-specific protein synthesis. Pulse-labeling techniques label an RNA fraction in the cytoplasm of vaccinia virus-infected HeLa cells. Within 30 seconds the labeled RNA combines with a 40S subribosomal particle and is subsequently seen in a polysome fraction (58). These polysomes have been shown to be the site of newly-synthesized, virus-specific protein (103), and the RNA on the polysomes forms hybrids with DNA from vaccinia virus and not with HeLa cell DNA (9).

RNA from a number of bacterial viruses can be extracted and used as an efficient message in the E. coli cell-free system. RNA from the f2 bacteriophage will form peptides identical with those of the coat protein (81). Virus-specific peptides are also seen when f2 RNA is incubated in cell-free extracts of Euglena gracilis (104), which supports the universality of the genetic code. Similar messenger activity is seen with bacteriophages R17 (21) and MS2 (80). The satellite tobacco necrosis virus, having a molecular weight of 4×10^5 daltons, is believed to contain only enough information to code for its protein coat; when incubated in the E. coli cell-free system, peptides are produced resembling those of the coat protein (26). Two other RNA-containing plant viruses, tobacco mosaic virus (13) and turnip yellow mosaic virus (122) are active in stimulating peptide formation in the E. coli system.

Although RNA's from bacteriophages, and plant viral RNA to a lesser extent, serve as natural messages *in vitro*, RNA's from mammalian viruses show only a slight stimulation of amino acid incorporation in the *E. coli* cell-free system. Incubated in the *E. coli* cell-free system, poliovirus RNA forms polysomes and shows amino acid incorporation which is not immunologically identifiable to poliovirus-specific antigens (112, 124). However, polysomes from the virus-infected cells are capable of producing virus-specific peptides in cell-free extracts of HeLa cells (103, 113). The possibility exists that necessary transfer RNA's are lacking in the *E. coli* cell-free system, and premature peptide chain termination occurs when RNA from higher cell types is used. The resulting peptides may be shorter than that required of antigens for antibody binding.

Other RNA's which appear to stimulate peptide synthesis are mengovirus RNA in L-cell cell-free extracts resulting in viral coat-specific peptides (37), and encephalomyocarditis (EMC) virus RNA which stimulates peptide bond formation in Kreb's tumor cell-free extracts (62).

Inhibition of Host Protein Synthesis

Besides replicating and synthesizing viral-specific protein, some animal viruses inhibit host DNA-dependent RNA polymerase

and host protein synthesis. Poliovirus infection leads to an inhibition of host protein synthesis only after a short period of protein synthesis (90). Replication of the viral RNA is not necessary. No RNA resembling that of poliovirus is seen in the nucleus (55). Migration of a virus-specific protein into the nucleus could be responsible for the inhibition of the RNA polymerase (54). The destructive rate of host polysome breakdown exceeds that produced by inhibition of RNA synthesis (91). Similar effects are seen after EMC virus infection (62) and mengovirus infection (37). This phenomenon is not limited to RNA viruses. The DNA-containing herpes simplex virus also inhibits host protein synthesis resulting in a breakdown of the host polysomes (114). The mechanism causing the breakdown of the host polysomes apparently is not active in preventing viral-specific polysome formation. Although myeloblasts can replicate after being infected with the avian myeloblastosis virus and active polysomes can be isolated, synthesis of specific proteins in the tumor cell may be inhibited. Such a mechanism could account for the failure of the myeloblast to maturate into granulocytes.

Messenger RNA-Ribosome Interactions

The current hypothesis for the mechanism of protein synthesis requires a messenger RNA-ribosome interaction. It is now believed by some investigators that messenger RNA is transported into the

nucleus by a 40S subribosomal particle (51) joining a 60S subribosomal particle in the cytoplasm to create a polysome in mammalian cells (59). The mechanism by which additional particles join the messenger strand has not been elaborated upon. However, it is believed 74S ribosomes do not dissociate into 60S and 40S subparticles and there is very little equilibration between the 74S ribosomes on the polysome and those free in the cytoplasm (59).

The removal of newly-formed messenger RNA from DNA by ribosomes may be an enzymatic process involving a DNA-associated enzyme which controls the rate of RNA transcription (93). It has been shown that RNA synthesis requires trace amounts of amino acids (87). Experiments suggest these amino acids act in protein synthesis and are not involved in the synthesis of nucleic acid building blocks. RNA-DNA ribosome complexes have been observed in an in vitro bacterial system coupling translation to transcription (20). Such ribosomal binding may be analogous to the transport of mRNA to the cytoplasm by 40S ribosomal subparticles from the nucleus of higher organisms.

Dependence upon a nuclear enzyme for ribosome-mRNA binding would not explain the formation of viral-specific polysomes in the cytoplasm shortly after infection. The two enzymatic factors found on the ribosome may be all that is necessary for ribosome attachment to viral RNA (17, 110). It has been shown that vaccinia

virus-specific RNA has a half-life of 30 seconds before it combines with a 40S particle. This can be followed into virus-specific polysomes (58). If ribosomes do not normally dissociate in the cytoplasm, the 40S and 60S subribosomal particles which account for 10% of the ribosomal particles in the cytoplasm (59) may have the capacity to bind viral RNA. Ribosomal subunits were identified in cell-free reticulocyte extracts under conditions in which the 74S monosome was completely stable (11). These subparticles were four times as active in poly U-dependent peptide synthesis as were the monosomes. The formation of viral-specific polysomes appears to occur by a mechanism different than that utilized in the formation of natural polysomes of the host.

Secondary Structure of Template RNA

There is considerable knowledge concerning the importance of secondary structure in ribosomal binding. Bacterial ribosomes can be dissociated into a 50S and 30S subunit by dialysis in low Mg^{++}. It is believed that only the 30S subunit contains sites for messenger binding (86, 117). Ribosomal binding to polyuridylic acid and *E. coli* RNA requires only 10^{-2} M Mg^{++} ion which is reversed when the Mg^{++} ion is lowered to 10^{-3} M (49). TMV RNA and TYMV RNA form only monosomes with *E. coli* ribosomes at 0° C (48), although a conflicting study reports as many as eight ribosomes are able to bind TYMV RNA

at 0°C (122). Kinetic data indicates poly U binds at a much higher rate to ribosomes than to the RNA of TYMV. The increasing secondary structure of the following polynucleotides reflects the decreasing ability to bind ribosomes: Poly U $<$ TYMV RNA $<$ TMV RNA ≈ MS2 RNA $<$ ribosomal RNA. Since poliovirus RNA can form polysomes containing at least 50 ribosomes in HeLa cells, the failure to form polysomes *in vitro* may be due to the loss of a membrane-orienting structure. During the process of mRNA synthesis, DNA extends RNA to a degree sufficient to form ribosomal complexes (48). Polyribothymidylic acid (poly rT) is a better template than poly U at elevated temperatures which randomize the secondary structure of poly rT. Polyadenylic acid (poly A) is a much better template for amino acid incorporation at 45°C than at 20°C (115). At 0°C poly A cannot bind ribosomes as it does at 37°C. The low incorporation of proline using polycytidylic acid (poly C) is due to its high degree of secondary structure (117). The template activity of various UG copolymers is correlated with the G content which reflects upon the secondary structure seen in polynucleotides with a high G content (109).

Differences in secondary structure can result in competition of polynucleotides for ribosomal sites. Poly U can replace poly C from ribosomes due to its higher affinity for ribosomes (76). Poly U can also inhibit incorporation of amino acids other than

phenylalanine when incubated in the reticulocyte cell-free polysome system (129). It is believed that ribosomes preferentially bind to poly U rather than reattach to reticulocyte messenger RNA.

Secondary structure and methylated nucleotides reflect the basic difference between ribosomal RNA and template RNA. Low-methylated rRNA can be prepared from E. coli. Cell-free extracts can be made from cells lacking mRNA but containing a large amount of ribosomal precursors. Incubation of this in vitro protein synthesizing system results in amino acid incorporation which may reflect the synthesis of ribosomal proteins (41, 78). Ribosomal RNA may serve as a messenger RNA shortly after its synthesis in the cell. Heated ribosomal RNA and transfer RNA can serve as templates for protein synthesis especially in the presence of the antibiotic neomycin. Neomycin may be causing miscoding or an alteration of the ribosome surface to increase the affinity for certain RNA or DNA molecules (56). Cleavage of ribosomal RNA in one or two positions greatly increases the template activity. Treatment of ribosomal RNA and phage T2-specific RNA with formaldehyde or heat followed by quick cooling causes an association with E. coli ribosomes not otherwise seen (86).

Heat and formaldehyde treatment renders DNA capable of serving as a messenger molecule (69, 79, 116). DNA from ΦX 174, a naturally-occurring, single-stranded DNA bacterial virus, can

bind two *E. coli* ribosomes (69). Formaldehyde treatment of ΦX 174 DNA, which destroys the secondary structure by reacting with amino groups, resulted in the binding of four to five ribosomes per DNA molecule. Bacteriophage T2 DNA after heat and formaldehyde treatment could also bind to ribosomes (116). DNA's from several sources, made single-stranded by heat treatment, were active in amino acid incorporation, especially in the presence of streptomycin or related antibiotics (69). Removal of purines from DNA by acid treatment resulted in the incorporation of only those amino acids whose known code words consisted solely of pyrimidines. Secondary structure may be used advantageously within the cell. The absence of secondary structure in ribosomal and messenger RNA may provide sites for ribosomal binding (47). Areas rich in G, containing a high degree of secondary structure, may specify the beginning and end of a cistron coding for specific proteins (109).

The Role of Polysomes in Protein Synthesis

It is believed that proteins are synthesized on polysomal clusters in which a large number of ribosomes are assembled on a messenger RNA molecule which codes for a specific protein. Newly synthesized RNA can be followed into polysomes by pulse-labeling RNA and isolating the polysome fraction at various times following the pulse. A known inhibitor of DNA-dependent RNA synthesis,

Actinomycin D (Act. D), prevents any new mRNA from being transferred into polysomal structures. Using Act. D, the half-life of RNA in the polysome can be determined. Although the half-life of mRNA in *Bacielus megaterium* is only 3.5 minutes (102), mouse liver mRNA has a half-life of eight to 12 hours (119). Actinomycin D treatment did not prevent the synthesis of previously induced amino acid-catabolic enzymes in rat liver (92). Cells which mainly make one kind of protein appear to have a stable messenger RNA (106). Included in this category are cells from skin, down, liver, muscle, connective tissue, reticulocyte, pancreas, thyroid and ocular lens.

Polysomes can be isolated and utilized in *in vitro* protein synthesizing systems. The polysomes from liver have been characterized (78, 129). They stimulate amino acid incorporation *in vitro* resulting in their breakdown within five minutes. In reticulocyte polysomes, one peptide chain was detected for every ribosome active in peptide formation (125). Using techniques which result in gentle breakage of the bacterial cell, active polysomes have been isolated which sediment faster than ordinary ribosomes (32, 98).

Following induction of the enzyme β-galactosidase in *E. coli*, polysomes containing nearly 40 ribosomes can be isolated (63). β-galactosidase activity was associated with the polysome region when the cell-free extract was fractionated on a sucrose density

gradient. Induction of β-galactosidase is coordinated with increased levels of galactoside permease and thiogalactoside transacetylase. The size of polysomes from 20 different _E. coli_ strains was measured after induction of β-galactosidase (64). Those strains which had deletions in the acetylase-permease region of the lactose gene had smaller polysomes than the wild type. Some strains with point mutations also had smaller polysomes. It was concluded that the message which codes for β-galactosidase is polycistronic. The length of the polysome appears to correspond to the length of the gene.

Other specific proteins have been identified on isolated polysomes. One, from muscle, consisting of 50-60 ribosomes, synthesized a protein which was identified by acrylamide gel electrophoresis as migrating the same as myocin, the fibrous protein of muscle (52). Reticulocytes, which synthesize hemoglobin almost exclusively, yield a polysome fraction in which the protein synthesized _in vitro_ can be identified by column chromatography as peptides contained in globin (67). Polysomes isolated from chick embryos after a [^{14}C] -proline injection contained a protein which was characterized by a high concentration of hydroxyproline, which is distinctive of collagen (70). This characteristic polysome contains 90 to 100 bound ribosomes.

Virus RNA is also seen in polysomal structures. Using Act. D

to prevent the synthesis of natural RNA, newly synthesized poliovirus RNA formed polysomes containing 60 ribosomes in HeLa cells (96). The largest, natural cellular polysome that could be isolated contained 40 ribosomes which disappeared prior to the formation of poliovirus-specific polysomes. These poliovirus-specific HeLa cell polysomes are unique in that they are associated with a membrane not seen in uninfected cells (89). Such a structure, containing nearly one-fourth of the cell's ribosomes, may protect the polysome from the agent which caused the breakdown of natural polysomes soon after viral infection. Using the same techniques, labeled mengovirus was seen in the polysomes of infected HeLa cells (118).

Mengovirus RNA is first seen associated with a 45S ribosomal subunit 45 minutes after infection. A 450-500S polysome structure containing mengovirus RNA is seen as early as one hour after infection. A recent discovery concerning the mechanism of interferon action is that the presence of homologous interferon prevents ribosomal binding to mengovirus RNA (22).

Initiation and Termination of Protein Synthesis

A current fervor in protein biosynthesis concerns initiation and termination of polypeptide synthesis. Early investigations led to the observation that after amino acid acylation of tRNA, methionine can become formylated in the α-amino position (71). The

addition of N-formylmethionyl-tRNA to a R17 virus RNA cell-free *E. coli* system resulted in the N-terminal addition of formylmethionine into several peptides (1). The absence of formylmethionine in *E. coli* proteins would suggest its enzymatic cleavage *in vivo*. Two methionyl-tRNA's have been isolated. Binding experiments suggest AUG is the codeword for the methionyl-tRNA which can be formylated (met-$tRNA_1$) (25). The non-formylating methionyl-tRNA binds well to AUG, GUG, and UUG (met-$tRNA_2$).

