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Factors Affecting Translation of Messenger RNA's In Vitro: Use of a GTP Analogue to Investigate Rates of Polypeptide Chain Elongation

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FACTORS AFFECTING TRANSLATION OF MESSENGER RNA'S IN VITRO: USE OF A GTP ANALOGUE TO INVESTIGATE RATES OF POLYPEPTIDE CHAIN ELONGATION

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ABSTRACT

The order of addition of amino acids to a growing protein is determined by the codon sequence of a messenger RNA molecule. This translation process was studied in vitro with a cell-free protein synthesis system derived from Escherichia coli. The rate of protein synthesis was proportional to the amount of messenger RNA added to the system. However, it was observed that different messenger RNA's were not equally effective in promoting protein synthesis. Experiments were conducted to determine why the rate of protein synthesis depends on the type of messenger RNA. These experiments employed a nucleotide analogue, 5'-guanylyl-β,γ-methylenediphosphonate, which was shown to be a non-competitive inhibitor of protein synthesis. The presence of this inhibitor increases the time required to hydrolyze a guanosine-5'-triphosphate molecule. Since this hydrolysis is one of the sequential steps which occur each time a codon is translated, the effect of the inhibitor is to increase the time it takes to translate each codon. It was shown that the effectiveness of the inhibitor in reducing the rate of protein synthesis varied with the type of messenger RNA added to the system. Through the use of messenger RNA's whose coding properties were well characterized, it was possible to demonstrate that the inhibitor also has a differential effect on different cistrons within the same messenger RNA, and on different codons within the same cistron. Since the inhibitor probably extends the time it takes to translate each codon by a uniform amount of time, it was concluded that the differential effectiveness of the inhibitor was caused by intrinsic differences in the time it takes to translate each type of codon. Thus, different types of codons are translated at different rates. Each messenger RNA can be characterized as a collection of codons, with some types of codons occurring often and others rarely. Thus, the overall translation rate depends on the "codon composition" of the messenger RNA; and this is why the rate of protein synthesis was observed to depend on the type of messenger RNA added to the system. The significance of this conclusion is that the substitution of one codon for another in a messenger RNA can affect the rate

at which that messenger RNA is translated. In particular, most amino acids can be specified by a number of different "synonym" codons. Though synonyms specify the same amino acid, the rate of translation probably varies from one synonym to another. Through the mechanism of natural selection, each amino acid in a protein could come to be specified by either a "fast" or a "slow" synonym codon. Thus, a gene might influence the amount of a protein synthesized, in addition to specifying the protein's amino acid sequence.

TABLE OF CONTENTS

RNA AND PROTEIN SYNTHESIS 1
Synopsis of Early Work Relating RNA and Protein Synthesis 2
Cell Fractionation and the Use of *in vitro* Systems 2
The Identification of Messenger RNA 4
An Apparent Exception to the mRNA Hypothesis 7
The Possibility of Stored mRNA 8
RNA Synthesis in the Amphibian Oocyte 9
The Difficulty of Detecting mRNA in Oocytes 11

THE *IN VITRO* DETECTION OF mRNA 14
Methods 15
Isolation of f2 RNA 15
Isolation of *Xenopus laevis* Oocyte RNA 15
Preparation of S-30 Extracts 20
The S-30 Cell-Free System 21
Response of the S-30 System to Exogenous RNA 22
Characteristics of the System 23
The Response of the System to *Xenopus* Oocyte RNA 26
Measuring Template Activity 30
Discussion 35

RELATIVE SPECIFIC TEMPLATE ACTIVITY 37
Differential Response to Different mRNA's Under Various Reaction Conditions 37
Effect of Altering Mg^{++} and NH_4^+ Concentration 37
Effect of Added Transfer RNA's 39
Effect of Preincubation 39
Codon Composition and the Rate of Translation 44
Codon Composition 44
Finding and Binding an Appropriate tRNA 44
Discussion 45

USE OF A GTP ANALOGUE TO STUDY THE RATE OF POLYPEPTIDE CHAIN ELONGATION .. 47

The Polypeptide Chain Elongation Cycle 47

Enzymes that Catalyze the Chain Elongation Cycle 49

The Sequential Nature of the Steps 49

The Role of GTP in Chain Elongation 51

GDPCP Inhibition of Protein Synthesis 53

The Competitive Nature of the Inhibition of GTP Hydrolysis 53

The Effect of GDPCP on Protein Synthesis in the Cell-Free System 55

Differential Effect of GDPCP on the Translation Rate of Different mRNA's 57

A Model of GDPCP Inhibition 60

Characteristics of GDPCP Inhibition 61

A Mathematical Description of GDPCP Inhibition 67

Possibility of GTP Hydrolysis Occurring between Binding and Bonding 73

GTP Hydrolysis during Chain Initiation 74

Effect of Mg^{++} Concentration on Inhibition by GDPCP 78

Discussion 80

TRANSLATION RATE AS A FUNCTION OF CODON COMPOSITION 82

Differential Effect of GDPCP on the Translation of Two Cistrons in the Same mRNA 82

Differential Effect of GDPCP on the Synthesis of Different Homopolypeptides in Response to a Ribopolynucleotide Containing a Repeating Trinucleotide Sequence 86

Discussion 92

SUMMARY 96

BIBLIOGRAPHY 97

LIST OF FIGURES

Figure		Page
1.	Fractionation of Xenopus Oocyte RNA on a Sephadex G-100 Column Run in 0.3 M NaCl	18
2.	Ultraviolet Absorption Spectra of Typical RNA Preparations	19
3.	Time Course of Incorporation of ^{14}C-Lysine into Proteins in Response to f2 RNA	24
4.	Diminishing Response of the S-30 System to Additional f2 RNA	25
5.	Time Course of ^{14}C-Lysine Incorporation in Response to Increasing Concentrations of f2 RNA	27
6.	Determination of Initial Incorporation Rates in Response to Xenopus Oocyte RNA	28
7.	Determination of the Specific Template Activity of Xenopus Oocyte RNA and f2 RNA	29
8.	Effect of Increasing RNA Concentration on the in vitro Response to Xenopus RNA Prepared by Two Different Methods	31
9.	Saturation of Endogenous Amino Acids with Added ^{14}C-Amino Acids	33
10.	Amino Acid Composition of Proteins Synthesized in vitro	34
11.	Dependence of Mg^{++} Concentration Optimum on the Type of mRNA	38
12.	Independence of Mg^{++} Concentration Optimum from f2 RNA Concentration	40
13.	Dependence of NH_4^+ Concentration Optimum on the Type of mRNA	41
14.	Differential Response to Xenopus RNA and f2 RNA upon Addition of Exogenous Transfer RNA's	42
15.	Differential Effect of Preincubation of the Reaction Mixture on the Subsequent Response to Xenopus RNA and f2 RNA	43
16.	The Polypeptide Chain Elongation Cycle	48
17.	5'-Guanylyl-β,γ-Methylenediphosphonate (GDPCP)	52
18.	Pestka's (1968b) Demonstration that GDPCP is a Competitive Inhibitor with Respect to GTP	54
19.	Effect of Increasing Concentrations of GDPCP on the Time Course of ^{14}C-Proline Incorporation	56
20.	Effect of Added GTP on ^{14}C-Proline Incorporation	58
21.	Independence of the Extent of Inhibition of Protein Synthesis by GDPCP from the Concentration of "GTP-like" Nucleotides at High Nucleotide Concentrations	59
22.	A Model of GDPCP Inhibition	62

Figure		Page
23.	Effect of GDPCP on the Template Activity of Xenopus RNA and f2 RNA	63
24.	Demonstration that GDPCP is a Non-competitive Inhibitor of Protein Synthesis	65
25.	Determination of the K_i of GDPCP Inhibition of Protein Synthesis	68
26.	Derivation of an Equation which Describes the Effect of GDPCP on the Translation of a Single Codon	70
27.	Derivation of an Equation which Describes the Effect of GDPCP on the Translation of Many Codons in an Entire mRNA	71
28.	Derivation of an Equation which Describes the Effect of GDPCP on the Translation of a Single Codon, in which Two Independent Periods of GTP Hydrolysis Occur	75
29.	Effect of Mg^{++} Concentration on Inhibition by GDPCP	79
30.	Acrylamide Gel Electrophoresis of f2 Proteins Synthesized in vitro	84
31.	Differential Effect of GDPCP on the Synthesis of f2 RNA Polymerase and f2 Coat Protein	85
32.	Two-stage Synthesis of Homopolypeptides	89
33.	Synthesis of Poly r-UUG by RNA Polymerase in Response to Poly d-TTG:CAA	90
34.	Chromatographic Analysis of the Reaction Products of the Reduction of ^{35}S-Cystine by Glutathione	91
35.	Differential Effect of GDPCP on the Synthesis of Homopolypeptides in Response to Poly r-UUG	93

Table		
	Nucleotide Composition of Xenopus laevis Nucleic Acids	12

CHAPTER ONE: RNA AND PROTEIN SYNTHESIS

Within the past two decades it has been shown that the primary control of growth, metabolism, differentiation, and genetic continuity is exercised by the chromosomes, utilizing information stored in the chemical structure of deoxyribonucleic acid (DNA). Reacting to stimuli from the cellular environment, the chromosomes are able to select appropriate portions of their coded information and translate them chemically into the physical intermediates necessary to continue the process of life.

Specifically, selected segments of DNA serve as templates for the synthesis of ribonucleic acid (RNA) molecules, whose chemical constituents, the nucleotides, are arranged in a linear order, homologous with the sequence of nucleotides in the DNA. These RNA molecules are transported from the chromosomes, out of the nucleus, to the cytoplasm. The cytoplasm contains the chemical aggregates responsible for the synthesis of proteins. These aggregates, called ribosomes, assemble amino acids into linear polypeptide chains, whose order is determined by the sequence of nucleotides comprising the RNA. This RNA is therefore a "messenger" molecule, serving as an intermediate between the chromosomes, where genetic information is stored, and the ribosomes, where genetic information is expressed, through the synthesis of specific proteins, whose form and function define the pageant of cellular processes.

The experiments to be described in this dissertation are concerned with the relationship between messenger RNA and protein synthesis. They focus on the ability of messenger RNA to direct the synthesis of proteins in a cell-free system. They suggest that the rate of protein growth on the ribosome depends on the frequency with which different code words are represented in the messenger RNA being "translated," and they explore the use of a protein synthesis inhibitor whose effectiveness is a function of the rate of messenger RNA translation.

Synopsis of Early Work Relating RNA and Protein Synthesis

Caspersson (1939), taking into account the characteristic spectral absorption of nucleic acids, was able to locate high concentrations of RNA in specific cell types, through the use of a microscope designed to detect ultraviolet light. He found RNA localized in cells actively synthesizing proteins. For instance, the gastric mucosa is composed of cells which synthesize hydrochloric acid and cells which secrete digestive enzymes. Only the protein-producing cells absorbed strongly in the ultraviolet. Similarly, pancreatic exocrine cells and liver cells show the same strong correlation between the presence of RNA and the synthesis of protein. However, cells which are not actively synthesizing proteins, but which are physiologically active, such as heart, kidney and muscle cells, do not absorb strongly at ultraviolet wavelengths (Caspersson, 1950). Similar results were obtained by Brachet (1941) with the aid of a histochemical technique specific for RNA. Both workers concluded that RNA was required for the synthesis of proteins (see Brachet, 1957, for review). This hypothesis received strong support from the work of Allfrey and his associates (1953), who demonstrated that ribonuclease inhibits the incorporation of labelled amino acids into proteins in a cell-free extract of hepatic tissue. This was confirmed *in vivo* by Brachet (1954), who used autoradiography to show that protein synthesis in root-tip cells was inhibited by ribonuclease.

Cell Fractionation and the Use of in vitro Systems

Further progress was facilitated by the use of the ultracentrifuge to prepare fractions of cell homogenates. As early as 1940, Claude was able to demonstrate that cellular RNA is localized in a particulate fraction which he characterized as a "phospholipid-ribonucleoprotein complex." The fractionation procedure was refined by Hogeboom and his co-workers (1948), who prepared four fractions which were identified by their most abundant component. The fractions were termed: nuclear, mitochondrial, microsomal, and supernatant. They found the majority of the cellular RNA of rat liver localized in the microsomal fraction. Borsook (1950) demonstrated that labelled amino acids are rapidly incorporated into proteins of this fraction and Hultin (1950) was able to show that this incorporation occurs in the microsomal fraction at earlier times and at a more rapid rate than in other

cellular fractions. Moreover, there is a correlation between the quantity of RNA and the rate of protein synthesis in the microsomal fractions of several tissues (Allfrey et al., 1953). Hence, the microsomal fraction appeared to be the site of RNA-dependent protein synthesis.

However, when cell-free systems, reconstituted from cellular fractions, were used, three fractions, the mitochondrial, microsomal, and supernatant, were necessary for protein synthesis to occur (Siekevitz, 1952). The mitochondrial fraction could be replaced with an adenosine triphosphate (ATP)-generating system (Zamecnik and Keller, 1954). The ATP was required for the enzymatic synthesis of carboxyl-activated amino acids (Hoagland et al., 1955), and this reaction is localized in the acid-precipitable components of the supernatant fraction (Hoagland et al., 1956). This finding fulfilled Lipmann's (1941) classic prediction that the potential of high-energy phosphate bonds would be utilized in the synthesis of proteins. The active components of the microsomal fraction were shown to be small, RNA-rich granules, called ribosomes (Littlefield et al., 1955; Palade, 1955). Thus, it seemed that ribosomal RNA (rRNA) was the only type of RNA participating in protein synthesis.

However, the supernatant fraction was shown to contain low molecular weight RNA's which rapidly bind labelled amino acids (Hoagland et al., 1957). Pretreatment of the supernatant fraction with ribonuclease results in the inability of cell-free systems to incorporate amino acids into proteins (Ogata and Nohara, 1957), even though ribonuclease has no effect on amino acid activation (Holley, 1956). Subsequently, Hoagland and his associates (1958) demonstrated that supernatant RNA's react with activated amino acids to form amino-acyl RNA's. Many supernatant RNA's exist, each specifically binding a particular type of amino acid (Schweet et al., 1958). Moreover, a corresponding set of amino-acyl synthetases exists (Berg and Ofengand, 1958), each of which catalyzes the activation of an amino acid and the subsequent formation of an acyl bond between that amino acid and a specific RNA (Berg et al., 1961). In short, supernatant enzymes use ATP to synthesize active amino-acyl-RNA intermediates. Amino acids are thus transferred to the ribosomal site of protein synthesis by these "transfer RNA's" (tRNA's).

Guanosine triphosphate (GTP), which is also required for protein synthesis (Keller and Zamecnik, 1956), but is not required for amino acid activation (Hoagland et al., 1958), participates in this transfer. The GTP is used by "transfer enzymes," which catalyze reactions central to the assembly of proteins from amino-acyl tRNA's. These important GTP-requiring reactions will be discussed later, when they are relevant to the experimental results.

Spiegelman (1956) proposed that amino acids were assembled into specific polypeptide sequences on an RNA "template" located on the ribosomes. The dissimilarity between RNA and amino acid structure led Crick (1958) to suggest the "adaptor hypothesis," in which specific amino-acyl tRNA's "recognize" complementary regions of this template RNA, thus providing a plausible physical mechanism by which RNA could specify the sequence of amino acids in a protein. This role for tRNA was elegantly confirmed by Chapeville and his associates, who converted the cysteinyl moiety of a cysteinyl-tRNA to an alanyl moiety, and then demonstrated that this altered intermediate could be used for the synthesis of proteins in which alanine was incorporated into positions normally containing cysteine (Chapeville et al., 1962; von Ehrenstein et al., 1963). It was thought that the RNA of the ribosomes could serve as the template molecules which tRNA's recognize (Hoagland, 1959).

The Identification of Messenger RNA

Genes control the synthesis of enzymes (Beadle and Tatum, 1941) and other proteins (Beadle, 1945) by specifying the identity and order of the amino acids (Ingram, 1958). This control is indirect, since hemoglobin synthesis can occur in non-nucleated cells (London et al., 1950), even though the structure of hemoglobin is genetically determined (Pauling et al., 1949). In light of the demonstration that DNA is the transmitter of genetic specifications (Avery et al., 1944; Hershey and Chase, 1952), and the discovery that DNA is a double-helical molecule (Watson and Crick, 1953), which replicates semi-conservatively (Taylor et al., 1957; Meselson and Stahl, 1958), it was reasonable to assume that DNA exerts indirect control over the structure of proteins through the production of nucleic acid segments, which are synthesized in a manner related to DNA replication, and which then serve as templates for the synthesis of proteins (Spiegelman, 1957). Moreover,

since DNA replication is not required for protein synthesis (Cohen and Barner, 1954), and since DNA-dependent RNA polymerase had been discovered (Weiss, 1960; Hurwitz et al., 1960), it was likely that the template molecules would be RNA. It was generally assumed that this was the role of the ubiquitous RNA found in the ribosomes.

However, isolated ribosomal RNA consists of two species, each of which is of uniform size (Hall and Doty, 1959) and possesses a characteristic nucleotide composition (Spahr and Tissières, 1959), which is the same in different tissues (Hoagland, 1960). It was difficult to understand how these uniform molecules could serve as templates, possessing a diversity of coded information, and reflecting the heterogeneous size of proteins. Jacob and Monod (1961), working on the mechanism of enzyme induction in bacteria, convincingly demonstrated that template molecules would be short-lived intermediates. They predicted that this "messenger RNA" (mRNA) would become temporarily associated with pre-existing ribosomes, where they would serve as templates for protein synthesis. As evidence in support of their model, they cited earlier work on the appearance of phage-specific RNA during the infection of bacteria by DNA-containing bacteriophages. Thus, despite there being no net synthesis of RNA after viral infection (Cohen, 1948), a small amount of short-lived RNA is synthesized (Hershey, 1953), whose nucleotide composition resembles that of viral DNA, rather than the bacterial DNA (Volkin and Astrachan, 1956). Moreover, this RNA was of heterogeneous size and could be found associated with bacterial ribosomes (Nomura et al., 1960). Jacob and Monod's hypothesis was reinforced by the finding that this short-lived RNA will form specific hybrid molecules with heat-denatured viral DNA (Hall and Spiegelman, 1961) and by the demonstration that this RNA becomes associated with ribosomes synthesized before infection (Brenner et al., 1961). Also, bacterial mRNA was identified in uninfected cells by its rapid rate of turnover, DNA-like nucleotide composition, and its ability to hybridize extensively with bacterial DNA (Hayashi and Spiegelman, 1961). Bacterial mRNA, like viral RNA, associates with pre-existing ribosomes (Gros et al., 1961). Moreover, higher organisms synthesize mRNA in the cell nucleus (Sibatini et al., 1962), and this RNA is rapidly synthesized and possesses a DNA-like nucleotide composition.

The presumed role of mRNA in protein synthesis was confirmed by experiments employing bacterial extracts able to synthesize proteins in vitro. Using a crude extract, Tissières and Hopkins (1961) demonstrated that the addition of DNA and ribonucleotide triphosphates resulted in the synthesis of RNA which had the characteristics of mRNA and promoted in vitro protein synthesis. Furthermore, the addition of DNA-dependent RNA polymerase, in conjunction with DNA, markedly stimulated the synthesis of proteins (Wood and Berg, 1962). Using a cell-free system, pre-treated with deoxyribonuclease (Matthaei and Nirenberg, 1961b) and preincubated to drastically lower the level of endogenous amino acid incorporation due to bacterial mRNA, Nirenberg and Matthaei (1961) were able to demonstrate the synthesis of polyphenylalanine in response to added polyuridylic acid, a synthetic mRNA. Also, a natural mRNA extracted from the RNA-containing bacteriophage, f2 (Loeb and Zinder, 1961), stimulated the synthesis of proteins in vitro, whose tryptic peptides were identical with those of f2 coat protein (Nathans et al., 1962). Moreover, the RNA from tobacco mosaic virus, which normally infects plants, directs the in vitro synthesis of polypeptides which may be precipitated by anti-tobacco mosaic virus serum (Tsugita et al., 1962); and lastly, RNA from rat liver nuclei strongly stimulates protein synthesis in the bacterial system, while RNA from rat liver microsomes is only slightly stimulatory (Barondes et al., 1962). Thus, the role of mRNA was demonstrated directly through its ability to serve as a template for the synthesis of proteins in vitro.