Two enzymatic factors have been found which stimulate the incorporation of formylmethionine into the N-terminal amino acid position (17, 110). These factors, which are removed from *E. coli* ribosomes by washing, are necessary for the translation of natural messages, and stimulate the binding of formylmethionyl-$tRNA_1$ to purified ribosomes in the presence of the coding triplet AUG (100).

Chain initiation in nucleated cells may not be analogous. Two methionyl-tRNA's have been found in mammalian liver (24). Although one of them can be formylated in the *E. coli* transformylase system, there is no evidence for the existence of such an enzyme in all mammalian cells. The hemoglobin molecule suggests analogous initiation reactions may occur using N-acetyl amino acids. Globin from tadpoles and adult bullfrogs contain two moles of N-terminal acetyl groups per 65,000 molecular weight unit (28).

Just as initiation codons exist, chain termination codons may

also occur. Reversions of a nonsense mutation of the phosphatase structural gene in _E. coli_ result in several amino acid substitutions in the alkaline phosphatase molecule (127). Coding assignments for amino acids can be related by single base changes only to a nonsense codon in RNA of a base composition of UAG. Such a non-sense codon could be recognized by specific tRNA's which do not carry an amino acid resulting in the release of an incomplete pep-tide (19). This amber codon, UAG, has also been seen in mutant RNA viruses which produce incomplete peptides of the coat protein. A similar mutant triplet, UAA, results in the release of incomplete peptides (19). This mutation, named ochre, is weaker and is a more likely candidate for the chain-terminating codon. Utilization of N-formylmethionine as an initiator would preclude the coupling of peptides in a polycistionic message (126).

The foregoing leads to the conclusion that template RNA is necessary for amino acid incorporation in protein biosynthesis. RNA from numerous tissues has been shown to stimulate amino acid incorporation in cell-free extracts for protein synthesis. Virus RNA from bacterial virus, plant virus and animal virus has been shown to have template properties for protein synthesis. Amino acid incorporation _in vitro_ does not necessarily represent the synthesis of biologically active proteins, especially when intra-specie cell-free systems are used. Information from other viral

systems would suggest a universal utility of function designed by the Creator, who never ceases to amaze us with the grandeur and design of biological systems.

Tumor Virus-Specific Protein

There is very little evidence that tumor virus RNA serves a role in the synthesis of specific proteins. No avian myeloblastosis virus-specific proteins have yet been isolated. The RNA virion is coated during cellular release by membrane budding (42). Associated with the purified virus is ATPase activity, chick tissue antigen and Forssman antigen which all are a part of the membrane component (35).

A defective strain of the Rous sarcoma virus (RSV) has led to the observation that antibodies can be made against avian myeloblastosis specific protein. The defective RSV strain can infect and transform chick cells without production of complete virus particles (43). Any of the avian leukosis viruses can act as helper viruses, superinfecting these cells to produce virus particles containing the RSV genome within an avian leukosis-specific coat protein. The RSV activated by various helper viruses has been shown to have the same sensitivity as the helper virus to inactivation by helper virus-specific antisera (43). In addition to this, antibodies have been prepared in hamster cells against the RSV. The antisera

does not neutralize infectious RSV but fixes complement with an antigen present in virus-free hamster tumors (121). The same antigenic response is seen in chicken cells infected with an avian leukosis virus capable of replication. The inability to neutralize virus infectivity indicates it is not a component of the viral envelope. This antigen, seen in non-producing cells containing defective RSV, may be an internal viral component of all avian tumor viruses. The antigen, absent in normal avian or mammalian cells, has been designated the group-specific antigen (61). Chickens are capable of producing type-specific antibodies, which are able to combine with an antigen situated in the viral envelope (61). The group-specific antigen is observed in infected cells, virus-free hamster tumors and fragmented virions using hamster antisera (27). The intact myeloblastosis virus is unable to remove group-specific antibody from immunized rabbit sera until fragmentation of the virus particle takes place. Structurally complete virus particles in chicken fibroblasts are seen after infection with defective RSV but are absent in normal cells. These particles are indistinguishable from complete avian myeloblastosis viruses in structure and location. Thus RSV RNA may be enclosed in a cellular membrane which contains a group-specific antigen but lacks components produced by the helper virus (27). The type-specific antigen and the group-specific antigen appear to be different. Excluding the possibility that these avian

tumor viruses have RNA base sequences which may be inducing a group-specific antibody, and that the type-specific antibodies represent an immunological reaction against the complete virion, the antigens may represent viral-specific proteins.

Since the virus infection results in the alteration of the myeloblasts potential for maturation, it is important to investigate any mechanism that may selectively block the expression of genetic elements.

The effect of avian myeloblastosis virus RNA on cell-free systems for protein synthesis from E. coli, liver and the leukemic myeloblast will be presented in this manuscript. A system, sensitive to the addition of messenger RNA, was developed from chick liver and the myeloblast tumor cell. Discussions are presented concerning the role that a virus RNA fraction may have in blocking the maturation of immature granulocytes at the stage of the myeloblast.

METHODS AND MATERIALS

Preparation of Qβ RNA

3XD medium (38) was inoculated with an overnight culture of E. coli 102 grown in L medium. The culture was shaken in a Gyrotory Shaker, New Brunswick Scientific Co., New Brunswick, N. J., at 37° C until the absorbancy reached 0.32 at 660 mμ. To the culture Qβ phage were added at a multiplicity of five and allowed to absorb for 30 minutes without shaking. The infected cells were shaken for 5½ hours at 37° C, the flasks iced and the cells removed by centrifugation at 5000 × g for 15 minutes. The supernatant fluid was iced overnight. $(NH_4)_2SO_4$ was added at a concentration of 311 g/l and chilled for three hours. The phage were centrifuged at 9000 × g for one hour and suspended in a small volume of TML (0.01 M tris buffer, pH 7.6, 0.001 M $MgCl_2$). The suspension was dialyzed overnight with two changes of TML. The dialysate was clarified by centrifugation at 10,000 × g for five minutes. To the supernatant sodium dodecyl sulfate was added to a concentration of 0.2% and allowed to stand ten minutes at room temperature. The solution was phenol extracted three times, ether extracted three times, and the alcohol precipitated three times with 1/10 volume of 20% potassium acetate, pH 4.8, and two volumes cold ethanol. The Qβ RNA

was stored at -70° C in BRS at a concentration of 5 mg/ml.

Isolation of Myeloblasts

Blood samples were obtained from leukemic chicks by heart puncture. The heparinized blood was centrifuged at 1000 × g in a model 2 International centrifuge. The supernatant plasma was removed for virus purification. The upper cell layer composed of leukemic myeloblasts was washed with 50% chicken serum-50% medium 199 and centrifuged at 100 × g. Two cell washes followed using Zamecnick's homogenizing medium (116) or 0.01 M tris buffer, pH 7.5, and 0.9% NaCl. The cells were stored at -20° C. One cubic centimeter of packed cells contains around 2×10^9 myeloblasts.

Isolation of a pH 5 Enzyme Fraction

The pH 5 enzyme fraction was prepared from liver and myeloblasts by a modification of an earlier procedure (60). Fresh liver or frozen myeloblasts were homogenized with two volumes of BRS containing 0.25 M sucrose and 10 μg/ml DNase. Following homogenization for two minutes in the VirTis Homogenizer, the preparation was centrifuged 15,000 × g for ten minutes. Ribosomes were removed by centrifugation at 105,000 × g for three hours. The ribosome-free supernatant was diluted two to three fold and adjusted to pH 5.2 with 1 M acetic acid. After 40 minutes at 0° C, the

the precipitated enzyme was centrifuged for ten minutes at 15,000 × g. The rinsed pellet, suspended in BRS, yielded a solution containing insoluble protein. The enzyme solution was diluted with BRS, stirred for 30 minutes at 2° C and the denatured protein removed by centrifugation. Precipitation to pH 5.2 and resuspension were repeated twice to obtain an active pH 5 enzyme fraction. The activity increased after each pH 5 precipitation and resuspension. The pH 5 enzyme fractions from liver gave twice the phenylalanine incorporation as that obtained from myeloblast pH 5 enzyme fractions when optimal enzyme concentrations were used with low-magnesium ion ribosomes using poly U as a synthetic message. Fractions obtained from young chicks gave the best activity.

Isolation of an Enzyme Fraction from the pH 5 Supernatant Fraction

Following removal of the pH 5 precipitate by centrifugation, the supernatant fluid was adjusted to a pH of 7.5 with KOH. Ammonium sulfate was added to a concentration of 35% and the resulting precipitate was centrifuged at 10,000 × g for ten minutes after standing 30 minutes at 2° C. The supernatant was brought to a concentration of 60% ammonium sulfate and treated in the same manner as the 35% ammonium sulfate fractionation.

The pellets obtained by 35% and 60% ammonium sulfate

fractionation were suspended in a buffer solution (0.04 M tris buffer, pH 7.6, 0.01 M $MgCl_2$, 0.04 M NH_4Cl, 0.001 M glutathione and 0.008 M 2-mercaptoethanol), reprecipitated with 50% and 70% ammonium sulfate respectively, dissolved and dialyzed against fresh buffer solution. These enzyme fractions were stored at -70° C.

Preparation of a 70% Ammonium Sulfate Enzyme Fraction

Fresh chick liver was homogenized with two volumes of BRSG (0.04 M tris buffer, pH 7.6, 0.03 M KCl, 0.01 M magnesium acetate, 0.008 M 2-mercaptoethanol and 0.001 M glutathione) containing 0.25 M sucrose and 10 μg/ml DNase in the VirTis Homogenizer. The homogenate was centrifuged at 15,000 × g for ten minutes and the supernatant centrifuged at 105,000 × g for three hours to remove the microsomes. Saturated ammonium sulfate was added to the supernatant to 40% saturation. After 30 minutes at 0° C the precipitate was removed by centrifugation and ammonium sulfate was added to the supernatant until a concentration of 70% was achieved. The resulting insoluble fraction was centrifuged 15,000 × g for ten minutes, dissolved in BRS and the enzyme solution dialyzed overnight against BRSG. The 40-70% ammonium sulfate fraction was stored at -70° C.

Isolation of Liver and Myeloblast Supernatant Enzyme Fractions

Livers were quickly removed from seven day-old chicks, rinsed, and placed in ice-cold BRSG sucrose (0.04 M tris buffer, pH 7.6, 0.03 M KCl, 0.01 M $Mg(CH_3000)_2$, 0.001 M glutathione, 0.008 M 2-mercaptoethanol, 0.25 M sucrose). The minced liver was homogenized with two volumes of BRSG sucrose containing 10 μg DNase/ml for 1.5 minutes. The upper portion of the supernatant was centrifuged at 105,000 × g for three hours at 0° C. The supernatant was dialyzed at 0° C against BRSG for 14 hours with fresh changes at two and 12 hours. The dialyzed supernatant retained its activity after storage at -70° C.

With frozen myeloblasts, the same procedure was used to obtain a myeloblast enzyme fraction. Protein concentrations were measured by absorbancy at 260 and 280 mμ. The 105,000 × g dizlyzed, myeloblast supernatant was concentrated two fold using Sephadex G25.

Isolation of Ribosomes in a Low Magnesium Ion Buffer

Fresh liver or frozen myeloblasts was homogenized in two volumes of 0.02 M tris buffer, pH 7.6, 0.25 M sucrose, 0.1 M potassium chloride, 0.006 M 2-mercaptoethanol and 0.001 M or

0.0001 M magnesium acetate containing 10 μg/ml of DNase for one minute in the VirTis Homogenizer. The supernatant fluid obtained after centrifugation for ten minutes at 15,000 × g was made to 1.3% deoxycholate (DOC) with a 10% solution in 0.05 M tris buffer. The supernatant was layered over a solution of 1 M sucrose in homogenizing buffer and centrifuged 105,000 × g for four hours. The pellet was suspended in the homogenizing buffer, clarified by centrifugation at 30,000 × g for ten minutes and stored at -70° C.

Isolation of Polysomes

Polysomes were isolated from liver and myeloblasts by the technique described by Wettstein *et al.* (129). Livers obtained from 20 day-old chicks were quickly removed, washed, and put into ice cold Hoaglund's medium A (0.05 M tris buffer, pH 7.6, 0.025 M KCl, 0.005 M $MgCl_2$, 0.25 M sucrose) (60). The livers were minced with a scissors and homogenized with two volumes of medium A in the VirTis Homogenizer, for one minute. The homogenate was centrifuged ten minutes at 0° C at 15,000 × g. The supernatant fluid was made to 1.3% with sodium deoxycholate (DOC) by the addition of fresh 10% DOC in 0.05 M tris buffer. The solubilized polysomes were isolated by centrifugation through several layers of sucrose. The DOC-treated homogenate was layered over 3 ml of 0.5 M sucrose in medium A buffer, which itself was layered over 3 ml of 2 M

sucrose in medium A buffer. The polysome pellet was obtained by centrifugation at 0° C for three hours at 105,000 × g. The sucrose layers were removed and the clear pellets dissolved in medium A buffer. The solution was clarified by centrifugation at 0° C for five minutes at 15,000 × g. The absorbancy was read and corrections were made for the absorption of ferritin (130). All ribosome preparations were flash-frozen and stored at -70° C, retaining their stability for many months. The 2 M sucrose layer contained ribosomes with less endogenous activity than present in the polysome fraction. The 2 M sucrose solution was diluted with medium A buffer and the ribosomes centrifuged through IM sucrose in medium A buffer for three hours at 105,000 × g. The ribosome pellet was suspended in medium A buffer, clarified by centrifugation at 10,000 × g and stored at -70° C.