Messenger RNA can be seen in electron micrographs of "polyribosomes," which consist of a number of ribosomes linked together by a thin, ribonuclease-sensitive thread (Warner et al., 1963a). Each ribosome moves along this mRNA thread, translating the coded information into a growing polypeptide chain (Goodman and Rich, 1963).

The genetic code was deciphered by characterizing the polypeptide products resulting from the addition of defined synthetic mRNA's to cell-free systems. The code words consist of non-overlapping nucleotide triplets (Nishimura et al., 1965a,b). A "dictionary," relating the 64 possible triplets (codons) to the amino acid each specifies, was developed through the use of random polyribonucleotides of known nucleotide composition (Speyer et al., 1963; Nirenberg et al., 1963), specific ribotrinucleo-

tides (Nirenberg et al., 1965), and repeating polyribonucleotides (Khorana et al., 1966). A few codons serve as "commas," specifying the initiation (Clark and Marcker, 1966a) and termination (Kössel et al., 1967) of protein synthesis. Using block polynucleotides with specific codons at the 5'- or 3'-hydroxyl end, and taking into account the finding that proteins are synthesized linearly from the amino to the carboxyl end (Naughton and Dintzis, 1962), it was demonstrated that the coded message is translated linearly from the 5'-hydroxyl end of the mRNA (Salas et al., 1965). Also, the code is "degenerate," in that different codons may specify the same amino acid (Nirenberg et al., 1966). And most importantly, the code is universal, in the sense that the dictionary is the same regardless of which organism is used in the preparation of the cell-free system (Marshall et al., 1967).

These coding experiments clearly established mRNA as the key intermediate in the synthesis of proteins. Its discovery stimulated new discussion on the molecular basis of gene expression, resulting in the common belief that, although each cell contains a complete complement of genomic DNA (Vendrely and Vendrely, 1948; Pollister et al., 1951), only some genes are "active" (Allfrey et al., 1963), due to a "genetic control mechanism" which selects particular segments of the DNA for use as templates in the synthesis of specific mRNA's. Because bacterial mRNA's are short-lived intermediates, bacterial protein synthesis was thought to continue only as long as mRNA is synthesized. Though it was known that this immediacy of gene control does not always apply to the higher organisms, in which cellular differentiation results in specialized cells which contain long-lived mRNA's (e.g., reticulocytes), it was still quite surprising to find that animal embryos actively synthesize a whole spectrum of proteins in the virtual absence of RNA synthesis.

An Apparent Exception to the mRNA Hypothesis

Protein synthesis begins only a few minutes after the sea urchin egg is fertilized. Unlike the quiescent egg, the cleaving embryo actively incorporates amino acids into proteins (Hultin, 1961a). This synthesis is dependent on mRNA, since puromycin, a specific inhibitor of mRNA-dependent protein synthesis (Yarmolinsky and de la Haba, 1959; Traut and Monro, 1964), completely inhibits amino acid incorporation, resulting in the immediate arrest of cleavage (Hultin, 1961b). However, dactinomycin, a specific

inhibitor of DNA-dependent RNA synthesis (Reich et al., 1962), does not inhibit protein synthesis and cleavage is unaffected (Gross and Cousineau, 1963). Both inhibitors produce the same effects with amphibian embryos (Brachet and Denis, 1963; Brachet et al., 1964). Furthermore, the same type of proteins are synthesized during cleavage in the presence or absence of dactinomycin (Terman and Gross, 1965). Thus, RNA-dependent protein synthesis continues despite the presence of an inhibitor which blocks the synthesis of RNA. These observations were strengthened by experiments with amphibians and sea urchins showing that parthenogenetically-activated, anucleate egg fragments carry out cleavage (Harvey, 1936) and synthesize proteins as actively as fertilized, whole eggs (Brachet et al., 1963; Denny and Tyler, 1963; Smith and Ecker, 1965). These experiments suggested that early embryonic protein synthesis is directed by mRNA's synthesized before fertilization. The inhibition or absence of embryonic DNA would have no effect on protein synthesis if the required mRNA's were synthesized during oogenesis and stored for later use.

The Possibility of Stored mRNA

However, cell-free extracts of mature oocytes actively synthesize proteins in response to synthetic mRNA's (Nemer, 1962; Wilt and Hultin, 1962; Stavy and Gross, 1967, 1969). It was therefore difficult to explain the inactivity of mature oocytes, if they were to contain a large pool of "stable" mRNA's. If stored mRNA's are present in oocytes, why are they not used? And why, unlike bacterial mRNA's, are they not short-lived? An answer to both questions was suggested by the identification of ribonucleoprotein particles, which are not ribosomes, and which contain RNA that hybridizes extensively with genomic DNA (Spirin and Nemer, 1965). Perhaps mRNA, intended for use after fertilization, is stabilized and "masked" by a protein coat. This RNA would then be protected from ribonucleases and would not be able to serve as a template for protein synthesis. This hypothesis received support from the observation that trypsin, a potent protease, stimulates protein synthesis in crude extracts of unfertilized sea urchin eggs (Monroy et al., 1965). Perhaps a similar protease, introduced or activated during fertilization, "unmasks" stored mRNA's (Mano and Nagano, 1966). The prospect of exploring these hypotheses and of understanding the role RNA plays in early development led to the study of RNA

synthesis during oogenesis. The aim of these experiments was to detect the synthesis of mRNA and see if indeed it was "stored" for later use, during embryogenesis. Amphibian oocytes were studied because they contained extraordinarily large "lampbrush" chromosomes, which actively synthesize RNA.

RNA Synthesis in the Amphibian Oocyte

Amphibian oocytes grow slowly, within small sacs of the ovary. Each oocyte is surrounded by a sheath of follicle cells. Over a period of many months it grows from microscopic size to over a millimeter in diameter. Throughout this period, the chromosomes are in the diplotene stage of the first meiotic prophase. Therefore, homologous chromosomes are paired and their DNA has already replicated, resulting in a haploid number of "bivalent" chromosomes, each of which contains four times the haploid amount of DNA. During the oocyte's growth, these chromosomes also grow, until the middle of oogenesis, when they may attain the length of half a millimeter (Duryee, 1950). These giant structures were designated "lampbrush" chromosomes by their discover (Rückert, 1892), because the loops of material attached laterally to the central axis of each homologue resemble the tangled array of a brush. The growth of the chromosomes is accompanied by the appearance of hundreds of nucleoli, even though the tetraploid state of the chromosomes would ordinarily result in only four. Autoradiographs show that the loops of the lampbrush chromosomes and the nucleoli actively synthesize RNA (Gall and Callan, 1962). The asymmetric distribution of material composing the loops, combined with the correspondingly asymmetric pattern of RNA synthesis, suggests that the loops might be the site of synthesis of primary gene products, the thin end being the most recently exposed section of loop axis (Callan, 1963). The period of greatest metabolic activity occurs at the beginning of the second half of oogenesis, which is characterized by the accumulation of yolk and pigment. Towards the end of this period, the chromosomes shrink back to microscopic size and the loops disappear. The resulting mature oocyte is metabolically inactive, synthesizing little, if any, RNA (Brown and Littna, 1964b). The egg remains quiescent until ovulation, when the meiotic divisions are completed, resulting in the disappearance of the nuclear membrane and the nucleoli. The nucleoli do not appear again until

embryonic gastrulation (Brown, 1964). During cleavage, the fertilized egg is a closed system, encased in an impermeable membrane and jelly coat, and is therefore entirely dependent on substances accumulated during oogenesis. Very little, if any, RNA is synthesized during cleavage (Bieliavsky and Tencer, 1960). Early embryogenesis is characterized by rapid cell division, without growth, exactly the opposite of oogenesis, which is characterized by growth, without cell division (Brown and Littna, 1964a).

The vast majority of RNA found in the oocyte is ribosomal RNA (Brown and Littna, 1964b). This RNA is stable and is preserved, at least, until ovulation (Davidson et al., 1964). Since labelled RNA precursors are rapidly incorporated into nucleolar RNA (Gall and Callan, 1962), and since the nucleotide composition of RNA found in the nucleoli resembles the nucleotide composition of rRNA (Edström and Gall, 1963), it seemed reasonable to assume that nucleoli synthesize rRNA. This assumption was validated in an elegant series of experiments conducted by Brown and Gurdon (1964) with a strain of Xenopus laevis carrying a recessive mutation in which the homozygotes are unable to form nucleoli (Elsdale et al., 1958). They demonstrated that the homozygous embryos were unable to synthesize new rRNA at gastrulation, the time when nucleoli normally appear. This lack of rRNA resulted in the death of the embryo just after hatching, a stage which cannot be passed without the synthesis of additional ribosomes (Brown and Caston, 1962). Thus the nucleoli synthesize the RNA of the ribosomes.

Since rRNA hybridizes with normal genomic DNA (Yankofsky and Spiegelman, 1963), but fails to hybridize with mutant DNA (Wallace and Birnstiel, 1966), and since the "0-nucleolar" mutation also results in the loss of the chromosomal "nucleolar-organizer" constriction (Kahn, 1962), it was concluded that the mutation is a deletion of the genes coding for rRNA. Furthermore, the observation that dactinomycin inhibits nucleolar RNA synthesis (Izawa et al., 1963) and the discovery of a Feulgen-positive (Painter and Taylor, 1942), deoxyribonuclease-sensitive, circular structure in each nucleolus (Miller, 1966), led to the suggestion that the nucleoli each contain a small segment of DNA, complementary to the genes for rRNA. It was concluded that DNA-containing nucleoli are assembled on the lampbrush chromosome at the rRNA locus, by a process of selective "gene amplification"

(Brown and Dawid, 1968). Thus, the oocyte elaborates special structures, which are probably free of the constraints normally imposed on chromosomal genes, and which are designed to promote the efficient synthesis of a storehouse of ribosomes, which will be used in the synthesis of embryonic proteins (Brown, 1966).

The Difficulty of Detecting mRNA in Oocytes

The loops of the lampbrush chromosome also synthesize RNA (Ficq et al., 1958; Gall and Callan, 1962). Since dactinomycin inhibits this RNA synthesis and causes the loops to retract (Izawa et al., 1963), it is probable that the extended loop structure reflects a capacity for the continuing synthesis of RNA. Each loop contains DNA (Callan and MacGregor, 1958) arranged in an axial filament running throughout its length (Gall, 1963a; Miller, 1964). Moreover, the nucleotide composition of the loop RNA is similar to the nucleotide composition of somatic DNA (Edström and Gall, 1963). Taken together, these observations strongly suggest that mRNA is synthesized by the lampbrush chromosomes (Gall, 1963b).

However, in order to show that this chromosomal RNA is indeed mRNA intended for storage, its fate in the oocyte would have to be determined. This was a very difficult task, since almost all the RNA of the oocyte is rRNA. Thus, RNA extracted from Xenopus oocytes sediments in three major size-classes, identified as 28s and 18s rRNA's and 4s tRNA's (Brown and Littna, 1964a). Moreover, RNA, pulse-labelled during the period of maximal lampbrush chromosome activity, also sediments in these characteristic size-classes (Davidson et al., 1964), and, as illustrated in the table on page 12, I determined its nucleotide composition and found that it closely resembles the nucleotide composition of rRNA (cited in Davidson and Mirsky, 1965). Thus, if mRNA is present in the oocyte, and if it possesses a DNA-like nucleotide composition, it could at most account for only a few percent of the total RNA. There are so many active nucleoli in oocytes, that even if mRNA is synthesized, and regardless of whether it meets the criteria of heterogeneous size, rapid synthesis, and DNA-like nucleotide composition, its presence would be obliterated by the avalanche of ribosomal RNA synthesis.

In summary, messenger RNA is the key intermediate in the genetic control of protein synthesis. Surprisingly, protein synthesis in young embryos is not under immediate gene control, since it occurs in the absence of mRNA

RNA fraction	Nucleotide composition (mole per cent) CMP	AMP	GMP	UMP
pH 7.6 extract	27.2	18.2	37.7	16.9
pH 9.2 extract	27.0	20.2	34.9	18.0
28s RNA*	30	19	35	16
18s RNA*	28	22	32	18

*Brown and Littna (1964a)

	CMP	AMP	GMP	TMP
Somatic DNA‡	20.4	28.6	21.8	29.2

‡Dawid (1965)

Nucleotide composition of Xenopus laevis nucleic acids. RNA was prepared from lampbrush-stage oocytes which were labelled in vivo for 3 days with $Na_2H^{32}PO_4$. Details of the RNA extraction procedure are presented in Chapter Two. RNA was hydrolyzed in 0.3 M KOH at 37°C for 18 hours (Osawa et al., 1958). Since $^{32}PO_4$ is incorporated into RNA in the 5' position of nucleotides, and since basic hydrolysis results in a transfer of the 5' phosphate to the 2' or 3' position of adjacent nucleotide residues, the specific activity of each residue is approximately the same. Thus, the amount of radioactivity in each residue reflects the actual nucleotide composition of the RNA. The hydrolysate was neutralized with perchloric acid and the resulting precipitate was removed by centrifugation in the cold. Carrier 2', 3' nucleotides were added so that the final concentration of each nucleotide was approximately 1 μg/μliter. The nucleotides were separated by the electrophoresis of 10-μliter samples on 1" x 8-1/2" cellulose acetate strips (Sepraphore III, Gelman Instrument Co., Ann Arbor, Mich.), run at pH 3.42 in a 0.04 M sodium citrate buffer for 33 minutes at 420 volts and then for 13 minutes at 700 volts. The separated bands were located and cut out under ultraviolet light, dissolved in Bray's (1960) solution, and then counted in a Nuclear Chicago scintillation counter. The nucleotide compositions reported in this table are the average of measurements obtained from 8 parallel determinations.

synthesis. Significantly, intense RNA synthesis occurs months earlier on the loops of the lampbrush chromosomes. Most likely, this RNA is mRNA, and it is stored for use during embryogenesis. However, attempts at detecting this stored mRNA had failed. The experiments described in the next chapter were designed to detect this stored mRNA by its ability to direct the synthesis of proteins in a cell-free system derived from bacteria.

CHAPTER TWO: THE *IN VITRO* DETECTION OF mRNA

The cell-free protein synthesis system derived from *Escherichia coli* by Matthaei and Nirenberg (1961a) responds stoichiometrically to added messenger RNA (Nirenberg and Matthaei, 1961). This bacterial system has often been used to detect messenger RNA in exogeneous RNA preparations, since the amount of endogenous protein synthesis can be drastically lowered by preincubation. The "Nirenberg system" has been used to demonstrate the messenger role of viral RNA from tobacco mosaic virus (Tsugita *et al.*, 1962), f2 coliphage (Nathans *et al.*, 1962), turnip yellow mosaic virus (Ofengand and Haselkorn, 1962), polio virus (Warner *et al.*, 1963b), and satellite tobacco necrosis virus (Clark *et al.*, 1965). In higher organisms, the *in vitro* system has been used to detect mRNA in RNA preparations from various tissues. For example, "template-active RNA" has been detected in liver (Barondes *et al.*, 1962), reticulocytes (Shaeffer *et al.*, 1964), lymph nodes and spleen (Mach and Vassalli, 1965), secretory gland (Tanaka and Shimura, 1965), placenta (Silverstein and Bondy, 1966), brain (Bondy and Roberts, 1967), and tumor tissue (Jacob and Busch, 1967).

This chapter describes the detection of mRNA in RNA preparations from frog oocytes by its ability to direct the synthesis of proteins in the *E. coli* cell-free system. The bacterial system was chosen, rather than a mammalian reticulocyte preparation, because it has a lower endogenous background, and because it had been shown to respond stoichiometrically to exogenous mRNA's obtained from a wide variety of living tissue.

The response of the bacterial system to f2 coliphage RNA was used as a standard, against which the response to oocyte RNA was compared. Coliphage RNA serves as an effective template for the synthesis of phage-specific proteins (Nathans *et al.*, 1962; Ohtaka and Spiegelman, 1963; Nathans, 1965; Capecchi, 1966; Gussin *et al.*, 1966), and probably functions *in vitro* as a "natural" mRNA, in the sense that it is initiated, translated, and terminated with the same fidelity observed *in vivo* (Webster *et al.*, 1967).

Methods

Isolation of f2 RNA

f2 phage, grown and isolated as described by Webster and his co-workers (1967), was the gift of Dr. Norton Zinder. RNA was isolated from this virus by a modification of the procedure used by Gierer and Schramm (1956) to isolate RNA from tobacco mosaic virus. 5 mg of phage was suspended in 1 ml of water. 2 ml of water-saturated, pre-distilled phenol was added to the suspension and agitated at top speed on a "Vortex" mixer for three minutes. The mixture was separated into two phases by centrifugation at 33,000 g for 15 minutes at 2°C. The aqueous phase was saved and the phenol phase was re-extracted in the same manner with 1.5 ml of water. The resulting aqueous phase was combined with the first aqueous phase and further deproteinized, in the same manner, with 2.5 ml of water-saturated phenol. This final aqueous phase was made 0.1 M in potassium acetate and the RNA was precipitated by the addition of two volumes of ethanol. After standing 12 hours at -20°C, the precipitate was collected by centrifugation at 1,500 g for 10 minutes. The RNA was dissolved in 1 ml of water, lyophilized to remove traces of phenol and alcohol, redissolved in 1 ml of water, and stored at -87°C. The concentration of the RNA was determined by measuring the optical density at 260 mμ (24 OD units equals 1 mg of RNA, according to Nathans et al., 1962). A typical ultraviolet absorption spectrum is illustrated in Figure 2 on page 19.

Isolation of Xenopus laevis Oocyte RNA

Xenopus laevis, the South African clawed toad, was selected as a source of oocyte RNA, because of the ease with which it can be maintained in the laboratory, and because its oocytes do not contain the nucleic acid binding substance found in the common American frog, Rana pipiens (Brown and Caston, 1962). Medium-sized females were obtained from Jay E. Cook, Cockeysville, Md., and kept in sinks of cool, slowly moving water.

Females were anesthetized with gaseous ethyl ether and their ovaries were surgically removed and placed in embryological moist chambers at 4°C. Individual ovarian sacs were cut open and placed in small Petri dishes containing Barth and Barth's (1959) "solution X" to which 10.3 mg NaCl/ml had been added. This extra salt causes each oocyte to shrink, facilitating the isolation of the oocytes from their follicle-cell membranes. This step is

necessary since the follicle cells actively synthesize RNA (Ficq, 1961). Size and yolk content were used as indicators in the selection of oocytes in the same stage of oogenesis (Duryee, 1950). Selected oocytes were isolated by hand under a dissecting microscope and then rapidly frozen at -70°C. Approximately 1,000 oocytes were collected for each RNA preparation.

RNA was extracted from oocytes by a modification of the method of Davidson and Mirsky (1964), which is based on the technique of phenol deproteinization (Kirby, 1956). All glassware used in the preparation of RNA was pre-treated by heat at 110°C for 2 hours, in order to destroy ribonuclease. Phenol was always distilled before use.

The oocytes were thawed and homogenized at approximately 1000 rpm with seven strokes of a spinning Tenbroeck homogenizer in 5 ml of 0.1 M, pH 7.6 Tris-HCl buffer, which was 15 mM in naphthalene-1,5-disulfonate, and contained 8 μg/ml polyvinyl sulfonate, a competitive inhibitor of ribonuclease (Fellig and Wiley, 1959). 8 ml of phenol, saturated with the homogenization buffer, was added to the homogenate and stirred for 30 minutes at 20°C. The mixture was separated into two phases by centrifugation at 51,000 g for five minutes at 2°C. The aqueous phase was saved. Extraction at neutral pH may leave template-active material in the "interphase," between the buffer and the phenol (Sibatani et al., 1962). Re-extraction of the interphase at higher pH (Brawerman et al., 1963) releases this refractory RNA (Hadjivassiliou and Brawerman, 1967). Therefore, the phenol and interphase was re-extracted with 4.5 ml of homogenization buffer, adjusted to pH 9.2, using the same procedure employed at neutral pH. The two aqueous extracts were combined and further deproteinized by a water-saturated mixture of phenol, m-cresol, and 8-hydroxyquinoline (Kirby, 1965). This solution was prepared by mixing together 100 g crystalline phenol, 14 ml m-cresol, 100 mg 8-hydroxyquinoline, and 22 ml of water. The combined aqueous phases were twice deproteinized with 15 ml of this mixture in the same manner as in the initial extractions. The final aqueous phase was made 1% (w/v) in NaCl and the RNA was precipitated by the addition of two volumes of -20°C ethanol, which was then allowed to stand for 45 minutes at -20°C. The precipitate was collected by centrifugation at 63,000 g for 40 minutes at 2°C. The RNA was dissolved in 0.4 ml of a 15 mM naphthalene-1,5-disulfonate solution.

This extract was fractionated on a 0.9 x 37 cm column containing sieved (270-325 mesh) Sephadex G-100 (Pharmacia Fine Chemicals, Piscataway, N.J.), run in 0.3 M NaCl at 4°C (Galibert et al., 1965). A typical chromatogram is illustrated in Figure 1 on page 18. This procedure separated low molecular weight salts, transfer RNA's (Brown and Gurdon, 1966), and 5s ribosomal RNA (Brown and Littna, 1966) from high molecular weight RNA. The high molecular weight exclusion peak, though predominantly 18s and 28s ribosomal RNA, hopefully contained messenger RNA. Gel filtration, to remove lower molecular weight RNA's, was essential, since 5s ribosomal RNA may stimulate protein synthesis in vitro (Kirtikar and Kaji, 1968), and since the presence of Xenopus transfer RNA's would probably alter the response of the E. coli cell-free system (Marshall et al., 1967; Anderson, 1969).

The high molecular weight exclusion peak was treated with pronase (a mixture of bacterial proteases) to remove any remaining protein from the RNA (Berns and Thomas, 1965). In order to destroy any contaminating nucleases, pronase was pre-digested at 37°C for two hours in 0.3 M NaCl. Pre-digested pronase was added to the excluded RNA to a concentration of 500 μg/ml, and the resulting solution was incubated 10 minutes at 4°C, 10 minutes at 23°C, and 90 minutes at 37°C. The pronase was then removed by three deproteinizations, each with an equal volume of phenol saturated with 0.3 M NaCl. The RNA in the final aqueous phase was precipitated by the addition of two volumes of -20°C ethanol, and then redissolved and reprecipitated with 1% NaCl and -20°C ethanol. The RNA was dissolved in 1 ml of water and stored at -87°C. The concentration of the RNA was determined by the phloroglucinol method of Dische and Borenfreund (1957) and by spectrophotometry at 260 mμ. A typical ultraviolet absorption spectrum is illustrated in Figure 2 on page 19.

The nucleotide composition measurements were based on ^{32}P-orthophosphate incorporation into RNA extracted from lampbrush-stage oocytes. $Na_2H^{32}PO_4$ was injected into the dorsal lymph sac of Xenopus females (Kutsky, 1950), three days before the oocytes were collected. RNA was prepared by successive phenol extractions at pH 7.6 and pH 9.2. The pH 9.2 extract was not combined with the pH 7.6 extract, but, instead, was treated as a separate sample. After precipitation with alcohol, both extracts were desalted on

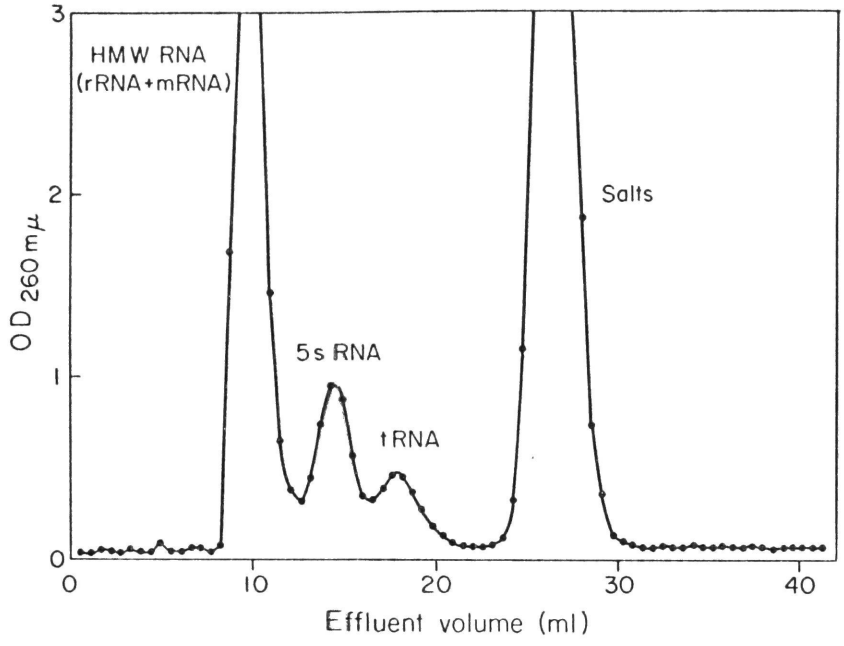

Figure 1. Fractionation of Xenopus oocyte RNA on a Sephadex G-100 column run in 0.3 M NaCl. The column was 0.9 x 37 cm, contained sieved Sephadex beads, and was run at 4°C. The RNA in the high molecular weight exclusion peak (HMW RNA) was tested in the cell-free system to see if it contained mRNA.

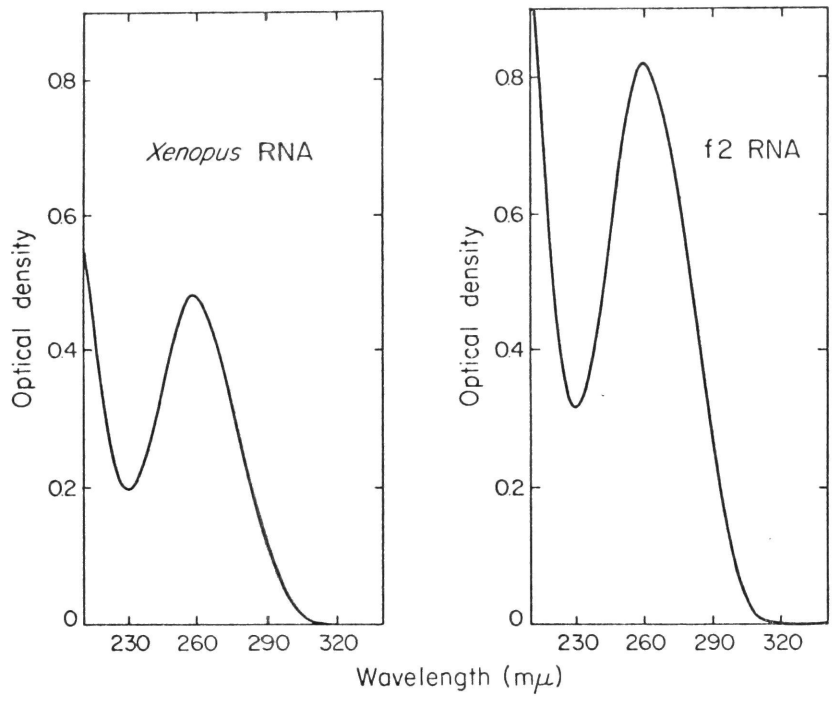

Figure 2. Ultraviolet absorption spectra of typical RNA preparations. The low 230 mμ/260 mμ ratios (0.42 for Xenopus RNA and 0.38 for f2 RNA) indicate that very little protein was present.

Sephadex G-25 columns, precipitated, and redissolved in a small volume of water. These preparations were not chromatographed on Sephadex G-100, nor were they treated with pronase.

Oocyte RNA was also prepared by a modification of the method of Brown and Littna (1964a). This procedure employs the detergent, sodium dodecyl sulphate, to extract the RNA, including the refractory "interphase" material, in one step at pH 5.1 (Scherrer and Darnell, 1962), instead of in two steps at pH 7.6 and pH 9.2. In addition, the negatively-charged hectorite, Macaloid (a sodium magnesium lithofluorosilicate obtained from the Baroid Division of the National Lead Company, Houston, Texas), was used as a ribonuclease inhibitor (Stanley and Bock, 1965), rather than polyvinyl sulfonate. Macaloid, which forms a suspension, was easily removed from solution by centrifugation, in contradistinction to the high molecular weight polyvinyl sulfonate, which remained in the RNA preparations.

In this procedure, oocytes were homogenized in a 0.1 M, pH 5.1 sodium acetate buffer containing 1/10% (w/v) Macaloid (pre-washed in buffer). Sodium dodecyl sulphate, twice recrystallized by the method of Mandel (1964), was added to the homogenate to a concentration of 1/2% (w/v). An equal volume of buffer-saturated phenol was then added to the homogenate and stirred for 30 minutes at $0^{\circ}C$. The procedure from this point on was essentially the same as in the two-pH method, except that Macaloid had to be added back to the aqueous phase after each centrifugation.

Preparation of S-30 Extracts

Bacterial extracts were prepared by a modification of the procedure of Nirenberg and Matthaei (1961), developed by Schwartz (1965), and described in detail by Webster and his co-workers (1967). An inoculum of *E. coli* S26 (K38) (Zinder and Cooper, 1964), a strain derived from *E. coli* K12 (Garen and Siddiqi, 1962), was introduced into a Biogen (American Sterilizer Co., Erie, Pa.) containing 40 liters of sterile Tryptone broth, pH 7.8 (Cooper and Zinder, 1962). The bacteria were allowed to grow at $34^{\circ}C$ until early log phase was reached (1.8×10^{8} cells/ml, as determined with a Petroff-Hauser counting chamber). Cells from early log phase have the highest ratio of ribosomes to endogenous mRNA, thus providing excellent starting material for cell-free protein synthesis systems (Li and Umbreit,

1966). The cells were harvested by passage through a cooling coil into a cold Sharples continuous-flow centrifuge. The collected cells were then washed twice by suspension and centrifugation in an isolation buffer, which was 10 mM Tris-HCl, pH 7.8, containing 11 mM magnesium acetate, 6 mM 2-mercaptoethanol, and 1 mM ethylenediaminetetraacetate.

The washed cells were rapidly frozen and then broken open under 20,000 lb/in^2 pressure at -25°C in an Edebo press (Biochemical Processes, Inc., Islip, N.Y.). This procedure is based on cell breakage due to the explosive change in the size and shape of ice crystals, which occurs when they are subjected to a rapid pressure drop, as the frozen cells pass through a small orifice in the press (Edebo, 1960, 1961). The disintegrated cellular material was thawed in 20 ml of isolation buffer, containing 20 μg/ml deoxyribonuclease (electrophoretically freed of ribonuclease by the supplier, Worthington Biochemicals, Freehold, N.J.), and allowed to stand for 90 minutes at 0°C. The resulting solution was centrifuged at 15,000 g for 10 minutes at 2°C to remove cell walls and unbroken cells. The supernatant was then centrifuged at 30,000 g for 30 minutes at 2°C. The resulting supernatant, termed the "S-30," contained ribosomes and soluble substances. This extract was then preincubated for 30 minutes at 35.5°C with 3 mM ATP, 10 mM phosphoenol pyruvate, 30 μg/ml pyruvate kinase (Boehringer Mannheim Corp., New York, N.Y.), 0.2 mM GTP, 20 mM reduced glutathione, 0.9 mM ethylenediaminetetraacetate, 63 mM ammonium chloride, 12.7 mM magnesium acetate, and 47 mM Tris-HCl, pH 7.8, in a total volume of 25 ml. The preincubated S-30 was then dialyzed for eight hours at 4°C in 1/4" Visking tubing (which had previously been boiled in glass-distilled water) against four changes of six liters of dialysis buffer. This buffer was 11 mM magnesium acetate, 30 mM ammonium chloride, 6 mM 2-mercaptoethanol, 1 mM ethylenediaminetetraacetate, and 10 mM Tris-HCl, pH 7.8. The dialyzed extract was centrifuged at 15,000 g for 10 minutes at 2°C to remove precipitated material. The extract was then rapidly frozen in 0.5 ml aliquots and stored at -87°C. At this low temperature, extracts were stable for more than a year. The protein concentration of each S-30 extract was determined by a micro biuret procedure developed by Goa (1953).

The S-30 Cell-Free System

Before each incubation, a portion of the S-30 extract was thawed and again preincubated at 35.5°C for five to 15 minutes, depending on the extract,

in the presence of all the reaction components enumerated below, except the labelled amino acid, the exogenous mRNA, and any component being tested. This mixture was chilled and then added to reaction tubes containing the excluded components. Unless otherwise specified, the final reaction mixture contained 6.0 to 7.5 mg S-30 protein/ml, 3 mM ATP, 10 mM phosphoenol pyruvate, 30 μg/ml pyruvate kinase, 0.2 mM GTP, 100 μM of each unlabelled amino acid, 20 to 100 μM of the radioactive amino acid (usually uniformly labelled with carbon-14, whose specific activity was approximately 40 mC/mA), 20 mM reduced glutathione, 12.7 mM magnesium acetate, 60 mM ammonium chloride, and 50 mM Tris-HCl, pH 7.8. In any given experiment, each reaction tube contained the same volume of reaction mixture. Ordinarily, volumes between 50 and 200 μliters were used. Incubations were carried out at 35.5°C.

The amount of labelled amino acids incorporated into protein was determined by the method of Mans and Novelli (1961). As the reaction proceeded, 20 μliter aliquots of the incubation mixture were absorbed onto numbered Whatman 3MM filter paper discs and plunged into 10% (w/v) trichloroacetic acid (TCA) at 0°C. Each reaction tube was sampled from three to eight times in the course of an incubation. When all the samples had been collected, they were placed in 5% TCA at 90°C for 15 minutes, in order to hydrolyze, and thus solubilize, the transfer RNA's carrying labelled amino acids. Unincorporated amino acids and other low molecular weight reaction components were then washed away with four rinses of 5% TCA. The samples were dehydrated in a 1:1 (v:v) mixture of ethyl ether and ethanol, immersed in ethyl ether, and dried. The radioactivity in each dried disc was measured in a Nuclear Chicago scintillation counter. Each counting vial contained 15 ml of a toluene-based scintillation fluid composed of 4 g/liter 2,5-diphenyloxazole and 50 mg/liter p-bis-[2-(5-phenyloxazolyl)]-benzene.

For every tube containing exogenous RNA, an identical tube was prepared, containing water in place of the RNA. Incorporation due to the endogenous background could then be subtracted to obtain the net incorporation in response to the exogenous RNA.

Response of the S-30 System to Exogenous RNA

The following terms will be used to interpret the results:

"Template activity" is the rate of incorporation of a labelled amino acid into TCA-insoluble proteins. It reflects the rate of protein synthesis.

"Specific template activity" is the amount of template activity measured per microgram of added RNA. It reflects the ability of an RNA preparation to promote the synthesis of proteins in vitro.

"Relative specific template activity" is a ratio, in which the specific template activity of one RNA preparation is divided by the specific template activity of another RNA preparation. It is a comparison of the abilities of two different RNA preparations to promote the synthesis of proteins in vitro.

Characteristics of the System

The time course of incorporation of labelled amino acids into proteins, in response to exogenous RNA, is illustrated in Figure 3 on page 24. The time course is composed of three characteristic periods. The first is a short lag period during which the temperature of the incubation mixture rises from $0^{o}C$ to $35.5^{o}C$. The reaction then proceeds at an essentially linear rate, throughout the second period. During the last period, the reaction tapers off, until protein synthesis ceases.

The decline of the amino acid incorporation rate is not due to a general breakdown in the system's ability to synthesize proteins. Figure 4, on page 25, illustrates an experiment in which additional f2 RNA was added to reactions in the terminal phase of incorporation. New bursts of amino acid incorporation were observed. Though the ability of the in vitro system to respond to additional RNA does diminish with time, this is not the primary cause of the termination of protein synthesis. The cause lies with the messenger RNA. At least two explanations are possible, both of which could be true. The first is that the mRNA is destroyed by ribonuclease (Tissières and Watson, 1962). The second is that the particular ribosomes translating the mRNA become unable to continue synthesis. These ribosomes might abruptly cease translation, or they might slow down gradually, until translation stops. No matter what the cause, the observed rate of protein synthesis does slow down with time of incubation. For this reason, only the initial rate of amino acid incorporation, measured during the linear period, was used to determine template activity.

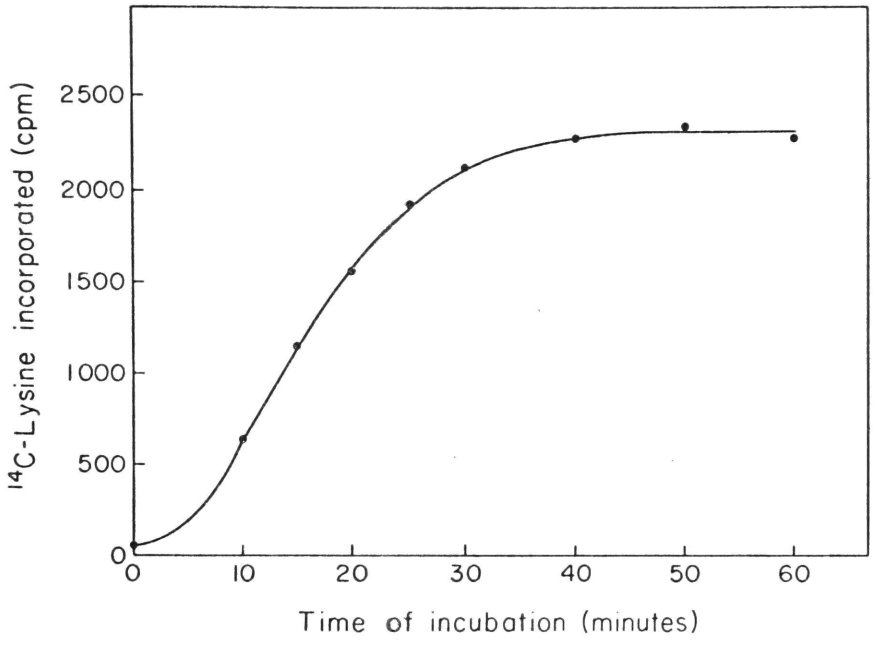

Figure 3. Time course of incorporation of ^{14}C-lysine into proteins in response to f2 RNA. 20 μliter samples were taken at intervals from a 200 μliter reaction mixture, containing 55 μg f2 RNA/ml. The reaction mixture was 20 μM in ^{14}C-lysine (247 mC/mM). The ordinate is the incorporation, measured as counts per minute, in each 20 μliter sample.