Polysomes were isolated from washed, frozen myeloblasts by the procedure used for liver except only 0.5% DOC was required for solubilization of the 15,000 × g homogenate. Myeloblasts showed very little contamination with ferritin.

The ribosome concentrations were calculated from the absorbancy at 260 mμ assuming a value of 11.3 A_{260} units per mg of ribosomes (120).

Isolation of Nuclear RNA

To obtain a nuclear fraction, myeloblasts were homogenized in ten volumes of a solution containing 0.25 M sucrose, 0.05 M tris buffer, pH 7.6 and 0.03 M $CaCl_2$. Breakage was checked by microscopy, using trypan blue as a nuclear stain. The nuclei, after centrifugation at 450 × g, were resuspended in three volumes of 2.2 M sucrose containing 0.01 M tris buffer, pH 7.6 and 0.003 M $CaCl_2$. The nuclei were purified by centrifugation in the Spinco 30 rotor for one hour at 45,000 × g, and further purified by resuspension in 1 M sucrose containing 0.0001 M $CaCl_2$ and subsequent centrifugation for 15 minutes at 3,000 × g to remove cytoplasmic contaminants. The nuclei were extracted with a solution containing 0.14 M NaCl, 0.05 M tris buffer, pH 7.6, 0.001 M magnesium acetate, and 0.1% Macaloid, and the homogenate was centrifuged at 4,000 × g for ten minutes. The supernatant was adjusted to pH 5.1 with acetic acid and SDS was added to a concentration of 0.3%. The SDS-treated solution was extracted at 65° C for ten minutes with phenol containing 0.1% hydroxyquinoline followed by a 15 minute extraction at room temperature. The aqueous solution was ether extracted and the RNA precipitated with alcohol.

Source of Bulk RNA, Liver and Myeloblast tRNA, and Virus RNA

Bulk RNA from E. coli, the 30,000 $\times$ g supernatant fraction (S30) from E. coli, and bulk RNA from liver were prepared by Dr. G. S. Beaudreau. Transfer RNA from liver and myeloblasts was a gift from J. W. Carnegie. Purified avian myeloblastosis virus RNA fractions were prepared by Dr. A.O'C. Deeney.

Determination of Protein and RNA Concentrations

Protein concentrations were obtained by measuring the absorbancy at 260 and 280 mμ. The concentration of dilute protein solutions was obtained using the nomograph of Warburg and Christian (123). The concentration of dilute solutions of RNA was determined by measuring the absorbancy at 260 mμ and using the value of 20 A_{260} units for the absorbancy of one mg of RNA. Good RNA preparations had A_{260}/A_{280} ratios which were approximately two.

Cell-free Protein Synthesizing System

The E. coli amino acid incorporating system consists of an energy generating system containing phosphoenolpyruvate, 6 mM; 2.5 μg pyruvate kinase; ATP, 1.5 mM: GTP, 0.3 mM; magnesium acetate, 10 mM; potassium chloride, 30 mM; tris buffer, pH 7.6,

40 mM; 2-mercaptoethanol, 8 mM; amino acids minus the one isotopically labeled, 4×10^{-5} M; 0.16 μC [^{14}C] -leucine, or 0.16 μC [^{14}C] -phenylalanine, 1.6 A_{260} units of an E. coli S30 fraction, and 50 μg of Qβ RNA or polyuridylic acid which were used as messenger molecules.

The 0.05 ml to 0.10 ml reaction volumes were incubated at 35° C for 30 minutes, stopped with ice cold H_2O followed by ice cold trichloracetic acid (TCA) to five percent. The tubes were heated at 90° C for 15 minutes, chilled and filtered onto membrane filters. Each filter was washed 13 times with 4 ml of 5% TCA, dried and counted in a Packard scintillation counter in vials containing toluene with either POPOP, 0.1 g/l, and PPO, 2.0 g/l, or BBOT, 4.0 g/l. Counting efficiency was 78%.

The liver and myeloblast cell-free systems contained, instead of the E. coli S30 fraction, 0.2-0.5 mg of ribosomes, 0.1-0.65 mg enzyme fraction, and 0-15 μg of tRNA in a volume of 0.10 to 0.20 ml.

Chemicals

Uniformly [^{14}C] -labeled L-leucine (251 mC/mmole) and L-phenylalanine (351 mC/mmole) were obtained from New England Nuclear Corporation. Phosphoenolpyruvate, pyruvate kinase, reduced glutathione, adenosine triphosphate (ATP), and poly U were obtained from Sigma Chemical Company. Poly A and poly C were

obtained from Mann Research Laboratories. Deoxyribonuclease, ribonuclease-free, was obtained from Worthington Biochemical Corporation. Guanosine triphosphate (GTP) was purchased from Schwarz BioResearch Incorporated. Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corporation. E. coli B sRNA was purchased from Calbiochem. 1,4-bis[2-(phenyloxazolyl)]-benzene (POPOP), 2,5 diphenyloxazole(PPO), and 2,5-bis-[2-(5-tert-butylbenzoxazolyl]-thiophene (BBOT) were obtained from Packard Instrument Company.

RESULTS AND DISCUSSION

Fractionation of Avian Myeloblastosis Virus RNA

Avian myeloblastosis virus RNA can be fractionated into a high molecular weight RNA fraction and a low molecular RNA fraction by sucrose density gradient centrifugation, shown in Figure 1 (15, 99). The high molecular weight RNA peak, sedimenting around 65S, may represent the viral genome. RNA, sedimenting in the 4S region, could consist of contaminating tRNA from the myeloblast, low molecular weight RNA of unknown function or fragments of high molecular weight RNA. To ascertain whether virus particles contain adsorbed tRNA from the cell, [^{3}H] -tRNA from the myeloblast was added prior to purification of the virus, and the usual procedures for isolation were followed. The purified RNA had only a few cpm associated with it, indicating absorbed tRNA would be removed during the purification procedure. Membrane budding may trap tRNA within the virus particle. If so, a full complement of tRNA's would be present. Using individual [^{14}C] -amino acids and a synthetase enzyme fraction, the complement of tRNA's in the low molecular weight fraction was examined. Several tRNA's were absent. Valine and lysine, both present in significant quantities in a myeloblast

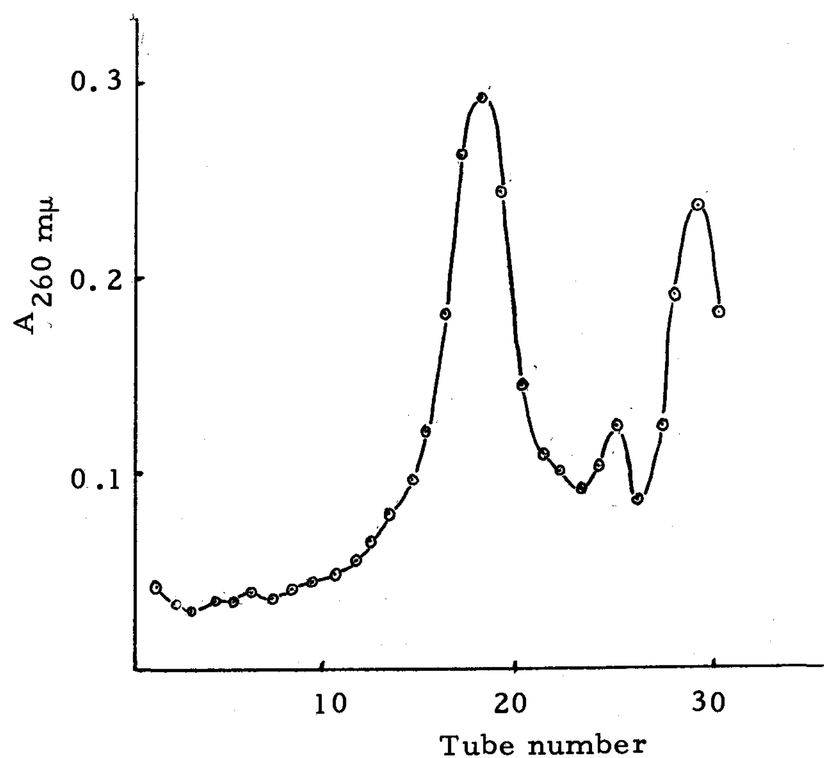

Figure 1. Fractionation of myeloblastosis virus RNA by sucrose density gradient centrifugation. Myeloblastosis virus RNA was layered on top of a 5-20% sucrose gradient (sucrose solution was in 0.01 M tris buffer, pH 7.8, 0.1 M NaCl, 0.001 M EDTA) and centrifuged in a SW 25.1 rotor, Spinco Model L, for four hours at 25,000 rev./min.

tRNA preparation were absent in virus RNA preparations.[2] Speculations concerning the nature of the 4S RNA will be discussed in this manuscript.

Activity of Low Molecular Weight Virus RNA in the *E. coli* Cell-free System for Protein Synthesis

Low molecular weight virus RNA has two biological properties of interest. It can accept amino acids in the presence of aminoacyl synthetases (7, p. 791-802) and it is a potent inhibitor of protein synthesis. Figure 2 shows the absorbancy profile of the low molecular weight RNA peak after 24 hours of centrifugation in a sucrose density gradient. Pool 1 represents high molecular weight RNA, pool 2 represents RNA heterogeneous with respect to molecular weight and pool 3 represents RNA having a Svedberg value of about 4S. These fractions were concentrated by alcohol precipitation and tested for inhibition in an *E. coli* S30 cell-free system for protein synthesis using Qβ phage RNA as a messenger RNA molecule. The results are shown in Table 1. Pool 2 RNA, expected to contain fragmented RNA, showed no inhibition. Although pool 1 RNA did inhibit 15%, pool 3 RNA inhibited 96% at a concentration of 1.7 μg. Unfractionated RNA at the same concentration resulted in a 65% inhibition

[2] J. W. Carnegie, personal communication.

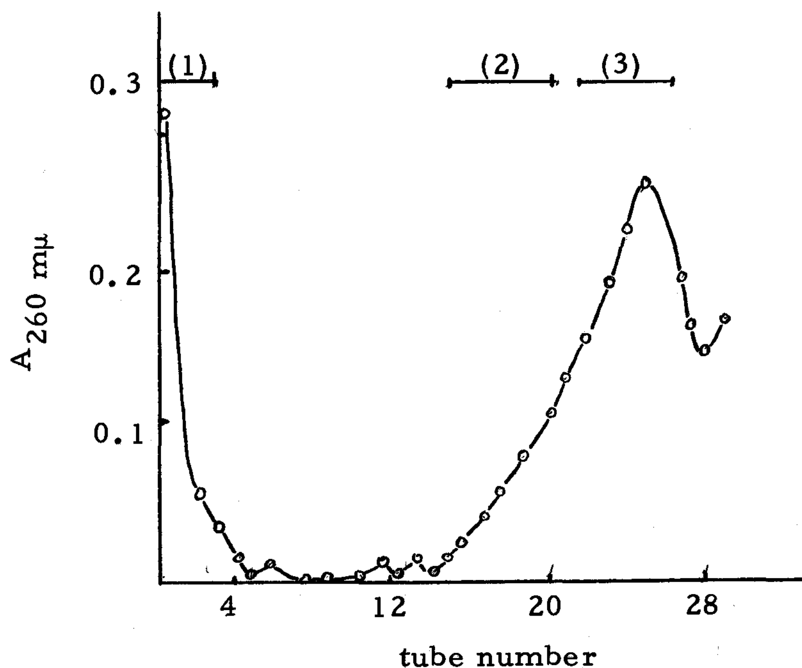

Figure 2. Separation of myeloblastosis virus RNA by sucrose density gradient centrifugation. Virus RNA isolated by phenol extraction was centrifuged for 24 hours in a 5-20% sucrose gradient at 25,000 rev./min. Three RNA pools were made as indicated in the figure. The RNA concentration in the pools were adjusted to 170 μg/ml and their effect on protein synthesis was studied. Pool 1 contains high molecular weight virus fraction and Pool 2 and 3 contain the low molecular weight virus RNA fraction.

Table 1. The effect of RNA fractions from myeloblastosis virus on protein synthesis.

Additions	Counts/min [^{14}C] -leucine incorporated	% inhibition
Qβ RNA	13,036	---
Qβ RNA + Pool 1 RNA (1.7 μg)	10,981	15.8
Qβ RNA + Pool 2 RNA (1.7 μg)	17,935	0
Qβ RNA + Pool 3 RNA (1.7 μg)	521	96.0
Qβ RNA + unfractionated RNA (1.7 μg)	4,565	65.0

Pooled fractions from the sucrose density gradient (Figure 1) and the original virus RNA preparation were added to an E. coli S30 fraction. The template RNA from Qβ phage was added at 50 μg/ reaction mixture. The percent inhibition was calculated from the average of the duplicate assays. Appropriate blanks for each set of conditions were subtracted from the reported values. [^{14}C] - leucine (0.2 μc) having a specific activity of 198 μC/μmole was added to each reaction.

indicating this treatment of the virus RNA concentrates the inhibitor RNA in the 4S region. If fragmented RNA accounts for the inhibition, a large inhibition should have been seen using the pool 2 RNA fraction. The small amount of inhibition by pool 1 RNA may be the result of mixing the RNA from the top of the gradient when the pellet was resuspended, or by aggregation of the low and the high molecular weight RNA.