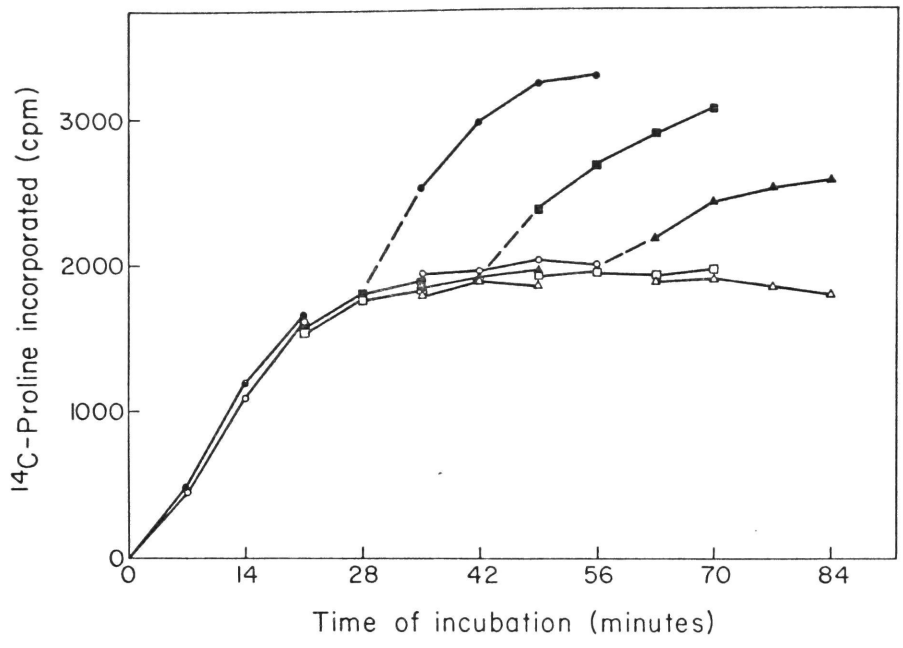

Figure 4. Diminishing response of the S-30 system to additional f2 RNA. Six identical reaction mixtures, each containing 23 μg f2 RNA/ml, were incubated at the same time. After 28 minutes, more f2 RNA was added to one of the reaction tubes, increasing the RNA concentration by another 23 μg/ml. At the same time, a control was prepared by adding an equal volume of water to a second tube. This procedure was repeated with two more reaction tubes at 42 minutes and with the last two reaction tubes at 56 minutes. The reaction mixtures were sampled before and after the additions at the times indicated in the figure. The values for samples taken after the addition of RNA or water were corrected for the dilution due to the resulting small increase in volume. Filled symbols represent the mixtures to which RNA was added and open symbols represent mixtures to which water was added. Despite a marked decline in the response to the original RNA, the system was able to respond anew to additional RNA. Though the system's ability to respond to additional RNA does decrease with time, it does not account for the pronounced cessation of synthesis in response to the original RNA.

The most important characteristic of the system is that the response is dependent on exogenous mRNA. This is illustrated, for f2 RNA, in Figure 5 on page 27. The more RNA that is added, the greater is the rate of amino acid incorporation. Thus, for this *in vitro* system, mRNA is a rate-limiting component. The initial rate of protein synthesis is linearly proportional to the concentration of mRNA.

The Response of the System to Xenopus Oocyte RNA

The same type of experiment was performed with *Xenopus* oocyte RNA preparations. *Xenopus* RNA did stimulate amino acid incorporation in the cell-free system. A typical experiment is illustrated in Figure 6 on page 28. Here again, the more RNA added to the system, the higher the rate of amino acid incorporation.

Specific template activity was determined by measuring the initial rate of incorporation at increasing oocyte RNA concentrations. (For example, the initial rates of incorporation for the experiment illustrated in Figure 6 were determined by measuring the slopes, between 4 and 12 minutes.) By plotting the initial incorporation rate against the RNA concentration, the specific template activity (incorporation rate/μg RNA) was determined. Figure 7, on page 29, illustrates an experiment with *Xenopus* RNA and f2 RNA, in which specific template activities were determined. As can be seen, the initial rate of incorporation is a linear function of the RNA concentration, for each type of RNA. Significantly, the *Xenopus* oocyte RNA had a much lower specific template activity than f2 RNA. This is due to the fact that oocyte RNA is mostly ribosomal RNA, which does not serve as a template in the *in vitro* system (Brawerman *et al.*, 1963).

Ribosomal RNA probably cannot serve as a template for protein synthesis because it is methylated (Gordon and Boman, 1964) and possessed a pronounced secondary structure. In order to serve as a template, rRNA must be heat denatured and translated in the presence of aminoglycoside antibiotics which distort the structure of the ribosomes (Holland *et al.*, 1966). Since it is not likely that high molecular weight ribosomal RNA's can serve as a template, and since the oocyte RNA preparations were freed of transfer RNA's and 5s ribosomal RNA, the template activity observed *in vitro* is most probably due to the presence of messenger RNA in the oocyte preparations.

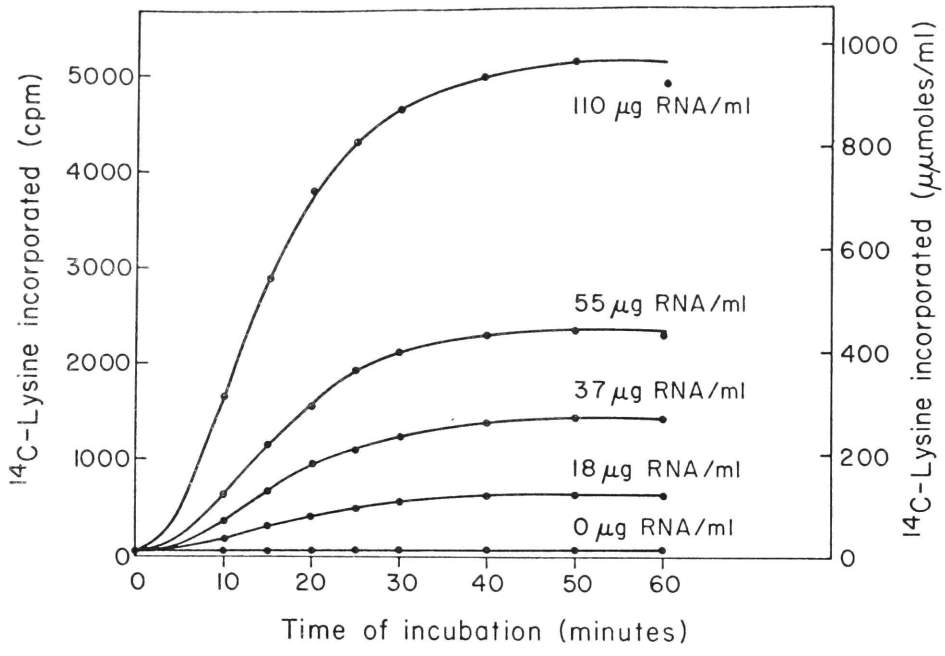

Figure 5. Time course of ^{14}C-lysine incorporation in response to increasing concentrations of f2 RNA. The response of the system is proportional to the amount of mRNA added. Thus, mRNA is a rate-limiting component of the system.

Counts were converted to μμmoles by first determining the number of counts per minute in a known amount of ^{14}C-lysine, and then using this ratio to convert the counts incorporated in each sample to μμmoles incorporated. Specifically, 5 μliter aliquots of each reaction mixture were counted on filter paper discs in the same manner as the samples, except they were not washed free of precursor amino acids. In this experiment, each 5 μliter aliquot contained 100 μμmoles lysine, and these "unwashed standards" averaged 26,500 cpm each. This ratio is equivalent to 5,300 cpm detected in each 20 μliter reaction sample for every 1,000 μμmoles lysine incorporated per ml of reaction mixture. This "internal standardization" method is independent of the counting efficiency of the scintillation counter.

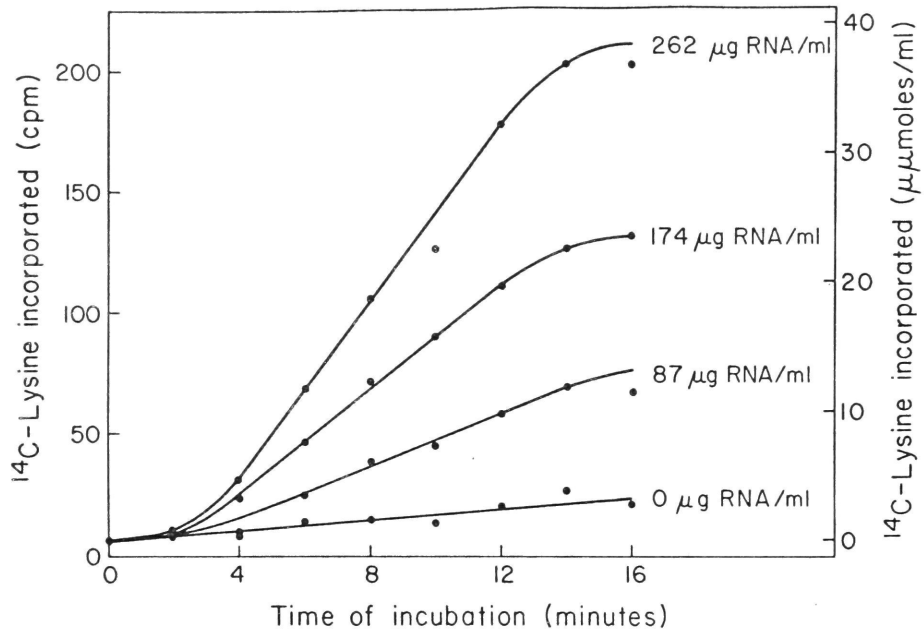

Figure 6. Determination of initial incorporation rates in response to Xenopus oocyte RNA. Note the low endogenous background. The initial incorporation rate for each concentration of RNA was determined by measuring the slope between 4 and 12 minutes (expressed as μμmoles lysine incorporated per ml per minute) and subtracting the slope due to the endogenous background.

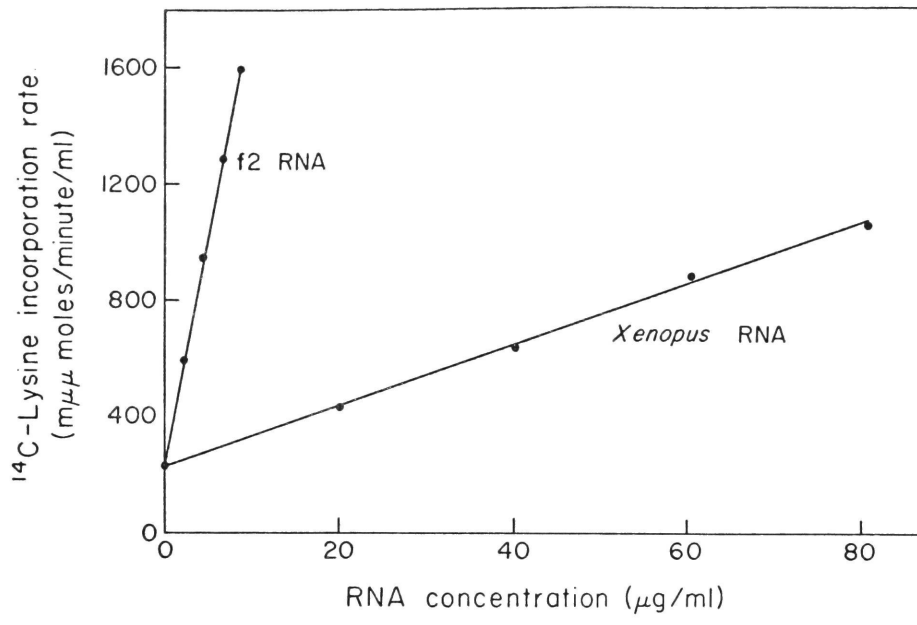

Figure 7. Determination of the specific template activity of Xenopus oocyte RNA and f2 RNA. The initial incorporation rate is linearly proportional to the RNA concentration, for both RNA's. By plotting rate against concentration and measuring the slope of the line, the specific template activity (expressed as μμmoles lysine incorporated per minute per μg RNA) can be determined. The absolute specific template activity has no meaning when taken alone, since the response varies from one S-30 extract to another. However, the relative specific template activity of the two RNA preparations is constant. Xenopus RNA has a low specific template activity relative to that of f2 RNA. This is primarily due to the presence of large quantities of inactive ribosomal RNA in the Xenopus RNA preparation. In this experiment, the relative specific template activity reflects the percentage of mRNA present in an oocyte RNA preparation. Thus, the amount of mRNA present in one Xenopus RNA preparation could be compared to another. However, the absolute percentage of mRNA present cannot be determined, since f2 RNA and Xenopus mRNA may be translated with different efficiencies.

The specific template activity of Xenopus oocyte RNA, relative to the specific template activity of f2 RNA, reflects the amount of mRNA present in the oocyte. RNA preparations from mature eggs had only a slightly higher relative specific template activity than RNA preparations from lampbrush-stage oocytes. Since RNA is not synthesized in mature eggs, these observations support the hypothesis that oocyte mRNA is stored in the mature egg. This work was reported by Davidson, Crippa, Kramer, and Mirsky (1966), and was confirmed by Cape and Decroly (1969). Furthermore, Xenopus oocyte RNA hybridizes with somatic DNA, and RNA extracted from embryos competes with oocyte RNA for binding sites on the DNA (Crippa et al., 1967; Crippa and Gross, 1969). Both the in vitro protein synthesis experiments and the hybridization experiments support the view that embryogenesis begins in oogenesis, when embryonic mRNA's are synthesized and stored for later use.

When oocyte RNA preparations were not treated with pronase, they failed to stimulate in vitro protein synthesis. This observation supports the hypothesis that oocyte mRNA is masked by a protein coat. Also, Mano (1966) demonstrated that mature eggs contain their own protease, which is activated by fertilization. This activated protease probably frees the stored mRNA from its protein coat. However, these observations offer no clue to the origin of the protein coat. One possibility is suggested by the work of Gall and Callan (1962), who used an autoradiographic technique to demonstrate that proteins are synthesized on the loops of the lampbrush chromosomes. Perhaps the lampbrush chromosomes are special structures designed to synthesize mRNA and "package" it in a protective protein coat.

Measuring Template Activity

When oocyte RNA was prepared in the presence of polyvinyl sulfonate, the assay of template activity was complicated by the inhibitory effects of this polyanion. Specific template activity could be determined only at low concentrations of oocyte RNA, because the presence of an increased amount of polyvinyl sulfonate at higher concentrations of oocyte RNA caused the specific template activity to drop. This is illustrated in Figure 8 on page 31. Polyvinyl sulfonate, which is a high molecular weight polyanion, competes with RNA for ribonuclease, and thus inhibits ribonuclease activity during the RNA extraction. However, it also competes with RNA for the ribosomes during

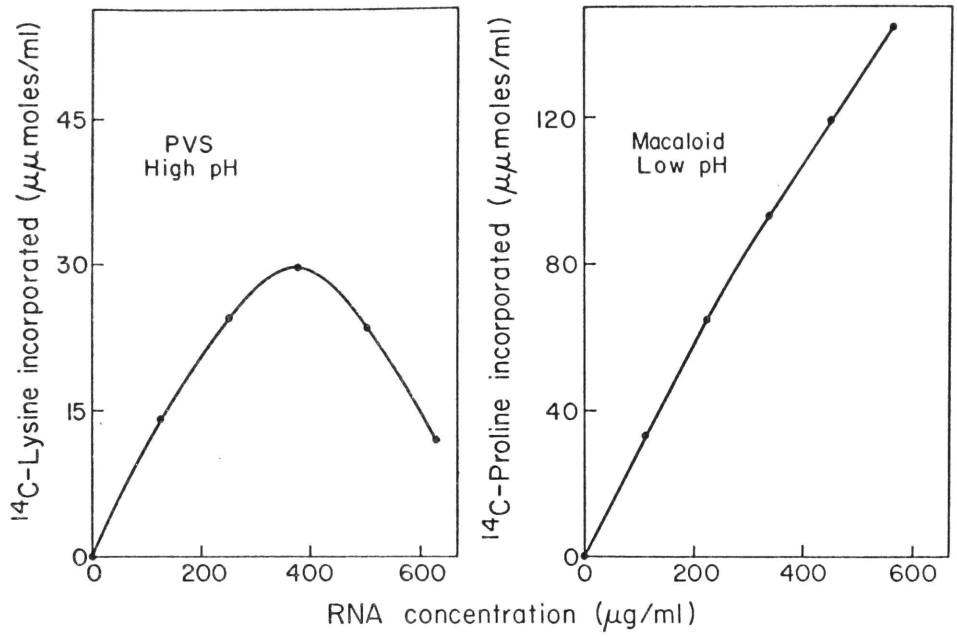

Figure 8. Effect of increasing RNA concentration on the in vitro response to Xenopus RNA prepared by two different methods. Polyvinyl sulfonate (PVS) is not removed from RNA preparations, and strongly inhibits the response of the cell-free system at high concentrations. RNA prepared with Macaloid is freed of Macaloid after each centrifugation step. Thus, there is no Macaloid in the final RNA preparation. The response to this RNA is not inhibited at high RNA concentrations. The absolute incorporation rates of these two experiments are not comparable, since each experiment was performed with a different S-30 extract.

incubation, and thus inhibits *in vitro* protein synthesis (Shinozawa *et al.*, 1968). When Macaloid was used as a ribonuclease inhibitor, specific template activity did not fall off at higher RNA concentrations, since this inhibitor is removed during preparation of the RNA.

Relative specific template activity depends on which amino acid is used as a label. For example, proteins synthesized in response to *Xenopus* RNA might contain a higher percentage of lysine than proteins synthesized in response to f2 RNA. On the other hand, there might be less arginine in *Xenopus* proteins than in f2 proteins. In short, template activities are best compared when they are based on the rate of protein synthesis, rather than on the rate of incorporation of one type of amino acid. Therefore, amino acid incorporation rates were converted to protein synthesis rates, by determining the amino acid composition of the proteins synthesized *in vitro*.

Incubations were carried out in the presence of a mixture of 16 ^{14}C-amino acids. The specific activity of each amino acid was known. These amino acids were added to the reaction mixtures at a concentration high enough to assure that unlabelled endogenous amino acids, not removed by dialysis, would not significantly alter the specific activities. A preliminary experiment, demonstrating the saturation of the endogenous amino acid pool by the labelled amino acids, is illustrated in Figure 9 on page 33.

Amino acid compositions were then determined for the proteins synthesized *in vitro*, in response to f2 RNA, *Xenopus* RNA, and water (to determine the amino acid composition of the endogenous background). After a 20-minute incubation, the proteins in each reaction mixture were washed free of precursors with 5% TCA, and hydrolyzed in 6 N HCl for two hours at 110°C. Only newly-synthesized proteins were labelled, and the unlabelled S-30 proteins served as carrier. Each amino acid mixture was separated by ion exchange chromatography (Moore *et al.*, 1958) on a Beckman 120B amino acid analyzer (Spackman *et al.*, 1958). The radioactivity of each amino acid peak was determined by passing the effluent from the analyzer columns through a solid-state flow cell in a Nuclear Chicago scintillation counter. Since the radioactivity of each amino acid was measured, and since the specific activity of each amino acid was known, the number of moles of each labelled amino acid could be determined. The number of moles of each amino acid incorporated was used to calculate the amino acid composition of the proteins synthesized *in vitro*. Figure 10, on page 34, illustrates the results of this experiment.