One μg of low molecular weight RNA inhibits amino acid incorporation 50-100% when Qβ RNA or E. coli RNA are used as a message. Synthetic polynucleotides were also used as messages in cell-free systems for protein synthesis. The translation of polycytidylic acid (poly C) in an E. coli cell-free system is inhibited 92% by the addition of 1.7 μg of low molecular weight RNA. This quantity of low molecular weight virus RNA does not hinder the translation of polyuridylic acid (poly U) or polyadenylic acid (poly A). The low molecular weight virus RNA appears to be selective in the inhibition of protein synthesis. The base composition of virus RNA may influence the translation of mRNA. Inhibition could take place by a hybrid formation between bases in the message and those in the virus RNA. The base composition of the low molecular weight RNA contains 34% guanine (99). The binding of guanine to cytidine is one of the strongest interactions formed by base-base hydrogen bonding. Hybridization, near the beginning of the genetic message, would

inhibit translation of the RNA. Double-stranded RNA serves as a very poor mRNA (86). This inhibition may be similar to that seen when synthetic nucleotides prevented amino acid incorporation by binding to MS2 phage RNA (75). Inhibition could also be taking place if the synthetic polynucleotides competed with MS2 RNA for ribosomal sites. However, the order of inhibition by several polynucleotides was the reverse of that which would be seen if competition for ribosomal sites was taking place.

To determine if there is a competition for ribosomal sites at low concentrations of poly U, LMWt RNA was incubated with low concentrations of poly U in the _E. coli_ cell-free system. The effect of low molecular weight virus RNA on the template activity of small concentrations of poly U is shown in Table 2. Not only did the LMWt RNA fail to inhibit, but it stimulated the incorporation of phenylalanine directed by poly U. Either the low molecular weight virus RNA does not inhibit peptide synthesis by binding to the ribosome, or poly U has a much greater affinity for ribosome sites. However, at low concentrations of poly U, competition for ribosomal sites should be accentuated. Stimulation of phenylalanine incorporation is seen frequently by virus RNA and by myeloblast tRNA. Because myeloblast tRNA does not inhibit peptide synthesis, stimulation of polyphenylalanine formation is likely unrelated to inhibition of peptide synthesis and needs further investigation.

Table 2. Effect of myeloblastosis virus RNA on phenylalanine polymerization.

poly U added (μg)	virus RNA added (μg)	phenylalanine incorporated (Counts/min x 10^{-3})	% change with added virus RNA
5	0	15.1, 13.2	--
5	1.7	14.7, 12.8	-3
10	0	17.2, 15.3	--
10	1.7	20.2, 18.2	+19
20	0	23.1, 21.2	--
20	1.7	26.7, 24.7	+15
30	0	25.0	--
30	1.7	36.8	+47
50	0	51.5, 49.6	--
50	1.7	43.4, 41.5	-16
50*	0	53.8, 45.9	--
50*	1.7	56.0, 54.8	+11

*Separate experiment.

The indicated quantities of poly U and LMWt virus RNA were incubated with 1.6 A_{260} units of an E. coli S30 fraction. Incubation at 35° C was for 30 minutes.

Influence of Ribosomes upon the Inhibition by Virus RNA

If either fragments of HMWt RNA or LMWt inhibitor RNA are inhibiting protein synthesis by ribosomal binding, increasing the quantity of ribosomes should provide sufficient ribosome sites for both RNA species. The ribosome concentration was increased four-fold in the *E. coli* S30 fraction by the addition of washed ribosomes. and their effect upon the inhibition by LMWt RNA is shown in Table 3. Assuming an average molecular weight of 25,000 for the inhibitor RNA, there were 3.5 times the number of ribosomes as LMWt RNA molecules. The concentration of Qβ RNA was kept low so any release of inhibition was due to the ribosome concentration only. It can be seen in Table 3, that in spite of the large number of ribosomes, inhibition remained very high. Increasing the ribosomes in a cell-free system containing fragmented R17 RNA in the presence of R17 phage RNA reversed the inhibition by fragmented RNA (12). It appears that inhibition is by other than fragmented RNA.

Messenger:Inhibitor Ratio Effect

If inhibition is occurring by a mechanism of hybrid formation, increasing the number of molecules of Qβ RNA to a number greater than the number of molecules of inhibitor RNA may result in the translation of free mRNA. Qβ RNA was increased in the S30

Table 3. Influence of ribosomes on inhibition of peptide synthesis by virus RNA.

Relative conc. *E. coli* ribosomes	Counts/min [^{14}C] -leucine incorporated		%
	minus virus RNA	plus virus RNA	inhibition
0.5X	4020	32	99
1.0X	6264	217	97
1.5X	6822	47	99
2.0X	6819	169	97
3.0X	8812	974	89
4.0X	8238	894	89

Ribosomes were separated from the S30 enzyme fraction and added back at relative concentration indicated in the table. 1.0X is equivalent to the normal concentration of ribosomes found in the S30 enzyme preparation from *E. coli*. This corresponds to 263 A_{260} units/ml. Qβ RNA was added at 25 μg/reaction volume. Avian myeloblastosis virus LMWt RNA was added at 1.77 μg/reaction volume.

cell-free system in the presence of LMWt virus RNA. The results are presented in Figure 3. Inhibition decreased as the ratio of template RNA molecules to LMWt RNA molecules increased and was completely reversed when five template RNA molecules for every virus RNA molecule had been added. It is difficult to obtain a stoichiometric value of the number of Qβ RNA molecules/inhibitor RNA molecules since the true molecular weight of the inhibitor RNA is not known, as well as the likelihood that not all the RNA in the LMWt fraction inhibits Qβ RNA translation. This template RNA concentration effect is also seen using poly C.

Evidence for the Involvement of a Supernatant Factor

If Qβ RNA and LMWt virus RNA are competing for a third component in the protein-synthesizing system, increasing the concentration of Qβ RNA could reverse the inhibition. *E. coli* ribosome-free supernatant was prepared and added to the S30 cell-free system containing Qβ RNA and LMWt virus RNA. The results are shown in Figure 4. As the supernatant concentration was increased three-fold, inhibition decreased from 97% to 54%. A still further increase in the concentration of the supernatant fraction produced a decrease in protein synthesis in the control reaction, but the inhibited reaction showed an increase in amino acid incorporationabout equivalent to that observed by the two-fold and three-fold level of supernatant

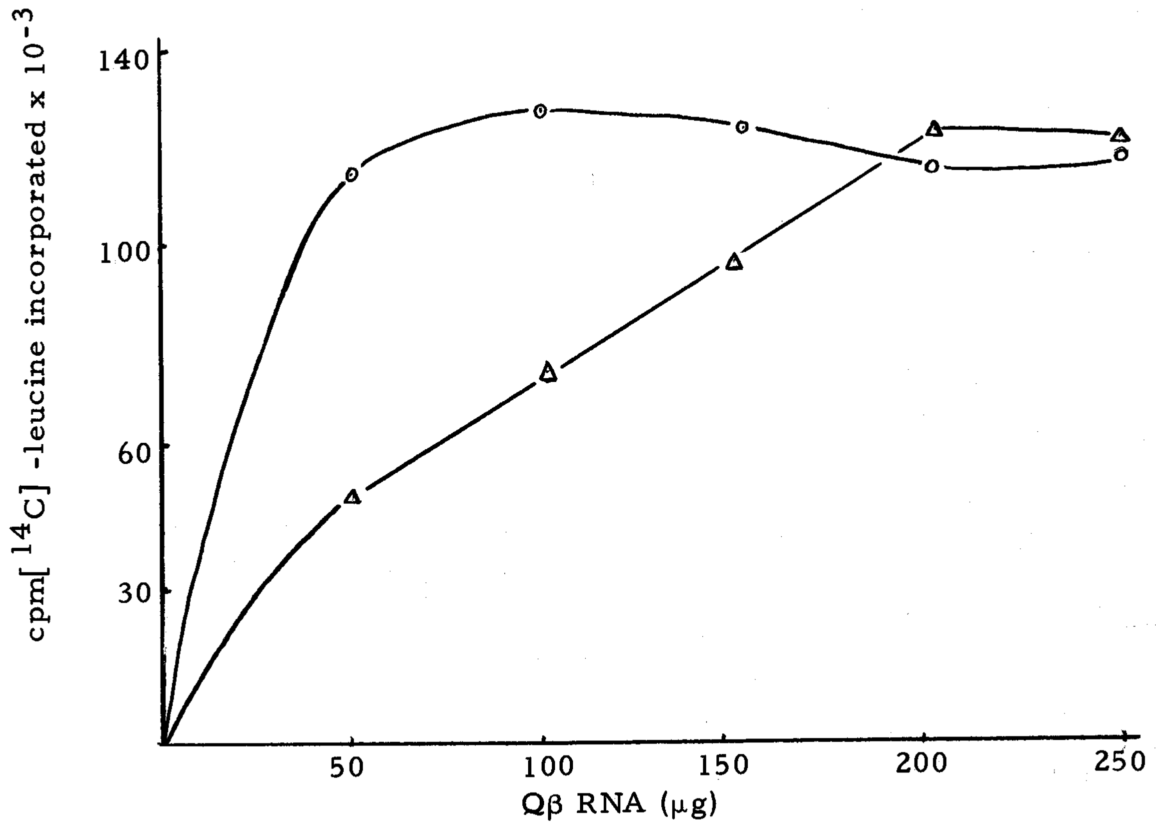

Figure 3. Incorporation of [^{14}C] -leucine into protein at different concentrations of Qβ RNA in the presence and absence of LMWt virus RNA. The reactions were incubated for 30 minutes at 35 ° C. o——o , Qβ RNA control. Δ——Δ , Qβ RNA plus 0. 89 μg LMWt virus RNA/ reaction volume.

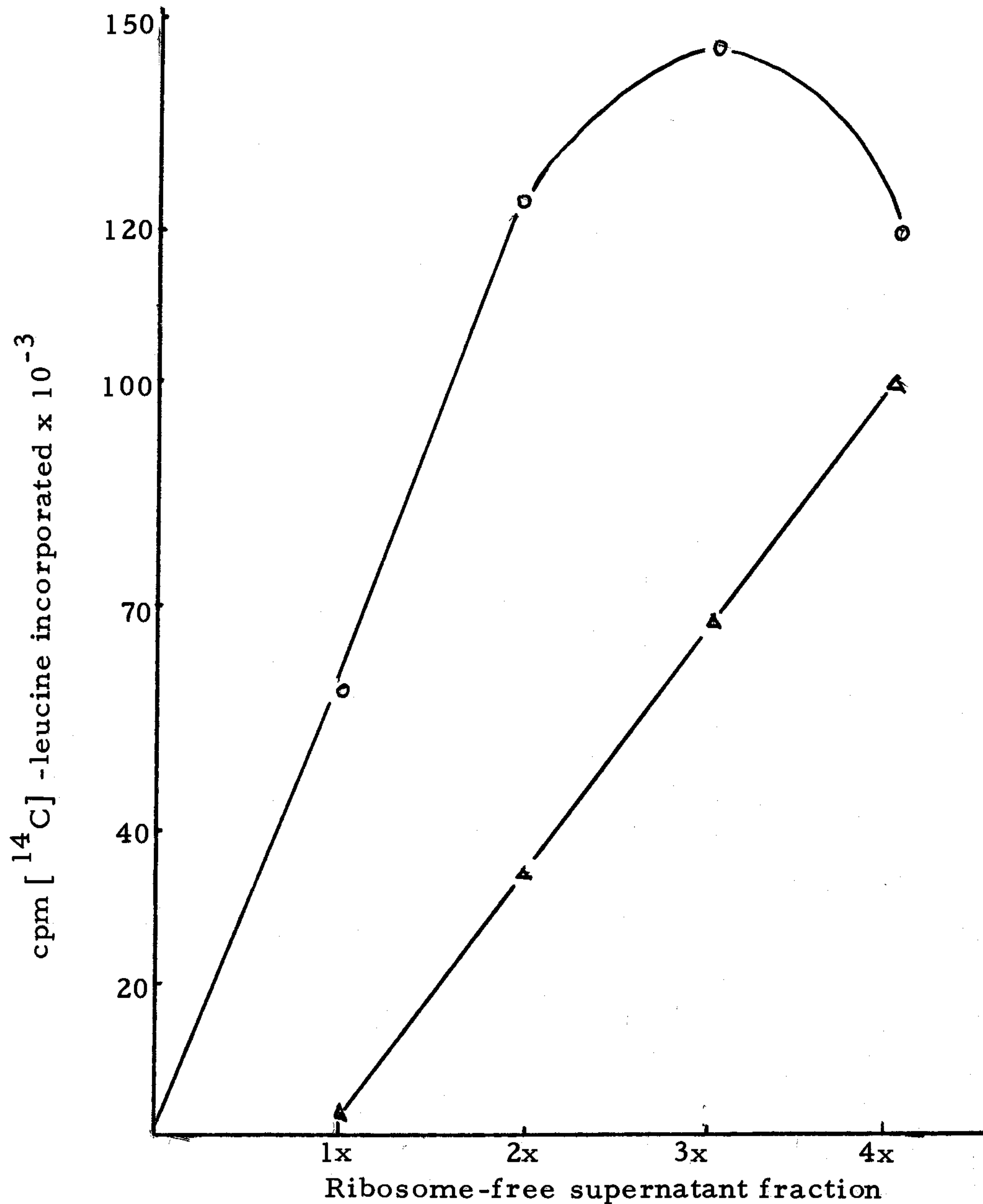

Figure 4. The effect of ribosome-free supernatant factors on inhibition by myeloblastosis virus RNA. The 1x supernatant fraction represents the enzymes and tRNA found in the S30 preparation. The S30 extract was centrifuged to remove the ribosomes and the supernatant fluid was added to the reaction mixture to give a 2, 3, and 4-fold increase in this fraction. Qβ RNA was added at 50 μg/ reaction tube and LMWt RNA was added at 0. 89 μg/ reaction tube. o—o, Qβ RNA control. Δ—Δ,Qβ RNA plus LMWt virus RNA.

factors in the Qβ RNA control. Inhibition by the LMWt virus RNA, by ribosomal binding, would not be expected to be affected by the addition of supernatant factors.

Competition for initiation factors could result in the inhibition of mRNA translation. Factors functioning in initiation have been found associated with DNA (93) and with the supernatant fraction (17). Poly U-dependent incorporation of phenylalanine was not enhanced by the initiation factor. If the LMWt RNA is inhibiting peptide synthesis by binding the initiation factor, inhibition of poly U translation would not be seen. The initiation factor promotes binding of mRNA to the ribosome. Interference with this mechanism would result in the inhibition of peptide synthesis. To determine if inhibition was taking place by preventing mRNA binding to the ribosome, RNA was added to a cell-free protein synthesizing system translating Qβ RNA. When LMWt RNA was added at 0 time, 99% inhibition of amino acid incorporation took place. However, when it was added after ten minutes of incubation, only 72% of the subsequent amino acid incorporation was inhibited. An inhibitor acting similarly to puromycin should result in immediate cessation of amino acid incorporation. The difference in the time of addition may be a consequence of the failure of LMWt RNA to inhibit translation of mRNA which had previously been initiated. After the ribosome completes the translation of a messenger RNA, it is available for reinitiation.

Inhibition of initiation factors would prevent any further reinitiation of polysome formation.

The formation of a complex between mRNA and inhibitor RNA could involve a supernatant factor. Binding experiments between Qβ RNA and [^{3}H] -virus RNA failed to show any complex formation. Three micrograms of [^{3}H] -LMWt RNA were incubated with 300 μg of Qβ RNA for five minutes at 25 °C in a buffered solution. The incubated solution was placed on a 5-20% sucrose gradient and centrifuged 25,000 rpm for 14 hours at 2 °C. Only 0.9% of the radioactivity of the LMWt RNA was associated with Qβ RNA. Assuming uniform labeling in the LMWt RNA fraction, the amount of RNA associated with Qβ RNA would be insufficient to inhibit amino acid incorporation. However this binding may require supernatant factors and needs further investigation.

Effects of tRNA upon Qβ RNA Translation

Because LMWt virus RNA has a sedimentation pattern and an amino acid acceptor activity characteristic of tRNA, its inhibitory role may be related to its tRNA activity. The effect of tRNA from myeloblasts, liver and *E. coli* upon Qβ RNA-directed amino acid incorporation is shown in Table 4. It is evident that inhibitor RNA is other than myeloblast tRNA. Five μg of tRNA failed to inhibit amino acid incorporation to a significant extent. Myeloblastosis

Table 4. Effect of tRNA upon amino acid polymerization in an E. coli cell-free system.

Source of tRNA	Amount added μg	[^{14}C]-phenylalanine incorporated (cpm x 10^{-3})	% Change in amino acid incorporation
Control (no tRNA added)	-	47.6	-
E. coli	1	51.3	+ 8
	2	54.3	+14
	5	54.5	+14
Liver	1	44.4	- 6
	2	38.9	-18
	5	42.2	-11
Virus-infected myeloblasts	1	49.9	+ 5
	2	45.6	- 4
	5	44.1	- 7

Different tRNAs were added to 1.6 A_{260} units of an S30 preparation from E. coli. Each tube was incubated 35 minutes at 35° C. Qβ RNA, 50 μg, was used as the template for protein synthesis.

virus RNA may contain tRNA's not present in the myeloblast tRNA fraction.

Two viruses have the capacity to produce new tRNA's. Phage T2 infection of _E. coli_ results in an alteration of an _E. coli_ leucyl-tRNA (111). Infection of mammalian cells with the DNA-containing herpes virus leads to the synthesis of viral-specific tRNA's (50). Synthesis of viral protein may require tRNA's not found in the host cell but which are produced by alteration of host tRNA or by synthesizing viral-specific tRNA. If altered tRNA's are utilized by the myeloblastosis virus for synthesis of viral-specific protein, they may be removed by membrane budding and concentrated in the virus particle. If inhibition of amino acid incorporation is due to the presence of tRNA's acting in peptide release, the addition of _E. coli_ tRNA should reverse the inhibition. Addition of a large excess of tRNA from _E. coli_ did not alter the translation of Qβ RNA. Thus, factors in the _E. coli_ supernatant reversing the inhibition by mRNA are probably enzymatic in nature.

High Molecular Weight RNA as a Source of Inhibitor RNA

Low molecular weight RNA has been described as fragments of HMWt RNA (99). However, no evidence has been presented demonstrating that HMWt RNA is the source of the LMWt RNA fraction. To determine if fragments of HMWt virus RNA are inhibitory,

fragments were prepared by heat and alkaline treatment and incubated in the QβRNA-*E. coli* S30 cell-free system. Table 5 summarizes the reactions using fragmented RNA. Treatments commonly used to fragment RNA did not produce a fraction inhibiting as the LMWt virus RNA does. Although the tube containing four μg of heat-treated RNA inhibited amino acid incorporation, no inhibition was seen with the reaction containing five μg. Alkaline treatment produced a slight inhibition not comparable to that produced by LMWt virus RNA. It appears that the LMWt RNA inhibitory fraction is other than fragments of HMWt virus RNA.

HMWt myeloblastosis virus RNA can be isolated directly by sucrose density centrifugation after SDS treatment, bypassing the need for phenol extraction.[3] The low molecular weight RNA obtained from the same sucrose density gradient cannot be fragments of HMWt RNA as a result of phenol extraction. This LMWt RNA was deproteinized by phenol and was applied directly to a DEAE column and fractionated. Only 45% of the RNA was eluted from the column. This RNA had significantly greater tRNA activity than unfractionated LMWt virus RNA.[4] One μg of RNA inhibited Qβ RNA translation 11% but two μg inhibited amino acid incorporation 73%. Fragmented RNA

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[3] Dr. A. O'C. Deeney. Personal Communication.

[4] J. W. Carnegie. Personal Communication.

Table 5. Effect of fragmented virus RNA upon Qβ RNA-directed peptide synthesis.

Untreated Virus RNA μg	Heat-treated Virus RNA μg	Alkaline-treated Virus RNA μg	[^{14}C] -leucine incorporated (cpm)
-	-	-	175, 940
1			176, 200
2			184, 891
4			180, 327
	2		181, 235
	4		99, 730
	5		175, 915
		2	167, 215
		4	143, 869

3.2 μg of the E. coli S30 fraction was incubated with 50 μg of Qβ RNA in the E. coli cell-free system. [^{14}C] -leucine having a specific activity of 251 μC/μmole was added to each reaction. Fragments of HMWt virus RNA were prepared by heating the RNA for five minutes at 98° C or incubating the RNA for 60 minutes in a pH 9 tris buffer solution.

having less intact secondary structure should have been retained on the column. Inhibitor activity was not removed from tRNA activity using DEAE Fractionation.

Low Molecular Weight Virus RNA as an Inhibitor in the Chick Cell-free System

Virus-infected leukemic cells can multiply but do not develop into mature leucocytes. The components of the virus structure do not inhibit protein synthesis to the extent that cell division is interrupted, but regulation of cell maturation while cells show exponential growth would imply an inhibition that is highly selective. Although it is not possible to detect a selective inhibition of particular proteins, the effect of virus RNA on protein synthesis can be measured using a chick cell-free system utilizing endogenous mRNA. The effect of inhibitor virus RNA on peptide synthesis directed by polysomal systems is shown in Table 6. Virus RNA inhibited amino acid incorporation for a greater degree in the liver cell-free system than in the myeloblast cell-free system. The addition of myeloblast tRNA showed no inhibition when one to four μg were added. The extent of inhibition is slight compared to the *E. coli* cell-free system. The inhibition by virus RNA in the two systems for protein synthesis may reflect recycling of ribosomes on mRNA. The interpretation was made in an earlier section that inhibition by the virus RNA involves events

Table 6. Influence of LMWt virus RNA on peptide synthesis by myeloblast and liver polysomes.

Experiment no.	Polysome system	Virus RNA added (μg)	[^{14}C] -phenylalanine incorporated (cpm)	% inhibition
1	myeloblast	-	7050	--
	myeloblast	1	6828	3
	myeloblast	2	6138	13
	liver	-	5770	--
	liver	1	5488	5
	liver	2	3951	32
2	myeloblast	-	6690	--
	myeloblast	2	6160	8
	myeloblast	4	4325	35

The chick cell-free system for protein synthesis consisted of 105,000 x g dialyzed supernatant enzyme fractions, a polysome fraction and an ATP-generating system incubated at 35° C for 60 minutes. [^{14}C] phenylalanine (0.25 μC) having a specific activity of 351 μC/μmole was added to each reaction. Each tube contained either 0.17 mg myeloblast enzyme fraction and 0.42 mg myeloblast polysomes or 0.63 mg liver enzyme fraction and 0.30 mg liver poly-some. The magnesium ion concentration was adjusted to an optimal concentration of 7.8 mM.

that occur prior to mRNA attachment. There is little evidence for recycling of polysomes in animal cell-free systems (95). Only a slight inhibition by virus RNA would be expected in this type of system if the LMWt RNA is inhibiting by preventing the reattachment of mRNA to ribosomes. Peptide synthesis is greatly reduced by omitting ATP and GTP or by adding puromycin. These controls in the experiment provide evidence that peptide synthesis by the polysomes is real and that the test is valid.

It is not possible to obtain a definite conclusion from these results with regard to selective control of protein synthesis in the virus-infected myeloblast. The experiments described are consistent with the interpretation that inhibition by virus RNA involves events that occur prior to mRNA attachment to the ribosome and has only a slight effect on peptide synthesis after the polysomes are formed.

Purity of Virus RNA

The LMWt virus RNA can be fractionated on a methylated albumin kieselguhr (MAK) column. Two RNA fractions were eluted at 0.43 M and 0.67 M.[5] No RNA appeared in the position characteristics of ribosomal RNA. Both RNA fractions strongly inhibited

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[5] J. W. Carnegie. Personal Communication.

amino acid incorporation although the 0.67 M fraction is more active. This fractionating procedure concentrates the transfer RNA activity in the 0.43 M fraction although some activity is seen in the 0.67 M fraction.

Inhibitor RNA is present in a bulk virus RNA preparation, in the 4S fraction after fractionation on a sucrose density gradient and in both fractions when LMWt RNA was fractionated on a MAK column.

There is no evidence for extraviral contamination. Chick plasmas were twice centrifuged to remove cells. Ribosomal RNA has never been seen in our virus RNA preparations using sucrose density gradient centrifugation or MAK column fractionation. Precautions have been taken to eliminate any contaminating protein. All the RNA preparations were phenol extracted three times and isolated after centrifugation on a sucrose density gradient. Inhibitor RNA was also isolated from a MAK column. This RNA was passed through a kieselguhr pad, which absorbs protein, before it was bound to the methylated albumin kieselguhr. No fragmentation of Qβ RNA was observed when incubated with the LMWt RNA fraction indicating it was free from nucleases. The sedimentation profile of Qβ RNA on a sucrose density gradient was unaltered after incubation with LMWt RNA. Although protein contamination cannot be completely eliminated, its presence is unlikely.

Inhibitor RNA has been present in the low molecular weight

fraction consistently in spite of the variety of methods used to prepare the RNA. The percentage of inhibitor RNA in the low molecular weight fractions shows a small variance which may be expected from one preparation to another. All attempts, including MAK chromatography, DEAE chromotography and sucrose density centrifugation to remove inhibitor RNA from the presence of tRNA have failed. It can be assumed until further separation is achieved that the LMWt virus RNA acting as an inhibitor is similar in structure and size to tRNA. The tRNA activity present in the virus RNA preparation may be a result of the transfer of cellular tRNA by membrane budding or it may represent the failure of tRNA to be deacylated from C-terminal amino acids after completion of viral coat protein.

Low Molecular Weight Virus RNA as a Transfer RNA Fraction

Transfer RNA accepts amino acids in the presence of aminoacyl synthetases and transfers them into nascent peptides. Myeloblastosis virus RNA has been shown to accept amino acids in the presence of an aminoacyl synthetase enzyme fraction (7, p. 791-803). A demonstration of transfer activity by virus RNA would completely characterize this viral RNA as transfer RNA.

To determine if the transfer function were present, low molecular weight RNA from the myeloblastosis virus was charged with

a [^{14}C] - amino acid mixture. The [C^{14}] -amino acid-virus RNA complex was incubated in the complete chick cell-free polysome system. The results are shown in Table 7. Some buffer hydrolysis of the [^{14}C] -amino acid-virus RNA complex took place. The possibility exists that myeloblast tRNA present in the chick cell-free system can transfer [^{14}C] -amino acids, hydrolyzed from the amino acid-virus RNA complex, to TCA-insoluble protein. However, the chick cell-free system lacks an amino acid requirement indicating there is a large amino acid pool present. Any hydrolysis of a [^{14}C] -amino acid-virus RNA complex would liberate a small quantity of labeled amino acids into a large amino acid pool and very little incorporation of amino acids would be seen because of this dilution. The results indicate there is an efficient transfer of amino acids from charged virus RNA. The lower percent of amino acid transfer at higher concentrations of charged virus RNA may be due to inhibition by additional tRNA on the previously tRNA-saturated cell-free system. Increasing the amino acid-virus RNA concentration results in an inhibition of amino acid transfer which is twice as great as that observed with a similar concentration change of liver aminoacyl-tRNA. The difference may be due to an inhibition of polysome-dependent amino acid incorporation by the low molecular weight RNA fraction described previously. Although ATP contamination prevented a determination of the actual virus

Table 7. Transfer of amino acids from virus RNA in the polysome system.