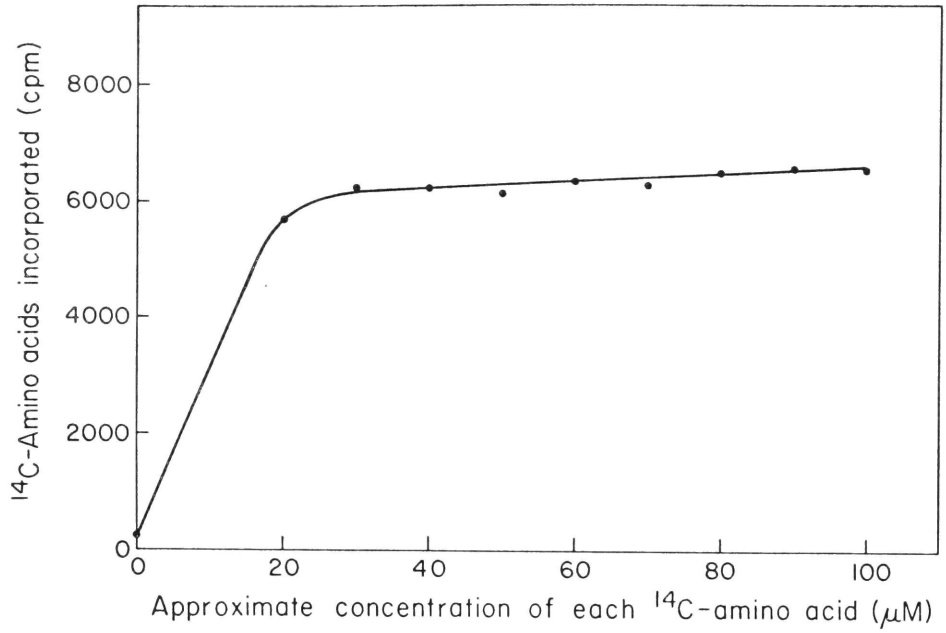

Figure 9. Saturation of endogenous amino acids with added ^{14}C-amino acids. A mixture of 16 labelled amino acids (reconstituted from algal protein hydrolysates by New England Nuclear Corp., Boston, Mass.), brought to pH 7.8 with Tris, and containing 4 unlabelled amino acids, was added in increasing amounts to reaction mixtures containing 208 μg/ml Xenopus oocyte RNA. The insensitivity of amino acid incorporation to the increasing ^{14}C-amino acid concentration, indicates that the cell-free system has been saturated and that the concentration of unlabelled, endogenous amino acids is very low. The amino acid composition experiment was carried out at the highest amino acid concentration shown in this figure, in order that the specific activity of each amino acid would remain essentially unchanged.

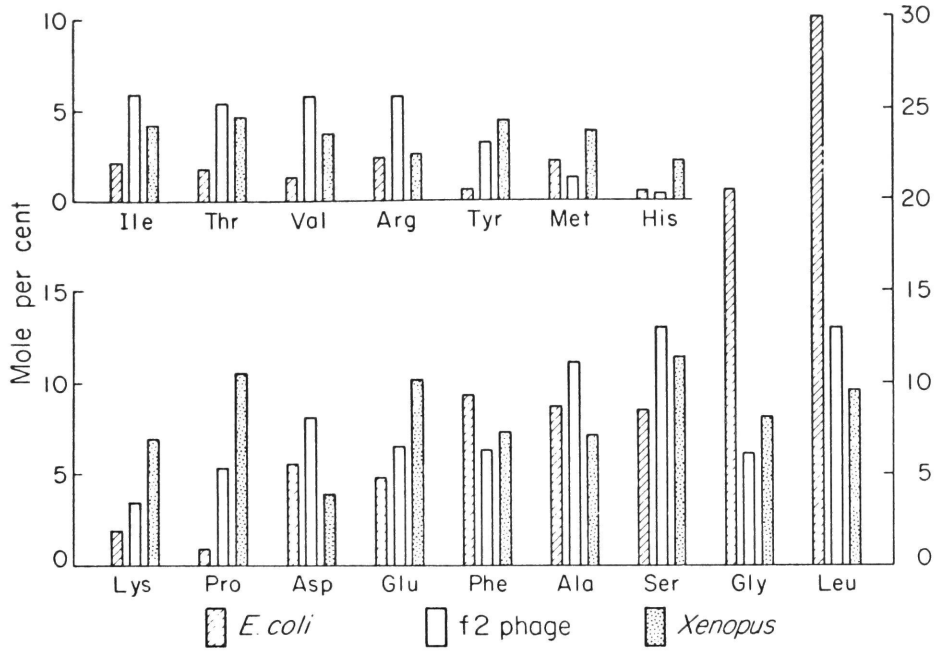

Figure 10. Amino acid composition of proteins synthesized in vitro. The amino acid compositions of Xenopus and f2 proteins were calculated after subtraction of the endogenous incorporation of each amino acid from the total incorporation of each amino acid observed in the presence of the exogenous mRNA. The amino acid compositions of the in vitro products synthesized in response to f2 RNA, Xenopus RNA, and E. coli (endogenous) mRNA, do not resemble one another. Therefore, the response to Xenopus RNA is not due to the stimulation of endogenous incorporation in a non-specific manner, as suggested by Drach and Lingrel (1966). On the basis of the results obtained for each amino acid, proline was selected as a useful label for following in vitro protein synthesis, since it is under-represented in bacterial proteins (unlike leucine or glycine), and since it is well represented in Xenopus and f2 proteins.

The results provide the basis of a more valid comparison between the specific template activities of f2 RNA and Xenopus RNA. For instance, there is twice as much lysine in Xenopus proteins, compared to f2 proteins; thus, the specific template activity of Xenopus RNA relative to that of f2 RNA (see Figure 7, for example), is actually half of what it appeared to be on the basis of lysine incorporation alone.

Discussion

Slater and Spiegelman (1966) used the E. coli S-30 system to demonstrate the existence of mRNA in unfertilized sea urchin eggs. They compared the specific template activity of sea urchin RNA with that of MS-2 coliphage RNA and found that sea urchin RNA was only 4-5% as active as MS-2 RNA. They concluded that "4-5% of the bulk RNA derived from unfertilized eggs possesses the ability to serve as templates for protein synthesis." Thus, they used relative specific template activity as a measure of the quantity of mRNA present in an RNA preparation. However, when comparisons are made between the template activities of heterologous RNA preparations, a number of factors will complicate the comparison.

For instance, Slater and Spiegelman assumed that viral RNA is "100 per cent translatable message." Although it is probably true that the entire length of coliphage RNA does serve as a template, some portions of this mRNA are translated more frequently than other portions. This is because coliphage RNA is "polycistronic," in the sense that it codes for three different proteins (Ohtaka and Spiegelman, 1963); and the synthesis of each protein may be initiated independently (Lodish, 1968; Webster and Zinder, 1969). Each protein is synthesized in different quantities in vitro, indicating that some sort of control mechanism exists, which regulates the frequency with which the different cistrons are translated (Zinder et al., 1966). An ideal mRNA standard would be a monocistronic mRNA which is translated in a manner common to all the mRNA's to which it will be compared.

A more fundamental complication is that it is not necessarily true that mRNA's from different sources are translated with the same efficiency. Stated another way, equal quantities of mRNA from different sources may not direct the synthesis of equal quantities of protein. Recognizing this objection, Slater and Spiegelman (1968), in their later work, only compared the

specific template activities of RNA preparations obtained from different developmental stages of the same animal. They did not make comparisons with a viral RNA. Thus, their comparisons were independent of assumptions requiring that heterologous mRNA's be equally efficient templates.

For these same reasons, the proportion of mRNA present in Xenopus RNA preparations can not be determined by a simple comparison of the specific template activities of Xenopus RNA and f2 RNA. Moreover, the presence of polyvinyl sulfonate in the Xenopus RNA preparations and the possible influence of the predominant rRNA molecules rule out the use of the cell-free system as a means of measuring the amount of mRNA in Xenopus RNA preparations.

In summary, mRNA can be detected by its template activity in vitro. However, quantitative comparisons of the template activity of heterologous mRNA's can not be meaningfully interpreted until the factors which influence specific template activity are explored. The remainder of this dissertation is concerned with the study of those factors which influence specific template activity. The next chapter describes experiments which were designed to determine whether equal quantities of different mRNA's promote the synthesis of equal quantities of protein, under a variety of experimental conditions.

CHAPTER THREE: RELATIVE SPECIFIC TEMPLATE ACTIVITY

If it is assumed that different mRNA's are translated in vitro with the same efficiency, then one would expect that their relative specific template activity would remain constant over a wide range of experimental conditions. For example, if the alteration of a reaction parameter lowered the specific template activity of one mRNA, the specific template activity of a different mRNA would be lowered to the same extent. This hypothesis was tested by comparing the template activities of f2 RNA and Xenopus oocyte RNA, in the presence of varying concentrations of several components of the cell-free system. As will be shown, the hypothesis is not sustained, since alterations in reaction conditions often had an opposite effect on the rate of translation of the two different mRNA's. Therefore, translation efficiency does depend on the type of mRNA being translated.

Differential Response to Different mRNA's under Various Reaction Conditions

Effect of Altering Mg^{++} and NH_4^+ Concentration

Figure 11, on page 38, illustrates an experiment which investigated the effect of magnesium ion concentration on the template activity of f2 RNA and on the template activity of Xenopus RNA. As can be seen, there was a magnesium ion concentration at which amino acid incorporation was optimal. Significantly, the optimal Mg^{++} concentration was different for the different RNA preparations. The template activity of Xenopus RNA, compared to the template activity of f2 RNA, increased as Mg^{++} concentration increased. Thus, relative template activity is a function of the magnesium ion concentration.

It was possible that the observed difference in the two Mg^{++} concentration optima was due to non-specific binding of magnesium ions by RNA. If this was the case, then increasing the concentration of RNA would result in the withdrawal of magnesium ions, causing an apparent shift of the Mg^{++} concentration optimum towards higher values, since more Mg^{++} would be required

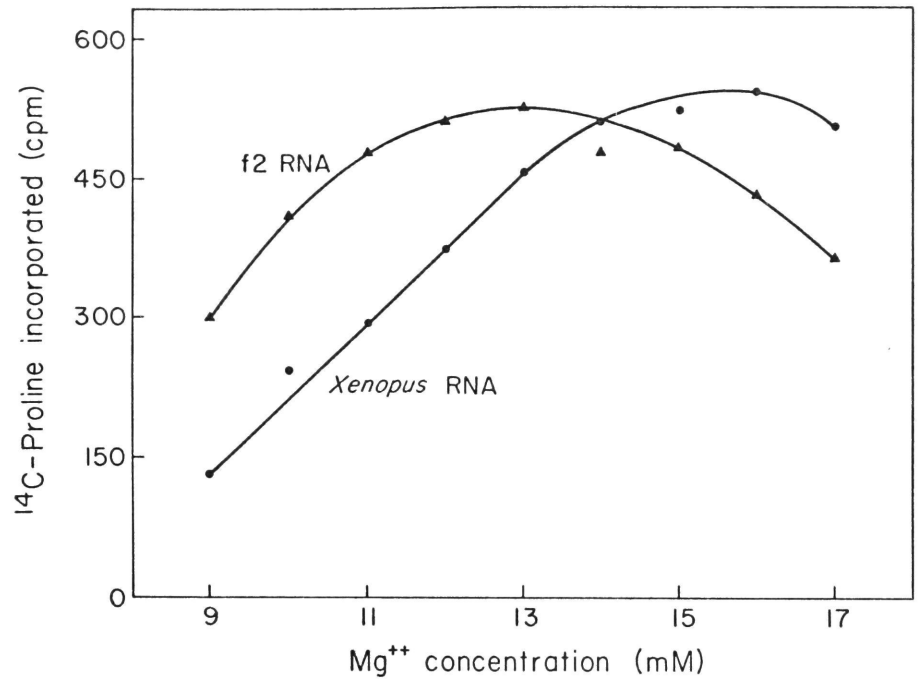

Figure 11. Dependence of Mg^{++} concentration optimum on the type of mRNA. The Xenopus RNA concentration was 160 μg/ml and the f2 RNA concentration was 19 μg/ml. The relative template activity of the two types of RNA was a function of the magnesium ion concentration.

to replace the bound magnesium. However, this proved not to be the case, since the optimum template activity at three different concentrations of f2 RNA, occurred at the same Mg^{++} concentration. The results of this experiment are illustrated in Figure 12 on page 40. Thus, the optimal concentration of Mg^{++} in the cell-free system is not a function of the amount of mRNA, but is a function of the type of mRNA.

The response of the system is also sensitive to ammonium ion concentration. Figure 13, on page 41, illustrates an experiment in which NH_4^+ concentration was varied. Again, the optimal concentration depended on which type of mRNA was being translated. Boedtker and Stumpp (1966) observed a similar difference in ammonium ion concentration optima with R17 coliphage RNA and tobacco mosaic virus RNA.

Effect of Added Transfer RNA's

The effect of adding additional transfer RNA's to the cell-free system was also investigated. These experiments are illustrated in Figure 14 on page 42. Transfer RNA's from E. coli K12 strongly stimulated the response to Xenopus RNA, while the response to f2 RNA was only slightly stimulated. Calf's liver tRNA's, on the other hand, were inhibitory, though the response to Xenopus RNA was inhibited to a lesser extent than the response to f2 RNA. Here again, relative template activity is a function of the reaction conditions, and can be altered by changing the concentration of a reaction mixture component.

Effect of Preincubation

The reaction mixture was usually preincubated, in order to lower the endogenous amino acid incorporation. Exogenous mRNA and the labelled amino acid were added after preincubation. Figure 15, on page 43, illustrates an experiment which investigated the effect of different preincubation times on the response to mRNA, which was added after the reaction mixture had been preincubated. Increasing the duration of preincubation, decreased the ability of the cell-free system to respond to Xenopus RNA, while the response to f2 RNA was only slightly diminished. Relative template activity is therefore a function of the duration of preincubation.

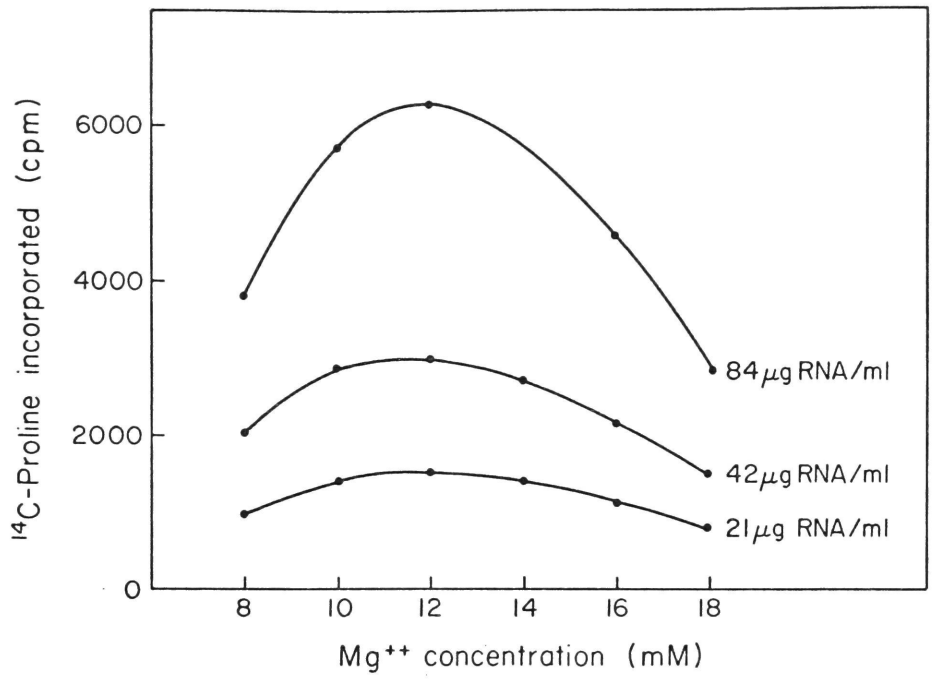

Figure 12. Independence of Mg^{++} concentration optimum from f2 RNA concentration. If mRNA had bound significant amounts of Mg^{++}, then the observed optimum would have been shifted towards the right at higher RNA concentrations. Therefore, the optimal concentration of Mg^{++} in the cell-free system is a function of the type of mRNA, rather than the amount of mRNA.

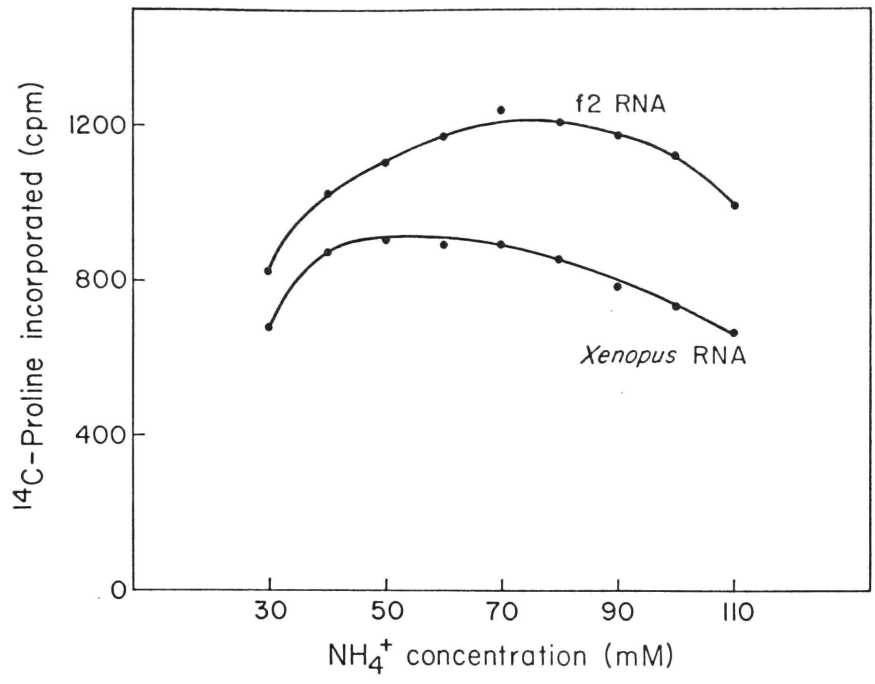

Figure 13. Dependence of NH_4^+ concentration optimum on the type of mRNA. The Xenopus RNA concentration was 160 μg/ml and the f2 RNA concentration was 19 μg/ml. The relative template activity of the two types of RNA was a function of the ammonium ion concentration.

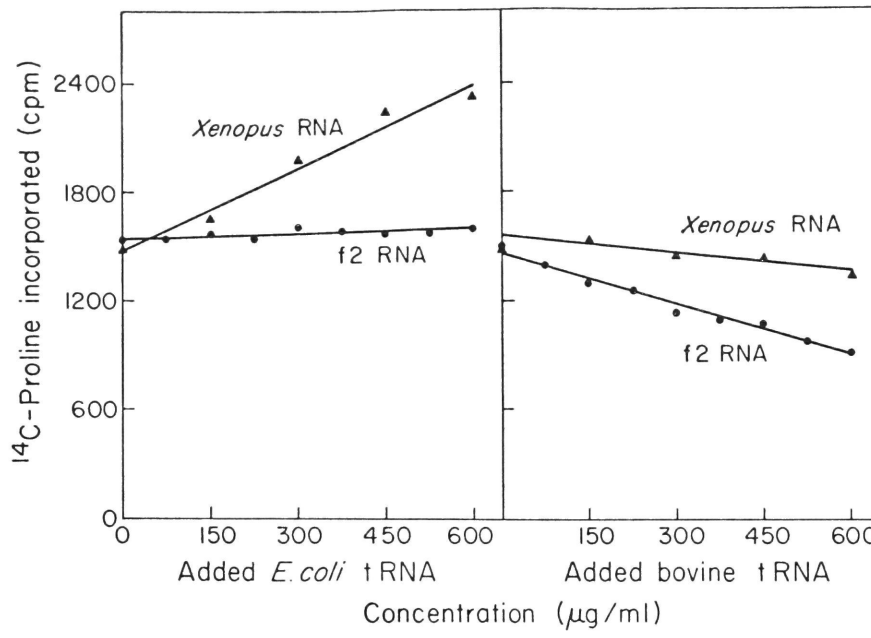

Figure 14. Differential response to Xenopus RNA and f2 RNA upon addition of exogenous transfer RNA's. The Xenopus RNA concentration was 96 μg/ml and the f2 RNA concentration was 22 μg/ml. Transfer RNA's were obtained from General Biochemicals, Chagrin Falls, Ohio. E. coli tRNA's from Strain K12 were isolated by the procedure of Holley and his co-workers (1961), and bovine tRNA's were prepared from calf's liver by the procedure of Brunngraber (1962). Both preparations were "stripped" by incubation in an alkaline buffer (Nathans and Lipmann, 1961; Wolfenden, 1963).