Experiment no.	Liver enzyme	Myeloblast enzyme	Myeloblast polysomes	Liver aminoacyl-tRNA (ml)	Virus aminoacyl-RNA (ml)	Percent amino acids incorporated	Percent amino acids hydrolyzed
1	-	-	-		0.03		41
	-	-	-	0.03			61
2		+	+		0.02	48	
		+	+		0.05	19	
	+		+		0.02	58	
	+		+		0.05	40	
3	+		+	0.03		54	
	+		+	0.06		46	

The tubes were incubated in the chick polysome cell-free system containing 0.15 mg ribosomes in experiment 1 and 2 and 0.42 mg ribosomes in experiment 3. Myeloblast enzyme was used at an optimal concentration of 0.18 mg and liver enzyme at a level of 0.63 mg. 0.03 ml of liver aminoacyl-tRNA contained 12.6 μg (2030cpm) and 0.02 ml of virus aminoacyl-tRNA contained approximately 4 μg of virus RNA (1088 cpm). To measure the quantity of unhydrolyzed aminoacyl-tRNA, the tubes in experiment 1 were not heated prior to filtration.

RNA quantities utilized, starting concentrations in the charging reaction would suggest up to 10 μg of LMWt virus RNA were present. This represents a high concentration of inhibitor RNA. These results demonstrate that virus RNA contains an RNA fraction which has biological activity identical to that of tRNA.

Development of a Chick Cell-free System for Protein Synthesis from Chick Cells

The *E. coli* cell-free system for protein synthesis is generally used, to the neglect of *in vitro* systems from other cell types. This has occurred partially because procedures for its isolation and utilization have already been developed (82). New, beneficial information, such as initiation of protein synthesis, is still being harvested from its utility. However, data obtained using the *E. coli* system may not be applicable to mechanisms occurring in vertebrate cell systems. A cell-free system for protein synthesis from reticulocytes has been very successful. Although RNA isolated from reticulocytes stimulates amino acid incorporation in both the reticulocyte and *E. coli* cell-free systems, newly synthesized, hemoglobin-specific peptides can only be identified in the reticulocyte cell-free system (66). Poliovirus RNA only produces poliovirus-specific peptides in cell-free extracts from HeLa cells and not in cell-free extracts from *E. coli* (103, 113). Specific factors are present in

certain cells favoring them as hosts for viral replication. Cells serving as hosts for viral replication may contain certain initiation factors necessary for viral-specific protein synthesis. They also may contain a more favorable complement of tRNA's for viral specific-protein synthesis. Liver and myeloblast cell-free systems for protein synthesis have been isolated to examine the influence of myeloblastosis virus RNA on protein-synthesizing systems and to examine the messenger RNA activity of myeloblastosis virus RNA.

Isolation of a Ribosome Fraction

Ribosomes were isolated from liver and myeloblasts according to the method of Korner (77) using a reduced magnesium concentration. Ribosomal aggregation can easily occur in the presence of magnesium ions, especially at concentrations greater than five millimolar during the isolation of ribosomes (108). Since it has been proposed that the ribosome-mRNA complex may solely be the consequence of magnesium ion bridges (76), isolation of ribosomes in low magnesium ion appears to result in an 80S ribosome fraction free of mRNA. A sucrose density gradient profile of these ribosomes is shown in Figure 5. These ribosomes had less endogenous activity than those isolated in higher magnesium ion concentrations and showed a much better stimulation of phenylalanine polymerization when poly U was added to the cell-free system for protein synthesis. The template activity of two RNA

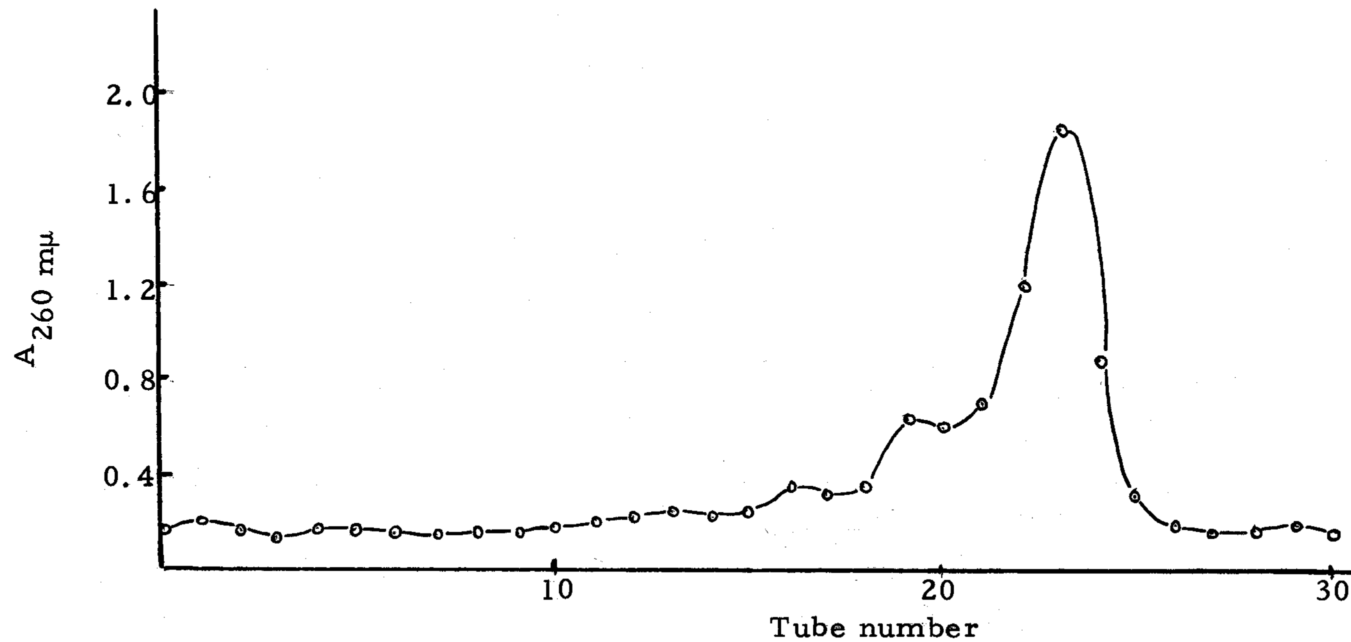

Figure 5. Sucrose density gradient profile of a ribosome preparation isolated using a low magnesium ion concentration. The ribosome solution was applied to a 0.3 M to 1.0 M sucrose gradient containing 0.02 M tris buffer, pH 7.6, 0.1 M KCl, 0.001 M magnesium acetate and 0.006 M 2-mercapto-ethanol and centrifuged three hours at 25,000 rpm in the Spinco SW 25.1 swinging bucket rotor. The centrifuge tube was punctured with a hypodermic needle and one ml fractions were collected. The absorbance of each fraction was measured using a Gilford spectrophotometer.

fractions and poly U is shown in Table 8. In search of a natural template RNA fraction, RNA extracted from the membrane fraction of the myeloblast cell was utilized. Such a fraction could contain viral RNA in small quantities since they are the site of virus replication in the leukemic cell. The results indicated that template RNA was present in quantities too low for stimulation, or that the cell-free system was inadequate to translate this RNA fraction. Qβ RNA stimulated amino acid incorporation when incubated with a low concentration of ribosomes, but no stimulation of amino acid incorporation by membrane RNA from the myeloblast was seen. Qβ RNA is an excellent template for protein synthesis in the <u>E. coli</u> cell-free system. Poly U stimulated phenalalanine incorporation in chick cell-free systems. Up to 525 μμ moles of [^{14}C] -phenylalanine/mg of liver ribosomes was incorporated using a pH 5 enzyme fraction and low endogenous ribosomes, and up to 472 μμ moles of [^{14}C] -phenylalanine/mg of myeloblast ribosomes was incorporated using the same enzyme fraction and low endogenous myeloblast ribosomes. Polymerization of phenylalanine occured at a nearly constant rate for 30 minutes.

Certain characteristics of these ribosomes are apparent. 1) Ribosomes isolated in 1.0 mM magnesium ion contained higher endogenous activity than the ribosomes isolated in 0.1 mM magnesium ion. Polymerization of phenylalanine was stimulated more by the addition of poly U using ribosomes isolated in the lower magnesium ion concentration. 2) Sonication reduced both the endogenous activity

Table 8. Template activity of different RNA fractions.

Experiment no.	pH 5 enzyme fraction	Ribosomes (mg)	Poly U (μg)	Qβ RNA (μg)	Membrane RNA (μg)	μμ moles incorporated/mg ribosomes
1	+	0.22	-	-		16
	+	0.22	250			159
	+	0.22		50		20
	+	0.11		-		21
	+	0.11		50		79
2	+	0.22			-	16
	+	0.22			50	16
	+	0.22			125	16

The tubes were incubated at 35° C for 45'. [^{14}C] -phenylalanine was incorporated using the above quantities of myeloblast ribosomes and 0.63 mg of a liver pH 5 enzyme preparation.

and the capacity to translate poly U. 3) Ribosomes were best able to translate template RNA and poly U when incubated in the reaction mixture at low concentrations. At low ribosome concentrations the binding constant for a ribosome-mRNA interaction is favored over ribosome-ribosome aggregation. Nuclease contamination in the ribosome fraction likely fragments poly U and mRNA at a faster rate when higher ribosome concentrations were used. Higher concentrations of ribosomes may form polysomes with poly U or mRNA containing too many ribosomes for optimal amino acid incorporation. 4) Linear incorporation of phenylalanine continues for 30 minutes directed by the addition of poly U. 5) Polyuridylic acid stimulates phenylalanine incorporation as much as 23 fold when incubated in a 80S ribosome cell-free system.

Isolation of Enzyme Fractions for Protein Synthesis

Various enzyme fractions were examined for activity for protein synthesis in cell-free systems. Liver and myeloblast pH 5 enzyme fractions were isolated by repeated precipitation and resuspension. The pH 5 enzyme fraction had a high concentration of tRNA and synthetase enzymes. It was the most efficient enzyme fraction catalyzing the incorporation of phenylalanine using ribosomes prepared with a low magnesium ion concentration and poly U as a template RNA. The full complement of aminoacyl synthetases

is not completely fractionated by pH 5 precipitation (2). Enzymatic factors catalyzing the transfer of amino acids from aminoacyl-tRNA to nascent peptides also remain in the pH 5 supernatant (36). An enzyme fraction was prepared from the pH 5 supernatant using ammonium sulfate fractionation techniques. When added to the pH 5 enzyme fraction, it stimulated poly U-dependent amino acid incorporation but only slightly stimulated Qβ RNA-directed amino acid incorporation. The pH 5 enzyme fraction was also supplemented with an enzyme fraction similar to that used in the hemoglobin cell-free system (68). Ribosome-free liver supernatant was precipitated by 70% ammonium sulfate, dissolved in BRS and dialyzed overnight at 2° C. This enzyme fraction when added with the pH 5 enzymes to a cell-free system for peptide synthesis directed by poly U also stimulated the incorporation of phenylalanine. Thus, the pH 5 enzyme preparation contains an incomplete complement of enzymes necessary for the synthesis of protein.

Because the enzyme fraction precipitated from the pH 5 enzyme supernatant contained excellent aminoacyl synthetase activity, it was used as the sole enzyme fraction in a cell-free protein-synthesizing system. When supplemented with tRNA it contains nearly the same activity as the pH 5 enzyme fraction.

To obtain a complete enzyme fraction, 105,000 x g supernatant fractions were prepared from liver and leukemic myeloblasts.

Although some preparations required tRNA for optimal activity, the S105 fraction resulted in the best incorporation in the natural polysome cell-free system. These ribosome-free supernatants also catalyze the poly U-directed phenylalanine incorporation. The incorporation is less, however, than that obtained with a pH 5 enzyme fraction or a 35-70% ammonium sulfate fractionated pH 5 supernatant preparation. The S105 enzyme fractions probably contain a smaller quantity of phenylalanine aminoacyl synthetase and phenylalanyl -tRNA than the pH 5 enzyme fractions, but a more complete complement of aminoacyl synthetases.