Here again, relative template activity is a function of the concentration of reaction mixture components. Perhaps Xenopus mRNA often requires tRNA's which are only present in the S-30 extract at low concentrations. The addition of exogenous tRNA's would increase the concentration of these rarer components.

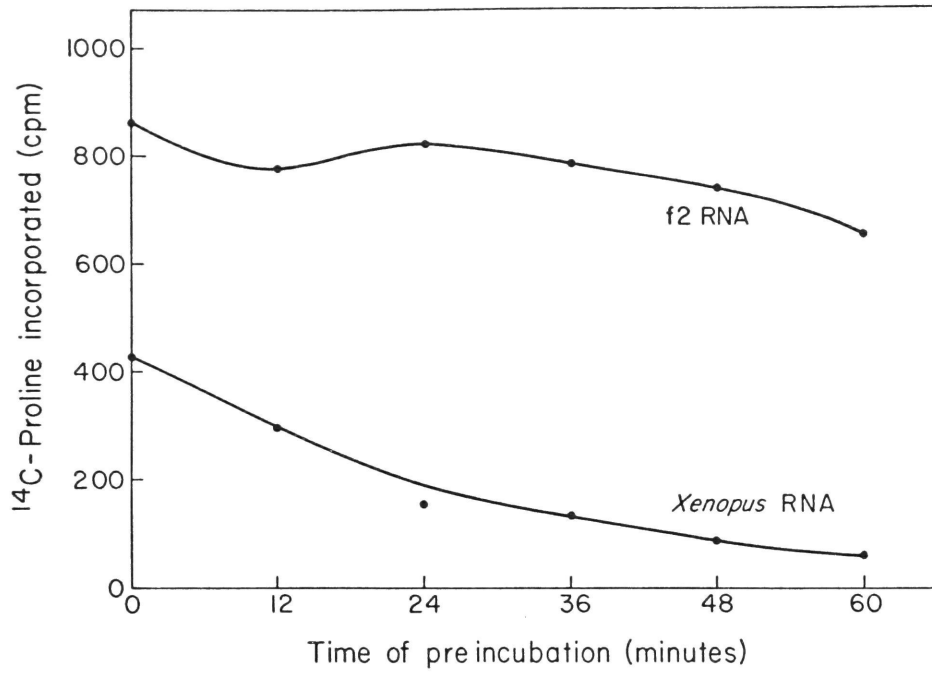

Figure 15. Differential effect of preincubation of the reaction mixture on the subsequent response to Xenopus RNA and f2 RNA. Exogenous RNA was not preincubated. It was added to preincubated reaction mixtures. The Xenopus RNA concentration was 160 μg/ml and the f2 RNA concentration was 25 μg/ml. The relative template activity of the two types of RNA was a function of the duration of preincubation.

Codon Composition and the Rate of Translation

Thus, in general, equal quantities of different mRNA's do not direct the synthesis of equal quantities of protein, since template activity is a function of the reaction environment, and alterations in the reaction environment have a differential effect on the response to different mRNA's. Therefore, relative specific template activity can not be used as a measure of mRNA concentration, when comparing heterologous mRNA's.

The intriguing question raised by these experiments is: why does template activity depend on the type of mRNA being translated?

Codon Composition

Messenger RNA's differ from one another in respect to the codons they contain. There are 64 possible codons. Some codons might occur frequently in one message, but might occur less frequently, or not at all, in another. Thus, each type of mRNA has its own characteristic "codon composition." Each mRNA is a linear array of codons, which serves as a sequential program of instructions for the synthesis of a specific protein. Each codon calls into play a particular subset of the protein synthesis machinery. Specifically, each codon can only bind those amino-acyl tRNA's possessing an anticodon region able to form hydrogen bonds with the codon, in accordance with precise pairing rules (Crick, 1966; Söll *et al.*, 1967; Söll and RajBhandary, 1967). My main hypothesis is that some codons are slow and some codons are fast, in respect to the time it takes to find and then bind an appropriate tRNA. Since different mRNA's have different codon compositions, the overall translation rate would vary for each type of mRNA.

Finding and Binding an Appropriate tRNA

The concentration of each tRNA species varies over a wide range. Some organisms may contain little or no amino-acyl tRNA for certain codons (Söll *et al.*, 1967; Muench and Safille, 1968; Caskey *et al.*, 1968). The time it takes to find an appropriate tRNA species probably depends on its concentration in the reaction mixture.

Once found, the tRNA must be bound to the ribosome-mRNA complex. Each tRNA possesses a secondary and tertiary structure which confers on it a specificity similar to that of the globular proteins (Fresco *et al.*, 1966).

The efficiency with which a tRNA binds to the ribosome-mRNA complex is dependent on the conformation of the tRNA (Doi and Goehler, 1966; Lindahl et al., 1967). Transfer RNA's are polyelectrolytes with high charge densities and are sensitive to the nature and concentration of neutralizing ions. Thus, the conformation of each tRNA is dependent on the presence of magnesium ions (Lindahl et al., 1966). It is therefore not surprising that the stability of the tRNA-ribosome-mRNA complex varies according to which tRNA is studied, and that this stability is a function of the Mg^{++} concentration (Nirenberg et al., 1966). Though the mechanism is not understood, it has been demonstrated that NH_4^+ concentration also affects the efficiency with which tRNA's bind to the ribosome-mRNA complex (Spyrides, 1964; Pulkrábek and Rychlík, 1968). Thus, the time it takes to bind an appropriate tRNA probably depends on the nature of the ionic environment of the reaction mixture.

The time it takes each type of codon to find and bind an appropriate tRNA has been studied directly by means of the "binding assay," developed by Nirenberg and Leder (1964). In this procedure, synthetic trinucleotide "codons" are used as templates to direct the binding of appropriate tRNA's to ribosomes. Each type of trinucleotide tested exhibited a characteristic "binding efficiency" when incubated with tRNA's from E. coli (Söll et al., 1965; Nirenberg et al., 1966). If tRNA's from Xenopus were used, the binding efficiency of each codon was altered in an unpredictable manner (Marshall et al., 1967), demonstrating that binding efficiency is a function of the tRNA's as well as the codons.

Discussion

The experiments described in this chapter have demonstrated that template activity is a function of Mg^{++} and NH_4^+ concentration, tRNA concentration, and time of preincubation. Significantly, alterations in these parameters did not have the same effect on protein synthesis promoted by f2 RNA as they did on protein synthesis promoted by Xenopus RNA. It is possible that these differences are the result of using a vertebrate RNA in a bacterial system, and that they reflect some basic structural difference between the RNA of microorganisms and the RNA of higher animals. Also, the differences might be due to the presence of rRNA or polyvinyl sulfonate in the Xenopus RNA preparations. However, a more plausible explanation (and one that is consistent with the experimental observations and with the observations of other

workers) is that the most significant difference between heterologous mRNA's is their codon composition. Since each type of codon calls into play a particular subset of the amino-acyl tRNA's, heterologous mRNA's (possessing differing codon compositions) will call into play some types of amino-acyl tRNA's more frequently than others. Any alterations in the reaction conditions which affect the ability of some amino-acyl tRNA's to bind to the ribosome, or which change the concentration of some amino-acyl tRNA's, are likely to have a differential effect on the translation of mRNA's possessing different codon compositions.

The implication of these experiments is that each type of codon has its own characteristic time of finding and binding an appropriate amino-acyl tRNA, under a given set of experimental conditions. Therefore, the time it takes to translate a mRNA will depend on the mRNA's codon composition.

The next chapter describes experiments designed to demonstrate that the time it takes to translate a mRNA is indeed different for different mRNA's; and it will also present a method by which the translation rates of different mRNA's can be compared.

CHAPTER FOUR: USE OF A GTP ANALOGUE TO STUDY THE RATE OF POLYPEPTIDE CHAIN ELONGATION

The mechanism of protein synthesis is complex and only partially understood. This chapter begins with a brief description of how polypeptide chains are thought to be assembled on the ribosomes. It describes the reactions that occur, the enzymes which catalyze the reactions, the sequential nature of the reactions, and the role that GTP plays in driving the reactions. The chapter then describes experiments (based on this model of protein synthesis), in which an analogue of GTP is used to investigate rates of polypeptide chain elongation. The remainder of the chapter discusses the mechanism of chain initiation and the role that GTP plays in the initiation reactions.

The Polypeptide Chain Elongation Cycle

Protein synthesis takes place on the ribosomes. Each ribosome is composed of two subunits (Tissières et al., 1959). Messenger RNA is attached to the smaller subunit (Takanami and Okamoto, 1963) and the growing polypeptide chain is covalently linked to a tRNA on the larger subunit (Gilbert 1963). Codon recognition occurs on the smaller subunit (Kaji et al., 1966), when an appropriate amino-acyl tRNA is bound to the ribosome (Kaji and Kaji, 1963, 1964). Thus, one mRNA, one polypeptide chain, and one or two tRNA's are bound to each ribosome, at any one time (Warner and Rich, 1964; Arlinghaus et al., 1964).

The most widely-accepted model of the mechanism of polypeptide chain growth was proposed by Watson (1964), and is illustrated in Figure 16 on page 48. In this model, each ribosome contains two tRNA binding sites, termed the "donor site" and the "acceptor site" (Heintz et al., 1966). Chain elongation is accomplished in three steps. First, an appropriate amino-acyl tRNA binds to the ribosome at the acceptor site. Then the nascent polypeptide chain, which is bound to the tRNA in the donor site, forms a peptide bond with the amino acid bound to the tRNA in the acceptor site. The result of this "bonding" step is that the nascent polypeptide chain is elongated by one amino acid and is bound to the tRNA in the acceptor site. The last step is the "translocation"

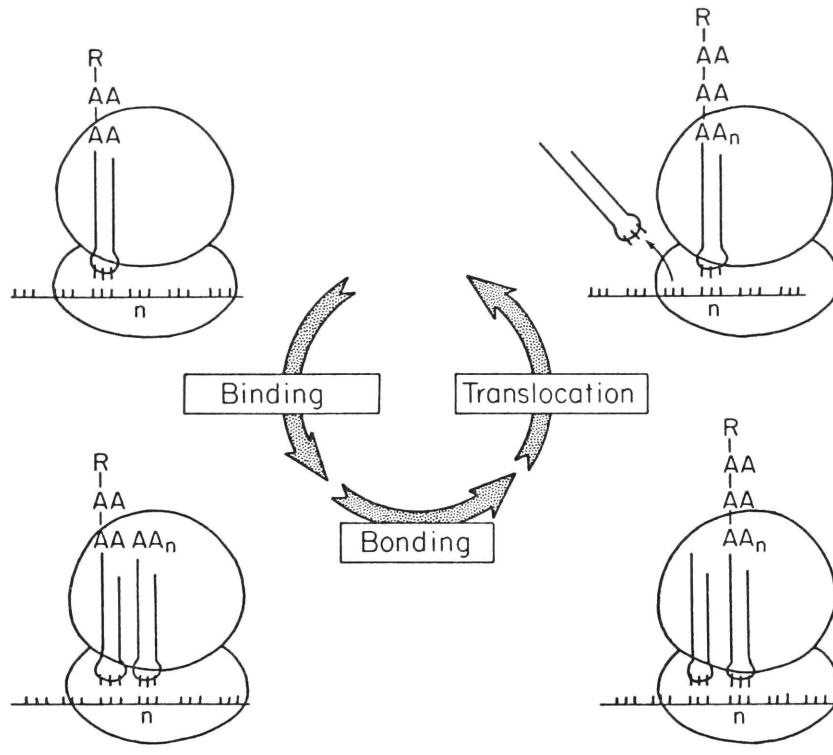

Figure 16. The polypeptide chain elongation cycle. The addition of an amino acid to a growing polypeptide chain requires three steps. First, an appropriate amino-acyl tRNA binds to the ribosome, and to the codon being translated, at the acceptor site. Next, the amino group of this amino-acyl tRNA condenses with the activated carboxyl group of the terminal residue of the polypeptide chain, which is bound to the tRNA at the donor site. As a result, a new peptide bond is formed and the elongated polypeptide chain is attached to the tRNA at the acceptor site. In the third step, the elongated peptidyl tRNA is translocated from the acceptor to the donor site, displacing the deacylated tRNA and moving the mRNA forward one codon. These three steps are independent and sequential.

The energy required to form the peptide bond resides in the potential of the ester bond joining the carboxyl end of the nascent polypeptide to the tRNA in the donor site (Jencks et al., 1960). The energy required for translocation is supplied by the hydrolysis of a GTP molecule during the translocation step.

of the peptidyl tRNA from the acceptor to the donor site, As a consequence of translocation, the deacylated tRNA, which was bound in the donor site, is ejected from the ribosome, and the mRNA, which is hydrogen-bonded to the peptidyl tRNA, is moved forward one codon, permitting the next codon to be recognized at the acceptor site (Watson, 1963). Thus, the addition of an amino acid to a polypeptide chain is thought to require three steps: binding, bonding, and translocation (Skogerson and Moldave, 1968a; Erbe et al., 1969; Lipmann, 1969). This sequence of steps forms a cycle, which is repeated each time an amino acid is added to a growing polypeptide chain.

Enzymes that Catalyze the Chain Elongation Cycle

Three enzymes have been identified, each of which catalyzes one step in the polypeptide chain elongation cycle. Two of the enzymes were first isolated from the supernatant fraction as soluble "transfer factors," which stimulate in vitro protein synthesis (Nathans and Lipmann, 1961; Allende et al., 1964; Arlinghaus et al., 1964). These enzymes were further purified and termed "T" and "G" by Nishizuka and Lipmann (1966a). The T enzyme was fractionated into two subunits (Lucas-Lenard and Lipmann, 1966; Ravel et al., 1968), both of which are required for full enzymatic activity (Ertel et al., 1968; Lucas-Lenard and Haenni, 1968). The isolated transfer enzymes have been crystallized (Parmeggiani, 1968; Kaziro and Inoue, 1968; Leder et al., 1969a). The third enzyme, "peptidyl transferase" (Monro et al., 1967), is one of the proteins of the larger ribosomal subunit (Monro, 1967). The T enzyme promotes the binding of the amino-acyl tRNA to the ribosome (Ravel, 1967; Ibuki and Moldave, 1968; Ravel et al., 1968; Lucas-Lenard and Haenni, 1968), peptidyl transferase catalyzes the formation of peptide bonds (Monro and Marcker, 1967; Gottesman, 1967; Maden et al., 1968; Pestka, 1968a), and the G enzyme is responsible for translocation (Nishizuka and Lipmann, 1966b; Pestka, 1968b; Skogerson and Moldave, 1968c; Haenni and Lucas-Lenard, 1968; Leder et al., 1969b).

The Sequential Nature of the Steps

Ribosomes can be washed free of transfer enzymes (Lucas-Lenard and Lipmann, 1966). These washed ribosomes retain the ability to catalyze peptide-bond formation (Monro and Marcker, 1967; Pestka, 1968a). The binding of amino-acyl tRNA's to washed ribosomes requires the T enzyme. If the donor site is occupied by a peptidyl tRNA or an N-acetyl amino-acyl tRNA (Haenni and

Chapeville, 1966), which serves as an analogue of peptidyl tRNA (Lucas-Lenard and Lipmann, 1967), the amino-acyl tRNA bound to the acceptor site by the T enzyme will immediately react to form a new peptide bond (Skogerson and Moldave, 1968a). However, if this reaction is carried out in the presence of sparsomycin, an inhibitor of peptidyl transferase (Goldberg and Mitsugi, 1967; Monro and Vazquez, 1967), binding occurs in the absence of peptide-bond formation (Lucas-Lenard and Haenni, 1968). Thus, binding and bonding are independent steps, though bond formation cannot occur until an amino-acyl tRNA has been bound.

Puromycin is a chemical analogue of the terminal amino-acyl adenosine of amino-acyl tRNA (Yarmolinsky and de la Haba, 1959). Thus, puromycin, by substituting for the amino-acyl tRNA at the active site of peptidyl transferase, can form a peptide bond with the nascent polypeptide chain. This puromycin polypeptide is then released from the ribosome (Morris et al., 1963), since it is no longer attached to a tRNA (Gilbert, 1963). Since puromycin competes with amino-acyl tRNA, which binds to the acceptor site, the release of a nascent polypeptide by puromycin indicates that the nascent polypeptide was bound to tRNA in the donor site (Traut and Monro, 1964).

Significantly, once bond formation has occurred on washed ribosomes, the nascent polypeptide chain cannot be released by puromycin (Skogerson and Moldave, 1968a). Thus, the elongated polypeptide chain is attached to the tRNA in the acceptor site. Moreover, once bonding has occurred, new amino-acyl tRNA's cannot be bound to the ribosome, and no further reactions can occur (Haenni and Lucas-Lenard, 1968). However, the subsequent addition of the G enzyme results in the translocation of the peptidyl tRNA to the donor site (Skogerson and Moldave, 1968c), where the nascent polypeptide chain may be released by puromycin (Haenni and Lucas-Lenard, 1968). The ejection of deacylated tRNA from the donor site is tightly coupled with this translocation (Lucas-Lenard and Haenni, 1969). Only after translocation has occurred, can a new amino-acyl tRNA be bound to the ribosome. This can occur in the absence of the G enzyme, but requires the T enzyme (Haenni and Lucas-Lenard, 1968). Thus, translocation must occur before the next codon can be recognized (Erbe and Leder, 1968; Erbe et al., 1969). In summary, the polypeptide chain elongation cycle consists of three independent and sequential steps, which are repeated each time an amino acid is added to a growing polypeptide chain.

The Role of GTP in Chain Elongation

The requirement for GTP in protein synthesis was first demonstrated by Keller and Zamecnik (1956). Recently, it has been shown that GTP is used in different ways in the various reactions involved in chain elongation. GTP is required in the binding step (Arlinghaus et al., 1964). Specifically, GTP binds to the T enzyme (Allende et al., 1967; Gordon, 1967, 1968; Ravel et al., 1968) and is an obligatory cofactor for the T-catalyzed binding of amino-acyl tRNA to the ribosome (Ravel, 1967; Lucas-Lenard and Haenni, 1968; Ertel et al., 1968; Erbe and Leder, 1968). GTP is also an obligatory cofactor for G-catalyzed translocation (Skogerson and Moldave, 1968c; Pestka, 1968b, Haenni and Lucas-Lenard, 1968; Erbe et al., 1969). However, GTP is not required for peptidyl transferase activity (Monro, 1967; Gottesman, 1967; Pestka, 1968a). Therefore, GTP serves as an enzymatic cofactor in the binding and translocation steps.

GTP is also hydrolyzed to guanosine diphosphate and orthophosphate during protein synthesis (Nathans et al., 1962; Conway and Lipmann, 1964). At least one molecule of GTP is hydrolyzed for every peptide bond formed (Webster and Whitman, 1962; Nishizuka and Lipmann, 1966a). In order to determine the step in which GTP hydrolysis occurs, reactions were carried out in the presence of the GTP analogue, 5'-guanylyl-β,γ-methylenediphosphonate (GDPCP), instead of in the presence of GTP. The structure of GDPCP closely resembles that of GTP, and is illustrated in Figure 17 on page 52. The only difference between GDPCP and GTP, is that the GDPCP has a methylene group, instead of an oxygen atom, between the β- and γ-phosphorus atoms, thus preventing hydrolytic cleavage at this position. Because it cannot be hydrolyzed, GDPCP inhibits protein synthesis (Hershey and Monro, 1966). However, since GDPCP closely resembles GTP, it can substitute for GTP in those reactions in which GTP is required as a cofactor. Thus, GDPCP will only inhibit reactions in which GTP is hydrolyzed.

T-catalyzed binding of amino-acyl tRNA to ribosomes occurs equally well in the presence of GDPCP as in the presence of GTP (Ertel et al., 1968; Skogerson and Moldave, 1968a; Ravel et al., 1968; Haenni and Lucas-Lenard, 1968). Therefore, GTP hydrolysis is not required for binding to occur. However, GDPCP inhibits G-catalyzed translocation, indicating that GTP hydrolysis is necessary for translocation to occur (Skogerson and Moldave,

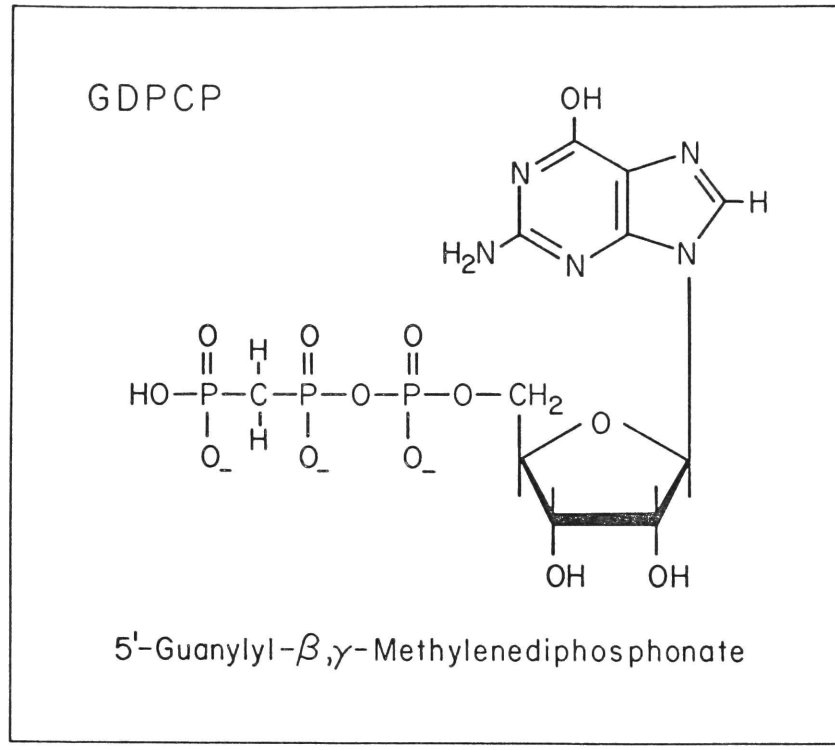

Figure 17. 5'-Guanylyl-β,γ-methylenediphosphonate (GDPCP). The structure of this molecule is very similar to that of GTP, so it can replace GTP in those reactions requiring GTP as a cofactor. However, it has a methylene group, instead of an oxygen atom, between the β- and γ-phosphorus atoms. Therefore, it cannot be hydrolyzed at this position and it will inhibit those reactions in which GTP is normally hydrolyzed.

1968a; Pestka, 1968b; Haenni and Lucas-Lenard, 1968; Erbe et al., 1969). Specifically, GTP is required as a cofactor for the binding of the G enzyme to the ribosome (Parmeggiani and Gottschalk, 1969), and GTP is hydrolyzed during the subsequent G-catalyzed translocation (Skogerson and Moldave, 1968b). This result confirms the earlier observation of Nishizuka and Lipmann (1966b), that the G enzyme exhibits GTPase activity, if, and only if, it is added to ribosomes; and the results support Watson's (1964) speculation that the hydrolysis of GTP would provide the energy required to accomplish translocation. In summary, GTP is a cofactor in the binding of amino-acyl tRNA to the ribosome; GTP is not required for bond formation; and GTP is hydrolyzed during the translocation step.

GDPCP Inhibition of Protein Synthesis

This section discusses the use of GDPCP as an inhibitor of protein synthesis. It will be shown that GDPCP is a competitive inhibitor of GTP hydrolysis, but does not inhibit reactions in which GTP is required as a cofactor. It will also be shown that, although GTP is a competitive inhibitor of GTP hydrolysis, it is a non-competitive inhibitor with respect to the overall process of protein synthesis. Moreover, since GDPCP probably interferes with only one of the three steps in the polypeptide chain elongation cycle, its effectiveness as an inhibitor of protein synthesis is dependent on the time it takes to complete the other two, unaffected steps. For example, if these two unaffected steps take a long time, the inhibitor will have a relatively small effect. On the other hand, if the two unaffected steps are short, the inhibitor will have a relatively large effect. Therefore, the effectiveness of GDPCP as an inhibitor of protein synthesis is a measure of the overall time it takes to complete the polypeptide chain elongation cycle.

The Competitive Nature of the Inhibition of GTP Hydrolysis

The inhibition of protein synthesis by GDPCP was studied by Pestka (1968b). He demonstrated that GDPCP is a competitive inhibitor of GTP hydrolysis. His experiment, in which the amino acid incorporation rate was determined at various concentrations of GDPCP and GTP, and then plotted according to the method of Lineweaver and Burk (1934), is illustrated in Figure 18 on page 54. Since the lines connecting the points obtained at the same inhibitor concentration could be extrapolated to the same point on the ordinate, GDPCP is a competitive inhibitor with respect to GTP.

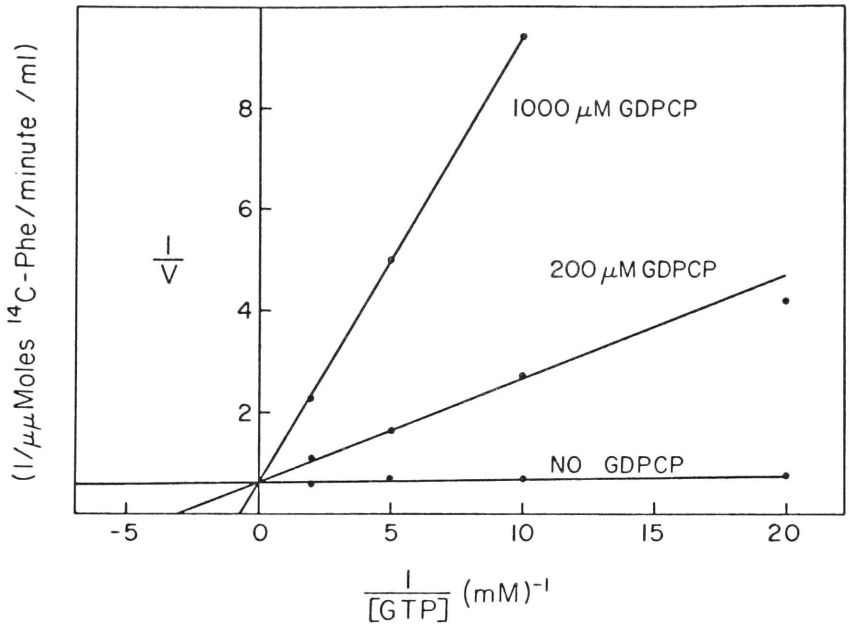

Figure 18. Pestka's (1968b) demonstration that GDPCP is a competitive inhibitor with respect to GTP. Washed ribosomes and purified enzymes (T and G) were used in the synthesis of oligophenylalanine in response to polyuridylic acid. V is the rate of phenylalanine incorporation. The lines connecting points at the same GDPCP concentration meet on the ordinate, indicating that the inhibition is competitive (Lineweaver and Burk, 1934).

The mechanism of competitive inhibition was first described by Michaelis and Menten (1913). They assumed that in an enzymatic reaction the enzyme and its substrate form a reversibly dissociable complex, and the rate of the conversion of substrate to products is directly proportional to the concentration of this enzyme-substrate complex. They also assumed that a competitive inhibitor can form a reversibly dissociable complex with the enzyme. Competitive inhibitors are distinguished from other types of inhibitors in that they bind to the enzyme at the same site as the substrate, rather than at some other site, as in the case of non-competitive inhibitors (Michaelis and Pechstein, 1914). Competitive inhibitors bind to the same site on the enzyme, since their structure is almost identical with that of the substrate. The enzyme-inhibitor complex cannot bind a substrate molecule, and cannot react to form products. Since the reaction rate is proportional to the concentration of the enzyme-substrate complex, and since, in the presence of the inhibitor, the number of enzyme-substrate complexes is lowered by the formation of enzyme-inhibitor complexes, the reaction rate is reduced in the presence of the inhibitor. The proportion of the enzyme molecules complexed with substrate, and thus able to catalyze the conversion of substrate to products, will depend on the relative concentrations of the inhibitor and the substrate, since they compete for the same site on the enzyme. Thus, the inhibition due to a fixed concentration of a competitive inhibitor can almost entirely be overcome by increasing the substrate concentration. Conversely, for a fixed substrate concentration, the addition of more inhibitor results in a reduction of the reaction rate.

The Effect of GDPCP on Protein Synthesis in the Cell-Free System

The remainder of this chapter describes experiments which investigate the effect of GDPCP on protein synthesis in the *E. coli* S-30 system. Figure 19, on page 56, illustrates the effect of increasing concentrations of GDPCP on the time course of amino acid incorporation in response to f2 RNA. The concentration of GTP was the same in each reaction tube. As expected, the higher the inhibitor concentration, the lower the amino acid incorporation observed at any given time. GDPCP does not act as a poison (*i.e.*, bind to an enzyme or substrate in an irreversible manner), since higher concentrations of the inhibitor did not shorten the time course of amino acid incorporation.

Presumably, the observed inhibition was due to the competitive inhibition of GTP hydrolysis, and not due to the inhibition of reactions in which

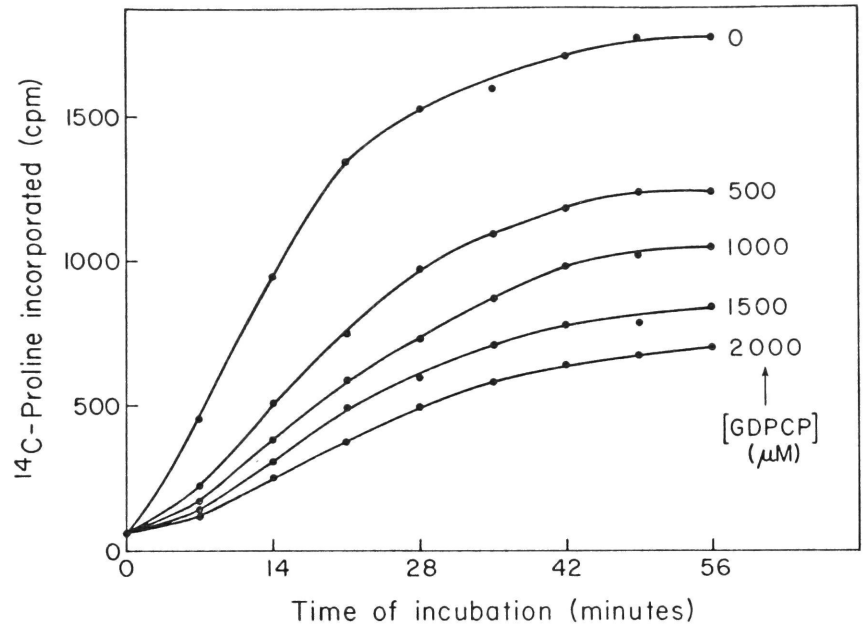

Figure 19. Effect of increasing concentrations of GDPCP on the time course of ^{14}C-proline incorporation. Each reaction mixture contained 28 μg/ml f2 RNA and was 200 μM in GTP. GDPCP was obtained from Miles Laboratories, Elkhart, Ind. GDPCP inhibited amino acid incorporation. The extent of inhibition was a function of the GDPCP concentration.

GTP is required as a cofactor. However, the addition of GDPCP does increase the concentration of "GTP-like" molecules. If any of the reactions in which GTP participates is sensitive to increased concentrations of GTP, the addition of GDPCP would affect them. Two experiments were performed in which the reaction rate was shown to be essentially insensitive to changes in the concentration of added GTP-like molecules, apart from the effect of GDPCP on hydrolysis.

The first experiment, illustrated in Figure 20, on page 58, shows that the endogeneous GTP (which could not be removed during the dialysis of the S-30, presumably because it was bound to proteins) is sufficient to saturate the system, and the addition of more GTP does not substantially alter the amino acid incorporation rate. Figure 21, on page 59, illustrates the second experiment, in which both GTP and GDPCP were added to each reaction tube. The ratio of GDPCP to added GTP was kept constant, though the total concentration of GTP-like molecules was varied. At low concentrations of added nucleotide, the reaction rate was higher than at higher concentrations of added nucleotide. This was due to the contribution of the endogenous GTP to the ratio of GTP to GDPCP, thus decreasing the inhibition. However, as the nucleotide concentration was increased, the contribution of the endogenous GTP became negligible, and the reaction rate was determined solely by the ratio of GDPCP to added GTP. The important point here is that once the dilution effect of the endogenous GTP was overcome the reaction rate was constant. Thus, in general, the ratio of GTP to GDPCP determines the rate of <u>in vitro</u> protein synthesis, and this rate is not affected by the overall concentration of GTP-like molecules. Therefore, at any fixed concentration of GTP, the addition of GDPCP will lower the reaction rate, since it inhibits GTP hydrolysis. However, the concomitant increase in the concentration of GTP-like molecules will not affect the reaction rate. In summary, GDPCP has no appreciable effect on reactions in which GTP is required as a cofactor, but does competitively inhibit reactions in which GTP is hydrolyzed.

<u>Differential Effect of GDPCP on the Translation Rate of Different mRNA's</u>

GDPCP reduces the rate of protein synthesis by increasing the duration of the translocation step. The higher the GDPCP concentration, the longer it will take a molecule of the G enzyme to bind a GTP molecule, since time will

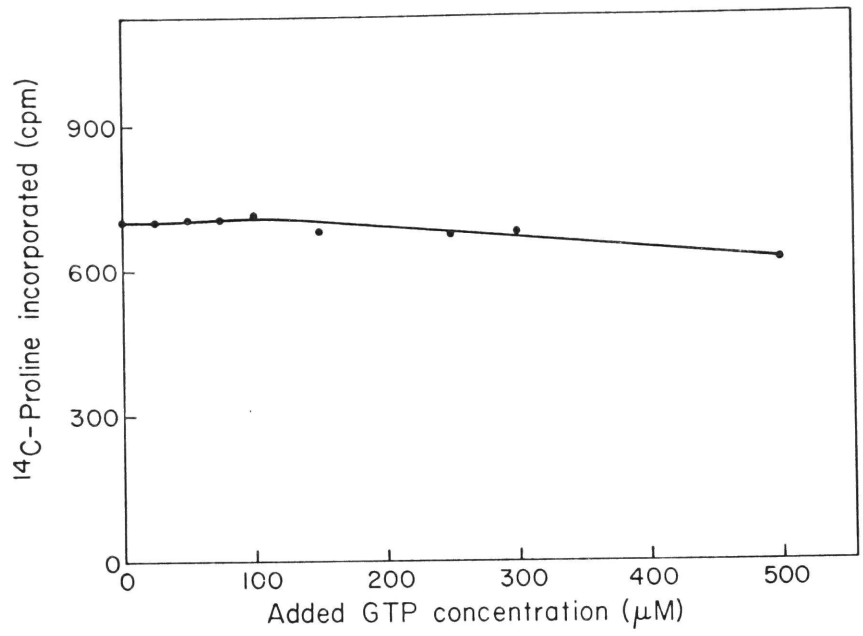

Figure 20. Effect of added GTP on ^{14}C-proline incorporation. Each reaction tube contained 34 μg/ml f2 RNA. The reaction did not require exogenous GTP. Apparently, the endogenous GTP in the S-30 extract was sufficient to sustain protein synthesis. The addition of exogenous GTP had a negligible effect on amino acid incorporation.

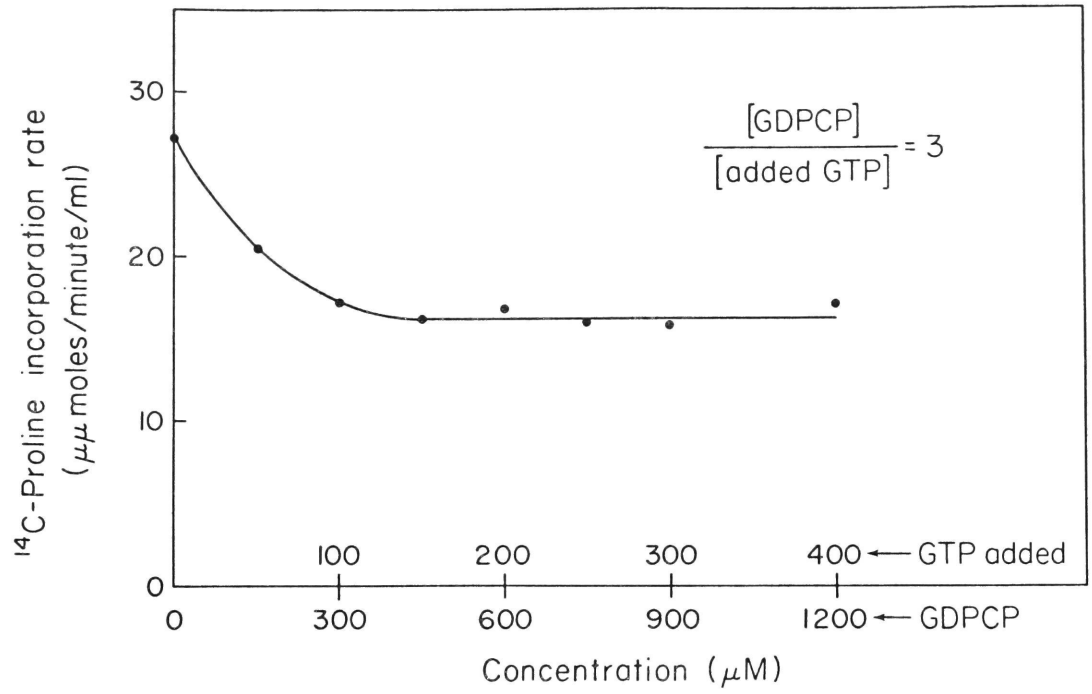

Figure 21. Independence of the extent of inhibition of protein synthesis by GDPCP from the concentration of "GTP-like" nucleotides at high nucleotide concentrations. Each reaction tube contained 56 μg/ml f2 RNA. The ratio of GDPCP to added GTP was the same in each reaction tube; only the total concentration was increased. At low concentrations, the endogenous GTP altered the ratio in favor of GTP, and consequently, in favor of a higher rate of protein synthesis. However, at higher concentrations, the contribution of the endogenous GTP was negligible, and the rate of protein synthesis was not effected by the increasing concentration of GTP-like molecules.

be "wasted" binding the deceptively similar GDPCP molecules. Since binding, bonding, and translocation are sequential steps, the time it takes to complete each step will contribute to the overall time it takes to translate a codon. Since GDPCP extends the time it takes to complete translocation, GDPCP extends the overall time it takes to translate each codon, and thereby decreases the rate of mRNA translation.