The Cell-free Polysome System

To obtain a cell-free system for protein synthesis containing mRNA, polysomes were isolated from myeloblasts (129). Polysomes are believed to be the functional unit in protein synthesis. Their isolation requires that only gentle breakage be used and that their natural union with the endoplasmic reticulum be disrupted. A sucrose density gradient profile of a polysome preparation from myeloblasts is shown in Figure 6. Comparing the analysis done for liver polysomes (129) with that of the myeloblast polysome profile, polysomes containing from one to 20 ribosomes appear to be present. In contrast to this, a similar analysis applied to ribosomes isolated in low magnesium ion indicates there is only one 80S peak which is

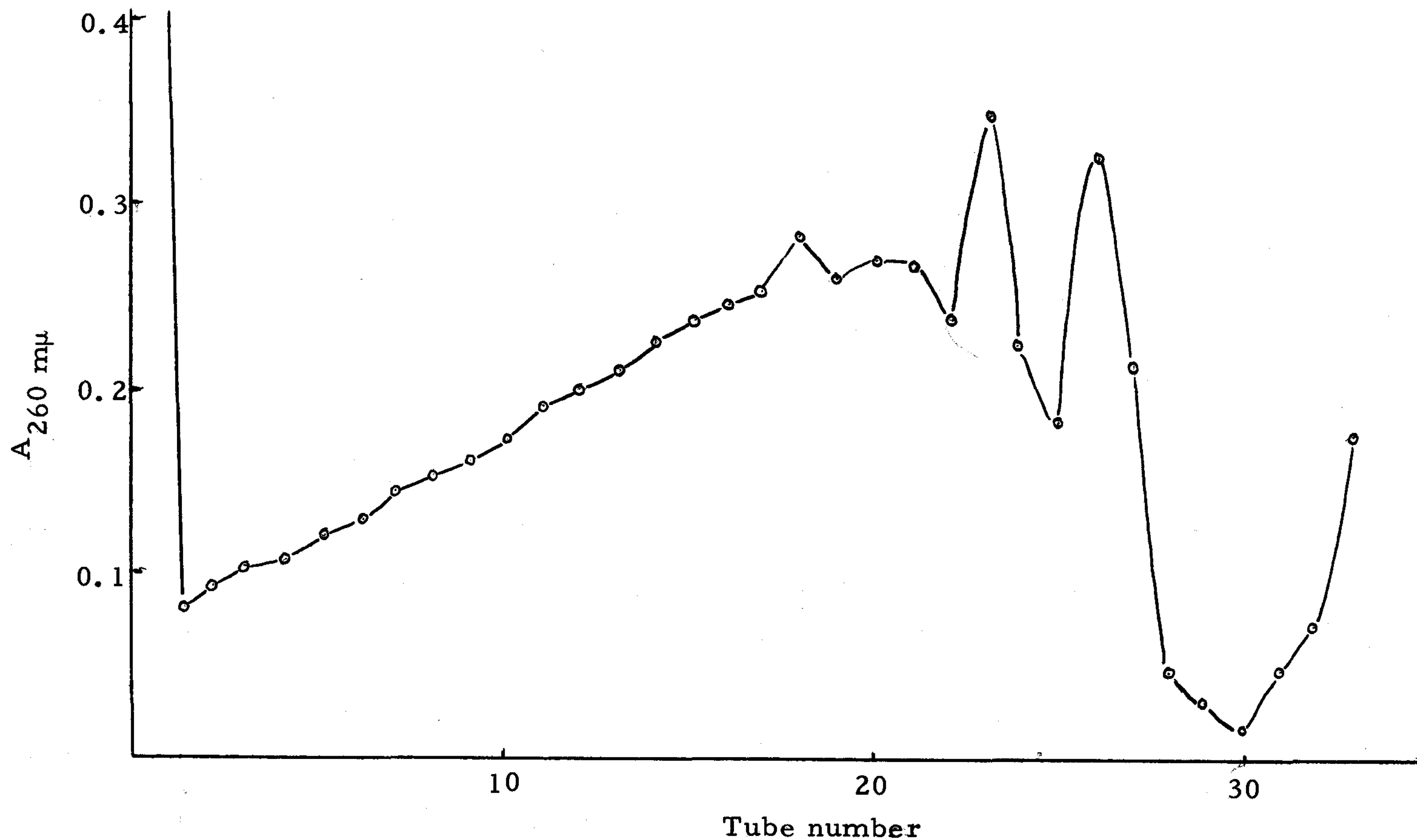

Figure 6. Sucrose density gradient profile of a polysome preparation. The polysome solution was applied to a 0.3 M to 1.0 M sucrose gradient containing 0.05 M tris buffer, pH 7.6, 0.025 M KCl and 0.005 M $MgCl_2$ and centrifuged 24,000 rpm for three hours in the Spinco SW 25.1 swinging bucket rotor. The temperature was held at 1 °C during the run. The centrifuge tube was punctured with a hypodermic needle and one ml fractions were collected. The absorbance of each fraction was measured using a Gilford spectrophotometer.

seen in Figure 5. Polysomes are characterized by high sedimentation values and a high content of endogenous mRNA giving a large incorporation of amino acids in comparison to the messenger RNA-independent activity of 80S ribosomes. Preincubation techniques were used to convert the messenger-bound ribosomes to those requiring mRNA for activity. Polysomes were a) incubated at 2° C with Qβ or membrane RNA 30 minutes prior to the addition of the components necessary for protein synthesis, b) incubated in a cell-free system for 30 minutes at 35° C prior to the addition of template RNA, and c) quick thawed, allowed to stand 30 minutes at 2° C and then incubated in the cell-free system. Preincubation at 2° C using method a) or c) resulted in very little change in the incorporation pattern from the normal procedure of preparing cell-free reactions. Although the stimulation of Qβ and membrane RNA was small, 195 μμ moles of [^{14}C] -phenylalanine was incorporated in response to the addition of poly U after a 30 minute preincubation. Addition of poly U at zero time resulted in the incorporation of only 49 μμ moles of phenylalanine. Current mechanisms in protein synthesis hypothesize that ribosomes leave the messenger RNA when a peptide is completed and begin synthesis of a new protein after rejoining the 5'-hydroxyl end of the messenger RNA. In an incubation mixture containing poly U, ribosomes would be unable to bind poly U until they were liberated from mRNA. Little incorporation took place after 30

minutes in a cell-free polysome system, suggesting that ribosome readout of template RNA was complete although ribosomes were fully competent to synthesize polyphenylalanine. The lower stimulatory activity of poly U when added at zero time may be due to the activity of nucleases prior to the formation of poly U polysomes. Ribosomes are probably able to bind poly U immediately after addition when added to a 30 minute preincubated reaction. Addition of poly U to preincubated polysomes showed the best biosynthesis of polyphenylalanine in a myeloblast cell-free system.

Polysomes isolated from liver were able to incorporate amino acids in a cell-free protein synthesizing system. Although some stimulation was seen with zero time addition of poly U, increased incorporation was absent when poly U was added at 30 minutes contrary to the myeloblast cell-free polysome system. Degradation of ribosomes may occur to a much greater extent than that in the myeloblast cell-free system. Ribosomes isolated in low magnesium ion remained responsive to the addition of poly U after a 20 minute incubation although some inactivation had occurred. Stimulation after 20 minutes was nearly 50% less than when poly U was added at zero time.

Polysomes isolated in the usual manner show the presence of a considerable amount of contamination by enzymes involved in the synthesis of protein. Contaminating enzymes may be bound to

the ribosomes themselves, failing to be removed by centrifugation through 2 M sucrose. The lack of a tRNA requirement would suggest that tRNA, too, is contaminating the polysome fraction.

Figure 7 indicates the dependence of amino acid incorporation upon ribosome concentration and enzyme fractions. Polysome-dependent amino acid incorporation has characteristics seen in other cell-free protein synthesizing systems. a) There is a strong dependence upon ATP and GTP. Omission of these triphosphates results in a 96% inhibition of amino acid incorporation. b) Puromycin, an antibiotic which results in premature peptide chain termination, inhibits 75% of amino acid incorporation. c) Radioactive amino acids are transferred to acid-insoluble protein when added to the reaction in the form of aminoacyl-tRNA.

Not only are polysomes contaminated by the enzymes required for protein synthesis, but certain phenomena suggest other contaminants may be present. The cell-free polysome system lacks an amino acid requirement. Although the enzyme fraction was extensively dialyzed, some amino acids may be constantly liberated by proteolytic enzymes or may be present as contaminants from the polysome fraction.

Incubation of polysomes in cell-free systems for protein synthesis results in an energy-dependent breakdown of polysomes (44, 67, 85). Analysis by sucrose density gradient centrifugation was

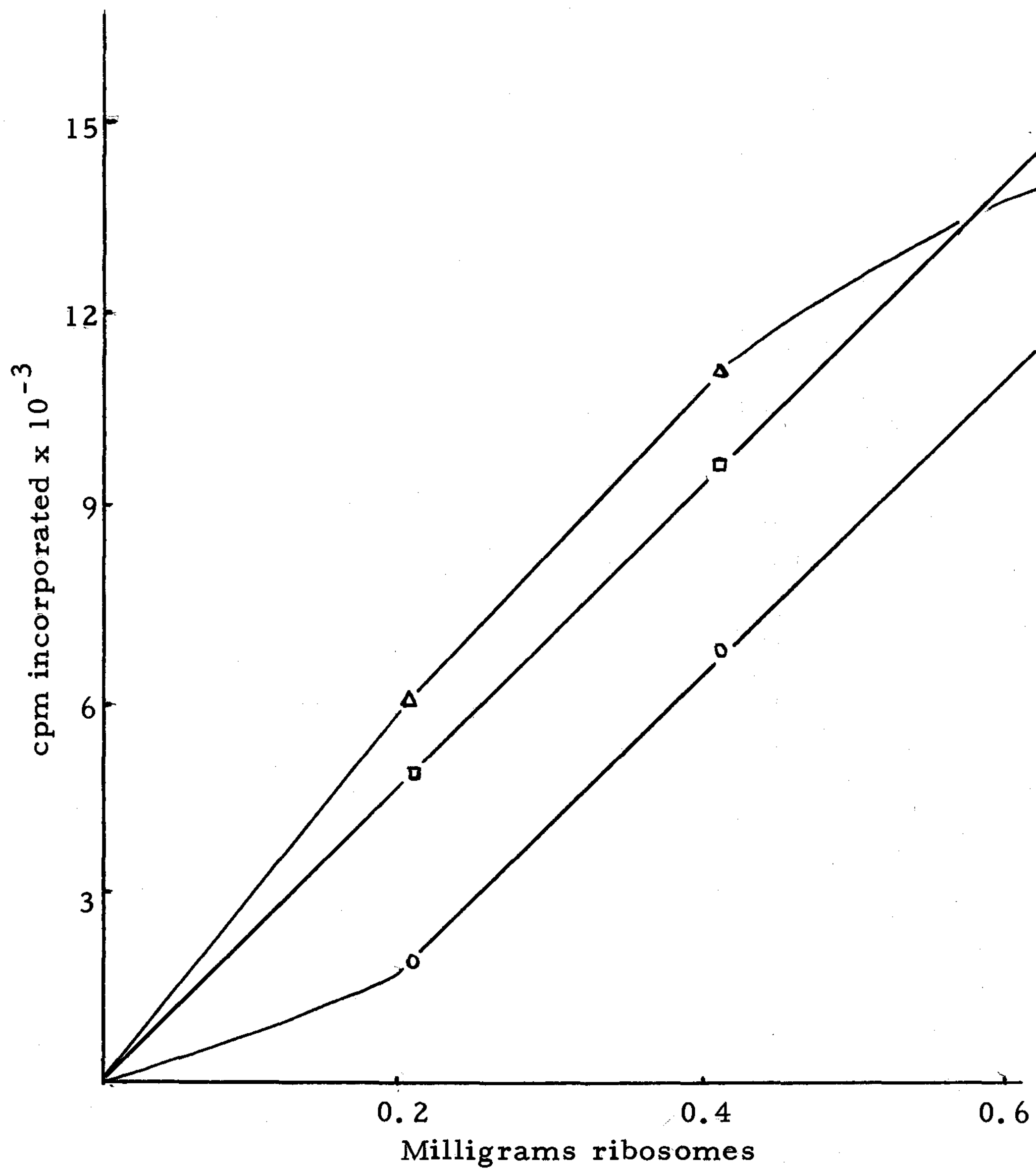

Figure 7. Incorporation of [^{14}C] -leucine in a polysome cell-free system. The indicated quantity of ribosomes were incubated in a protein-synthesizing system for 60 minutes at 35 ° C alone or with either 0. 63 mg liver supernatant enzyme or 0. 38 mg pH 5 liver enzyme. Polysomes alone, o——o ; polysomes plus supernatant enzyme, △——△ ; polysomes plus pH 5 enzyme, □——□ .

done on complete systemsafter 20 and 60 minutes. With time, a progressive breakdown of material occurred in the polysomal region. Breakdown of polysomes is generally followed by a quantitative increase in the 80S ribsosome peak. Because sucrose density gradient analysis of incubated reactions indicated that ribosomes were broken down to small fragments, proteolytic or RNase activity must be present, degrading ribosomes and preventing amino acid incorporation after 30 minutes.

The presence of RNase was confirmed using [^{3}H] -28S RNA isolated from myeloblasts. Following incubation of [^{3}H] -RNA with dialyzed liver supernatant or myeloblast polysomes for 45 minutes at 35° C, the incubation reaction was centrifuged in a sucrose density gradient. The gradients were fractionated and the RNA precipitated with trichloroacetic acid, filtered onto millipore filters and the radioactive RNA counted. Both the polysome and enzyme fractions shifted the RNA from the lower third of the gradient to the upper third as a result of specific cleavage of the 28S RNA. Several inhibitors of RNase failed to stimulate amino acid incorporation. Spermidine and protamine sulfate, while not inhibiting the formation of peptides, did not give increased incorporation. Macaloid, a sodium magnesium lithofluorosilicate similar to bentonite, did not increase amino acid incorporation in the polysome system.

The polysome-dependent cell-free system has a Mg^{++} ion

optimum between six and eight millimolar, a concentration optimum found with most natural cell-free amino acid incorporating systems. Differences were seen in the incorporation of different amino acids. Leucine was incorporated 82% more than phenylalanine on a per mole basis. A radioactive amino acid mixture showed greater incorporation over that obtained using phenylalanine or leucine alone. The failure to obtain an amino acid requirement would imply that there is a large amino acid pool diluting the specific activity of the radioactive amino acid. Peptide-synthesizing activity may be much greater than which has been observed.

The inhibition of [^{14}C] -leucine incorporation by poly U has been used as a measure of ribosome recycling in the reticulocyte cell-free system (44). In the myeloblast cell-free protein-synthesizing system, amino acid incorporation was not inhibited by poly U. This fact indicates that chain initiation may not be taking place.

It has been reported that chain initiation is a relatively rare event even in cell-free systems synthesizing hemoglobin peptides (10, 95). Polypeptide chain completion appears to be the major product of cell-free incorporation. Ribosome attachment to mRNA is very labile to proteolytic enzymes (105). Recycling of ribosomes may be inhibited by nuclease activity on mRNA and ribosomes and by proteolytic enzyme activity on enzymes involved in reinitiation of protein synthesis. *In vivo* systems may utilize circular polysomes

not requiring reattachment of ribosomes to mRNA (84).

If the large myeloblastosis virus RNA molecule binds ribosomes in proportion to its size, a large aggregation of ribosomes should be seen which would likely be in the pellet fraction of the centrifuged specimen. The absorbancy in this area contains 29% of the recovered material and could represent a large polysome like that seen with poliovirus RNA (96). To test whether this is an artifact, the supernatant fraction normally centrifuged to concentrate the polysomes was applied directly to a sucrose gradient and centrifuged. In addition to this, a similar supernatant fluid, which was obtained by breaking the myeloblast cell with a 0.1 M tris buffer-0.005 M $MgCl_2$ solution by stirring at 2° C for 30 minutes, thus avoiding any fragmentation of very large polysomes by homogenization, was applied to a sucrose gradient and centrifuged three hours. No large polysomes were seen after centrifugation, suggesting the pellet material similar to that seen in Figure 6 is non-specific ribosomal aggregates, a result of concentrating the polysomes by high speed centrifugation.

Template Activity of Nuclear and Cytoplasmic RNA

Stimulation of phenylalanine incorporation by poly U in a cell-free system does not signify that the system is capable of *in vitro* protein synthesis. Stimulation by natural mRNA would be a much

better criterion for peptide synthesis. RNA was isolated from liver and the nucleus of leukemic myeloblasts. Nuclear RNA should contain a higher concentration of mRNA. The activity of these RNA fractions in a ribosome cell-free system is shown in Table 9. Only a slight stimulation of amino acid incorporation is seen using two different RNA fractions. The activity of nuclear and cytoplasmic RNA in the *E. coli* cell-free system is shown in Table 10. Although no stimulation of amino acid incorporation by added nuclear RNA is seen at 10 mM magnesium ion, the presence of a small quantity of liver enzyme fraction allows the RNA to serve as a template for amino acid incorporation. The same phenomena occurs using liver RNA. These enzyme fractions may contain initiation factors necessary for the translation in the *E. coli* system of template RNA from higher cell types. Initiation of protein synthesis may be different in cells of higher organisms (83). These enzyme fractions may also contain factors which promote the binding of bacterial ribosomes to liver and myeloblast RNA. Table 11 summarizes the experiments in which RNA was added to the preincubated-polysome system. Stimulation of amino acid incorporation was obtained with both RNA fractions. However, the added RNA, in all of these systems, shows only a slight stimulation compared to that caused by the addition of poly U in the chick and *E. coli* cell-free systems and compared to the addition of Qβ RNA in the *E. coli* cell-free system. It is

Table 9. Template activity of nuclear and liver RNA.

Liver pH 5 supernatant fraction	Mg^{++} concentration (mM)	tRNA	Liver RNA	Nuclear RNA	μμ moles amino acid incorporated
+	10	+			6.3
+	13	+			6.0
+	13	+	+		7.4
+	13	+		+	7.2
+	10	+		+	7.5

The tubes were incubated for 30 minutes at 35° C. The following quantities were used: liver tRNA, 10 μg; liver RNA, 128 μg; nuclear RNA, 105 μg; myeloblast ribosomes, 0.18 mg; 35% AS pH 5 supernatant fraction, 0.15 mg; 35-60% AS pH 5 supernatant fraction, 0.17 mg.

Table 10. Activity of nuclear and liver RNA in the _E. coli_ cell-free system.

Experiment no.	70% AS enzyme fraction	S 105 enzyme fraction	Mg^{++} concentration (mM)	Qβ RNA	Liver RNA	Nuclear RNA	μμ moles [^{14}C] -leucine incorporated
1			10	+			52.0
			10				2.0
			13				1.3
			10			+	1.6
			13			+	1.9
	+		10			-	3.0
	+		10			+	4.4
2	+		10		-		5.7
	+		10		+		8.5
		+	10		-		6.0
		+	10		+		8.7

The _E. coli_ cell-free system, 1.6 A_{260} units, was incubated at 35° C for 35 minutes. In experiment 1, 50 μg of Qβ RNA, 50 μg nuclear RNA and 0.11 mg of the 70% AS fraction from liver were added to the appropriate tubes. Experiment 2 differed in that 0.22 mg of different liver enzyme fractions, and 64 μg of liver RNA were added.

Table 11. Stimulation of amino acid incorporation in a preincubated polysome system.

Experiment no.	S 105 enzyme fraction	pH 5 supernatant enzyme fraction	Myeloblast polysomes	Mg^{++} concentration (mM)	Nuclear RNA (μg)	Bulk RNA (μg)	$\mu\mu$ moles [^{14}C] -amino acid incorporated
1	+		+	9		-	0.5
	+		+	9		64	1.4
	+		+	6		-	0.1
	+		+	6		192	0.2
	+		+	9		-	0.6
	+		+	9		192	1.0
2	+		+	9.5	-		3.3
	+		+	8.5	100		2.9
	+		+	7.6	150		5.2
	+		+	9.5		-	3.3
	+		+	9.5		128	3.9
	+		+	9.5		256	4.1
3		+	+	10	-		1.4
		+	+	10	100		1.8
		+	+	13	-		1.4
		+	+	13	100		2.4

RNA was added to the preincubated cell-free systems after 20 minutes. Controls represent addition of buffer rather than RNA. Experiment 1 contained 0.63 mg protein and 0.42 mg ribosomes. Radioactivity represents incorporation of [^{14}C] -phenylalanine. Experiment 2 differed in the 0.42 mg of enzyme was used and [^{14}C] -leucine was added. Experiment 3 contained 0.15 and 0.17 mg of the 35% and 60% ammonium sulfate fractions of the pH 5 supernatant respectively.

probable that these RNA fractions have a low concentration of mRNA and that the RNA which is present has a high degree of intact secondary structure.

It is difficult to explain why Qβ RNA does not serve as efficiently as a template in the liver or myeloblast systems as it does in the *E. coli* system. Ribosomes from higher organisms may have their own initiation factors for which only certain RNA's contain the necessary starting sequence. Heterogenous cell-free systems may provide an answer. Neither Qβ RNA nor poly U served as a message in the cell-free system containing myeloblast ribosomes and *E. coli* ribosome-free supernatant. Since poly U can bind to myeloblast ribosomes, the tRNA-ribosome complex must not be functional in a heterogenous system. Results of incubating poly U in a cell-free system containing *E. coli* ribosomes and a pH 5 liver enzyme fraction also indicated a lack of transfer of amino acids to poly U-bound *E. coli* ribosomes.

In vitro protein synthesis from higher cell types is still at an early stage of development. There are a number of apparent reasons. 1) So many components are necessary for protein synthesis it is difficult to obtain fractions free from catabolic enzyme activity. 2) Evidence is accumulating that initial binding of ribosomes to mRNA may take place in the nucleus and not in the cytoplasm. Ribosomes may remove mRNA from DNA enzymatically and move

it into the cytoplasm forming a circular polysome not requiring continual reinitiation (84). These enzymatic factors which may require DNA for activity may be difficult to isolate. 3) The interaction of ribosomes with membranes appears to be a highly ordered process. The 50S ribosomal subunit appears to be embedded in a membrane (84). The membranes are removed in cell-free systems from higher organisms. Considering these are just a few of the differences between *in vivo* and *in vitro* systems it is not surprising that *in vitro* systems from vertebrate cells show little resemblance to the system functioning in intact cells.

Template Activity of High Molecular Weight Virus RNA

High molecular weight myeloblastosis virus RNA may represent the viral genome. The RNA is believed to have a molecular weight approaching 1×10^7 (15, 99). Such a large RNA molecule may have a high degree of secondary structure and not serve as a template for amino acid incorporation in cell-free systems. HMWt virus RNA was incubated in the *E. coli* cell-free system. The results are shown in Table 12. This purified virus RNA shows a definite stimulation of amino acid incorporation, better than that which is seen using myeloblast nuclear and membrane RNA, or with liver RNA. Sucrose density gradient fractions containing myeloblastosis virus RNA stimulated an *E. coli* cell-free system

Table 12. Messenger activity of high molecular weight virus RNA in the *E. coli* cell-free system.

Strain	HMWt virus RNA	Heated HMWt virus RNA	LMWt virus RNA	[^{14}C] -leucine incorporated cpm
K 12	-			1629
	+			3009
		+		3199
	+		+	1074
B	-			783
	+			2810

3.2 A_{260} units of the S30 fraction from strain K 12 or 2.9 A_{260} units of the S30 fraction from strain B were incubated for 30 minutes using 0.25 μc of [^{14}C] -leucine as the radioactive amino acid. 25μg of HMWt virus RNA or 25 μg of HMWt virus RNA which had been heated for five minutes at 90° C was added to the cell-free system. One HMWt RNA fraction was supplemented with 1.68 μg of LMWt virus RNA prior to incubation.

(139). The greatest stimulation was seen with the fractions containing HMWt viral RNA. Addition of liver and myeloblast supernatant did not stimulate template activity of the virus RNA. If the virus RNA has a complex secondary structure, fragmentation may result in a more efficient messenger RNA molecule. The virus RNA was heated for five minutes at 90° C, cooled and added to the cell-free system. Stimulation of amino acid incorporation was only increased slightly. Low molecular weight virus RNA appears to inhibit the template activity of high molecular weight viral RNA in the *E. coli* system. Inhibition by the LMWt RNA may prevent the formation of virus RNA-ribosome complexes as it appear to do with Qβ RNA. No conclusions can be drawn without further experimentation regarding the effect of the LMWt virus RNA on the template activity of the RNA viral genome *in vivo*. HMWt virus RNA was also incubated in a chick cell-free system. The results are shown in Table 13. The HMWt RNA also stimulated amino acid incorporation in chick cell-free system even better than Qβ RNA which serves as an excellent message in the *E. coli* system. These results indicate that high molecular weight virus RNA appears to have the capacity to code for protein. It is difficult to know how secondary structure may be affecting its template activity.

Preliminary results using sucrose density gradient centrifugation indicate that incubation of HMWt virus RNA with myeloblast

Table 13. Activity of high molecular weight virus RNA in the chick cell-free system.

Enzyme	Qβ RNA	HMWt virus RNA	Poly U	[^{14}C] -amino acid incorporated cpm
Liver pH 5	-	-	-	1140
	+			908
		+		1362
			+	28974
Liver pH 5 supernatant	-	-	-	3142
	+			3504
		+		4291

Various messenger RNA fractions were added to the chick cell-free protein synthesizing system: Qβ RNA, 25 μg; poly U, 250 μg; HMWt virus RNA, 25 μg. 0.22 mg of myeloblast ribosomes isolated in low magnesium ion were incubated in a cell-free system containing 1.0 mg of liver pH 5 enzyme or 0.34 mg of liver pH 5 supernatant enzyme. 0.25 μC of [^{14}C] -leucine was used as the radioactive amino acid except for the tubes containing poly U in which [^{14}C] - phenylalanine incorporation was measured. Incubation was for 30 minutes at 35° C.

ribosomes results in the formation of polysomes containing virus RNA. Further experimentation is needed to verify the presence of [^{14}C]-labeled peptides in the polysome region of the sucrose density gradient, showing that it is used as a template molecule.

SUMMARY

It is of interest that an RNA fraction from a tumor virus can strongly interfere with protein synthesis. Infection of myeloblasts with the myeloblastosis virus BAI strain A causes the cells to retain their primitive nature, while at the same time the cells are able to multiply. The inhibition of protein synthesis seen by using virus RNA may be relevant to viral oncology. Protein synthesis stimulated by various mRNA's was inhibited by low concentrations of low molecular weight virus RNA. The inhibition is effected by supernatant factors from the enzyme system and by the mRNA concentration in the *in vitro* reaction. The concentration of ribosomes does not greatly effect the inhibition. Inhibition appears to involve events prior to mRNA attachment to ribosomes. Peptide synthesis in the chick cell-free polysome system is also inhibited by virus RNA. Because myeloblast tRNA does not affect the formation of myeloblast peptides in the polysome system, the inhibitor RNA in the LMWt virus RNA fraction appears to be other than myeloblast tRNA and may be related to the infectious process.

Cell-free systems from chick tissue were developed to examine the biological activity of myeloblast virus RNA. The chick polysome cell-free system can incorporate amino acids added as a [^{14}C] -amino acid-virus RNA complex. Low molecular weight

virus RNA had transfer activity similar to that of liver RNA in the chick cell-free polysome system. The low molecular weight virus RNA fraction contains RNA with properties identical to tRNA.

The activity of high molecular weight virus RNA was determined for protein synthesis in cell-free systems. The inhibitor RNA activity present in the low molecular weight virus RNA fraction was not present in intact, heat-treated and alkaline-treated HMWt virus RNA, suggesting the low molecular weight inhibitor RNA is not fragments of the viral RNA genome.

High molecular weight virus RNA also served to stimulate incorporation of amino acids in cell-free systems from _E. coli_ and chick cells. Although the extent of stimulation was not great, certain comparisons suggest this fraction has template activity. 1) Stimulation was much better in the _E. coli_ system than that obtained using liver bulk RNA or myeloblast nuclear RNA. 2) Stimulation in the chick cell-free system was much better than with Qβ RNA, a natural mRNA. 3) Inhibition of template HMWt virus RNA activity was seen in the presence of LMWt virus RNA which appeared to inhibit by preventing the formation of an active mRNA-ribosome complex. High molecular weight virus RNA may serve as a template for viral-specific protein in the primitive myeloblast cell.

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