A Model of GDPCP Inhibition

The following section describes a model designed to show why GDPCP might inhibit the translation of one type of mRNA to a greater extent than another. The model is based on three assumptions. The first is that GDPCP inhibits GTP hydrolysis, but has no effect on reactions in which GTP is a required cofactor. The second is that the time it takes to translate a codon will vary, depending on which type of codon is being translated. This is due to variations in the time of finding and binding an appropriate tRNA, as outlined in the last chapter. It may also be due to variations in the time of bonding, since there is some evidence that the time it takes to form a peptide bond may depend on which amino acids are to be joined (Monro et al., 1968). The third assumption is that the time it takes to hydrolyze a GTP molecule is, on the average, the same for all codons, under a given set of reaction conditions. This last assumption is reasonable, since, as far as is presently known, translocation does not require the recognition of which type of tRNA, or which type of codon, is involved.

In the presence of GDPCP, the time it takes to hydrolyze a GTP molecule will be extended. Since GTP hydrolysis takes the same time for all codons, the time of extension of GTP hydrolysis will also be the same for all codons. Therefore, the effect of GDPCP is to extend the time it takes to translate each codon by a uniform time period. The more GDPCP that is added, the longer will be this extension. However, the effect of this extension will not be the same for all codons. For example, when a "fast" codon is being translated, the binding and bonding steps require only a short time. Therefore, the extension of the time of translocation by GDPCP would substantially increase the overall time of translation. On the other hand, when a "slow" codon is being translated, binding and bonding take a long time, and the extension of the time of translocation by GDPCP would have only a small effect on the overall time of translation. Thus, GDPCP would affect fast codons to a greater extent than slow codons.

Figure 22, on page 62, diagrammatically illustrates the effect of GDPCP on the overall time of translation of two codons which take a different amount of time to be translated. The white arrows in the figure represent the time it takes to translate a codon in the absence of GDPCP. The shorter white arrows represent a fast codon and the longer white arrows represent a slow codon. The time of GTP hydrolysis, h, is the same for both codons. The effect of GDPCP is to extend the time of GTP hydrolysis. This extension is indicated by the crosshatched arrows. The more GDPCP added, the longer it takes to hydrolyze a GTP molecule. As can be seen, the fast codon is strongly inhibited as the GDPCP concentration is increased, since GTP hydrolysis takes a substantial portion of the overall translation time of the fast codon. On the other hand, the slow codon is affected to a lesser extent by the same concentrations of GDPCP, since GTP hydrolysis is only a small portion of the slow codon's overall translation time.

Characteristics of GDPCP Inhibition

Since each mRNA has its own characteristic codon composition, and since some codons are fast and some codons are slow, it is likely that the translation of different types of mRNA would be differentially sensitive to inhibition by GDPCP. Figure 23, on page 63, illustrates an experiment in which this hypothesis was tested directly. This figure shows the effect of increasing concentrations of GDPCP on the template activity of Xenopus RNA and f2 RNA. Percent inhibition was determined by comparing the amino acid incorporation rate in the presence of GDPCP to the amino acid incorporation rate in the absence of GDPCP. For both types of RNA, the percent inhibition increased as the GDPCP concentration was increased. Significantly, the incorporation in response to f2 RNA was more sensitive to the inhibitor than was the incorporation in response to Xenopus RNA. Three different f2 RNA concentrations were used. Incorporation rates were proportional to f2 RNA concentration, both in the presence and the absence of the inhibitor. The effect of any given concentration of GDPCP was to reduce all three incorporation rates by the same percentage. Thus, percent inhibition was not a function of mRNA concentration. In summary, the sensitivity of protein synthesis to GDPCP inhibition is independent of the mRNA concentration, but dependent on the type of mRNA.

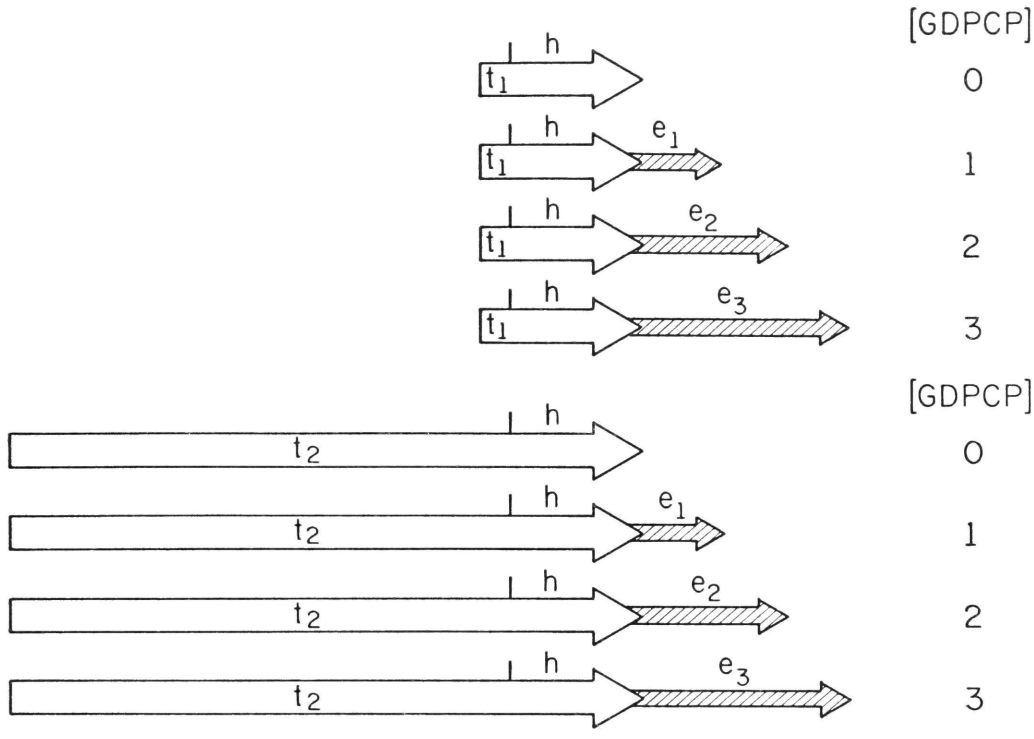

Figure 22. A model of GDPCP inhibition. The arrows represent the length of time required to translate a codon. The shorter white arrows represent the time it takes to translate a fast codon, t_1, and the longer white arrows represent the time it takes to translate a slow codon, t_2. The time of GTP hydrolysis, h, is the same for both codons. The time of GTP hydrolysis is extended by a time, e, in the presence of GDPCP, and is represented by the crosshatched arrows. The higher the GDPCP concentration (at a fixed GTP concentration), the longer is e. The time of translation of the fast codon is affected to a greater extent by GPDCP than the time of translation of the slow codon. For instance, it takes twice as long to translate the fast codon in the presence of 2 units of GDPCP, than it takes in the absence of GDPCP. On the other hand, it takes only 25% more time to translate the slow codon in the presence of 2 units of GDPCP, than it takes in the absence of GDPCP. Thus, the sensitivity of the translation rate to inhibition by GDPCP is a measure of the time it takes to translate a codon in the absence of the inhibitor.

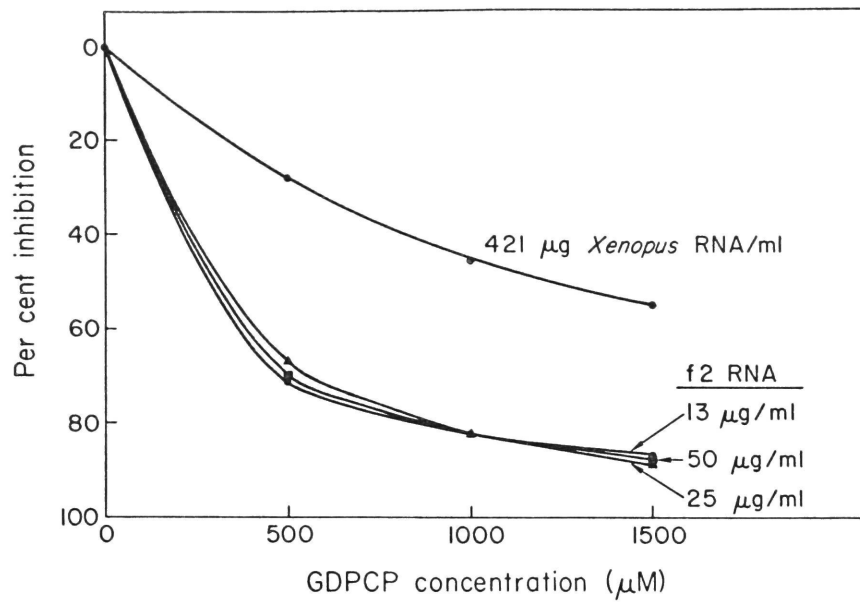

Figure 23. Effect of GDPCP on the template activity of Xenopus RNA and f2 RNA. The GTP concentration was the same in all the reaction tubes and was 200 μM. Percent inhibition was calculated by comparing the initial rate of amino acid incorporation in the presence of GDPCP to the initial rate of amino acid incorporation in the absence of GDPCP. The endogenous background at each GDPCP concentration was determined; and percent inhibition was calculated from the net incorporation due to the exogenous mRNA. The effectiveness of GDPCP is a function of the type of mRNA, but is not a function of the concentration of mRNA.

In order to demonstrate the nature of GDPCP inhibition, the same data were replotted according to the method of Dixon (1953). This is illustrated in Figure 24 on page 65. Since the lines representing the inhibition at three different f2 RNA concentrations meet on the abscissa, GDPCP fits one criterion of a non-competitive inhibitor of protein synthesis.

Non-competitive inhibition is observed when an enzyme requires both a substrate and a cofactor, which bind to separate enzymatic sites. If the binding of the cofactor to its site is blocked by an inhibitor, then the enzyme molecule is unable to convert the substrate into products. The effect of a non-competitive inhibitor is to lower the number of active enzyme molecules. This, in turn, lowers the reaction rate. Significantly, increasing the substrate concentration will not affect the number of inactivated enzyme molecules, since the substrate binds to a different site than the inhibitor. Therefore, the percent inhibition is independent of the substrate concentration.

The percent inhibition of protein synthesis by GDPCP is also independent of "substrate" concentration. Even though protein synthesis involves multiple sites on many enzymes, the effect of GDPCP can be explained in terms of the model outlined above. Thus, protein synthesis requires a substrate (mRNA) and a cofactor (GTP), both of which bind to separate enzymatic sites. mRNA is the substrate, in the sense that the rate of amino acid incorporation is a function of the mRNA concentration. GTP is the cofactor, since the reaction cannot proceed unless GTP is bound to a particular site on the G enzyme. Since GTP is hydrolyzed at this site, GDPCP cannot substitute for GTP. In fact, if GDPCP is bound at this site, mRNA-dependent protein synthesis will be inhibited. Thus, GDPCP reduces the number of enzyme molecules able to carry out a key step in protein synthesis. This inhibition is non-competitive, since an increase in the mRNA concentration does not affect the number of inactivated enzyme molecules. If the rate of GTP hydrolysis is observed, instead of the rate of protein synthesis, then the inhibition would appear to be competitive. However, if the rate of protein synthesis is observed (as was the case in Figures 23 and 24), then the inhibition would appear to be non-competitive. In summary, GDPCP is a competitive inhibitor of GTP hydrolysis, but is a non-competitive inhibitor of protein synthesis.

All non-competitive inhibitions obey Haldane's (1930) equation:

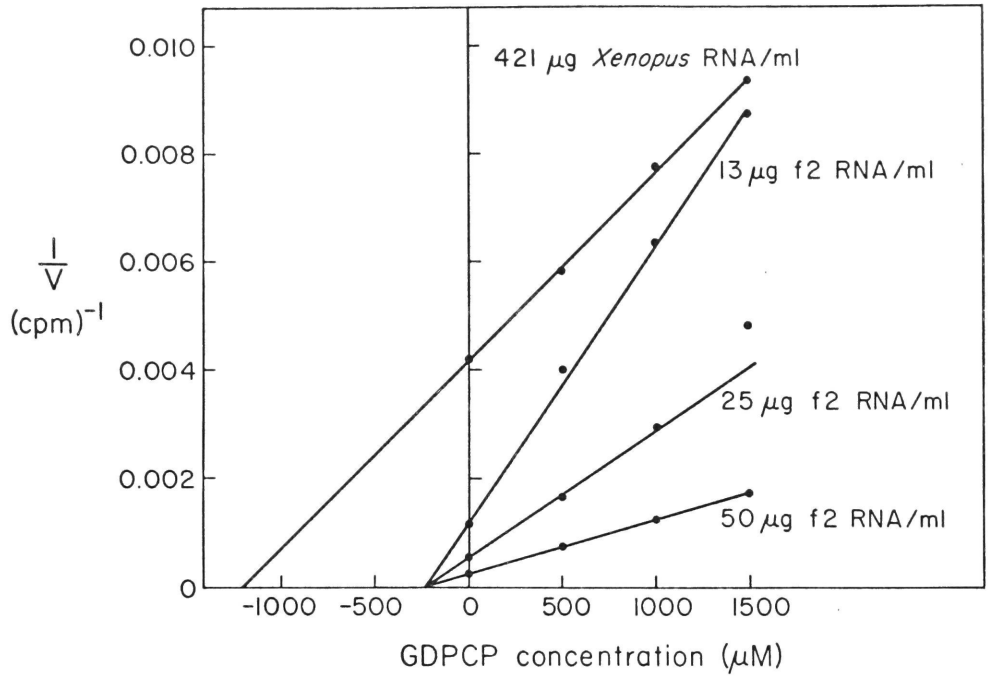

Figure 24. Demonstration that GDPCP is a non-competitive inhibitor of protein synthesis. The data from the experiment illustrated in Figure 23 were replotted according to the method of Dixon (1953). V is the initial rate of amino acid incorporation. Since the lines connecting the points at three different concentrations of f2 RNA meet on the abscissa, the inhibition is non-competitive.

$$[i] = K_i\left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 1}$$

V_o is the rate of the reaction in the absence of the inhibitor, and V is the rate of the reaction in the presence of the inhibitor. [i] is the concentration of the inhibitor, and K_i is the "inhibitor constant." This equation can be derived from the assumptions of Michaelis and Menten (see Friedenwald and Maengwyn-Davies, 1954). Also, the equation can be obtained from the reciprocal equation for non-competitive inhibition (Dixon, 1953) and the reciprocal equation for the uninhibited reaction (Lineweaver and Burk, 1934). Thus,

$$\frac{1}{V} = \left(\frac{K_m}{V_{max}} \cdot \frac{1}{[s]} + \frac{1}{V_{max}}\right) \cdot \left(1 + \frac{[i]}{K_i}\right) \qquad \text{Equation 2}$$

and,

$$\frac{1}{V_o} = \left(\frac{K_m}{V_{max}} \cdot \frac{1}{[s]} + \frac{1}{V_{max}}\right) \qquad \text{Equation 3}$$

[s] is the substrate concentration, V_{max} is the maximal reaction rate, and K_m is a constant. Dividing equation 2 by equation 3,

$$\frac{V_o}{V} = \left(1 + \frac{[i]}{K_i}\right)$$

which is algebraically equivalent to Haldane's equation (equation 1). This same equation was derived independently by Straus and Goldstein (1943) and Hunter and Downs (1945).

Since the inhibition of protein synthesis by GDPCP is non-competitive,

$$[GDPCP] = K_i\left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 4}$$

Equation 4 is algebraically equivalent to:

$$[GDPCP] = K_i\left(\frac{V_o}{V}\right) - K_i$$

which is the equation of a straight line. If [GDPCP] is plotted against $\left(\frac{V_o}{V}\right)$, the slope of the line will equal K_i.

The data of the experiment illustrated in Figures 23 and 24 were replotted, with [GDPCP] as the ordinate and $\left(\frac{V_o}{V}\right)$ as the abscissa. This is illustrated in Figure 25 on page 68. The slope of each line is equal to the inhibition constant, K_i. The slope of the line obtained with Xenopus RNA is higher than the slopes of each of the lines obtained with f2 RNA. However, the slopes of the lines at three different concentrations of f2 RNA were essentially the same. Thus, the inhibition constant is independent of the mRNA concentration, but dependent on the type of mRNA.

The inhibition constant may be interpreted as being equal to the concentration of inhibitor which causes a 50% inhibition, since, in equation 1, if $\left(\frac{V_o}{V}\right) = 2$, then, $[i] = K_i$. Therefore, it took more GDPCP to halve the template activity of Xenopus RNA, than it did to halve the template activity of f2 RNA. In terms of the model, illustrated in Figure 22, this would mean that the codons of Xenopus mRNA take a longer time to be translated, on the average, than the codons of f2 RNA.

A Mathematical Description of GDPCP Inhibition

The relationship between K_i and the time it takes to translate a codon can be expressed mathematically. Let t be the overall time it takes to translate a particular codon; let h be the time it takes to carry out GTP hydrolysis; and let e be the time of extension of GTP hydrolysis in the presence of GDPCP. Two relationships can be expressed.

The first is that the ratio of the time of hydrolysis in the presence of GDPCP, h + e, to the time of hydrolysis in the absence of GDPCP, h, is equal to the ratio of the concentration of GTP-like molecules to the concentration of GTP:

$$\frac{h + e}{h} = \frac{[GTP] + \alpha[GDPCP]}{[GTP]} \qquad \text{Equation 5}$$

(α is a constant, probably close to 1, which represents the relative affinity of GDPCP, compared to GTP, for the site of GTP hydrolysis). For example, it will take approximately five times as long for GTP hydrolysis to occur if only one out of every five GTP-like molecules is actually GTP. Equation 5 is algebraically identical to:

$$e = \alpha h \frac{[GDPCP]}{[GTP]} \qquad \text{Equation 6}$$

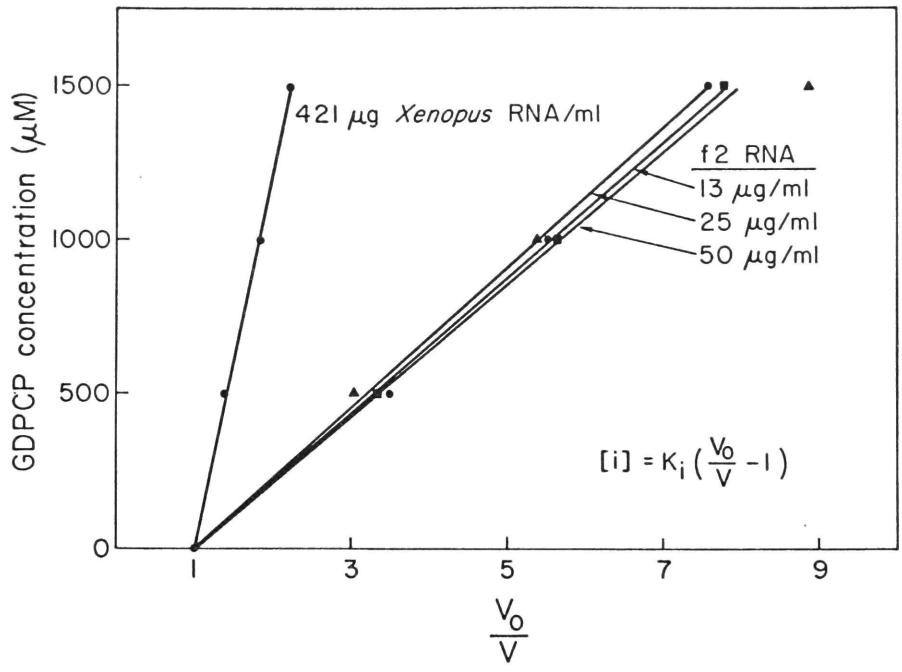

Figure 25. Determination of the K_i of GDPCP inhibition of protein synthesis. Since GDPCP is a non-competitive inhibitor of protein synthesis, $[GDPCP] = K_i\left(\frac{V_o}{V}\right) - K_i$, where V_o is the rate of protein synthesis in the absence of GDPCP, and K_i is equal to the concentration of GDPCP at which V is one half of V_o. K_i is therefore a measure of the sensitivity of the reaction to the inhibitor. The data from the experiment illustrated in Figures 23 and 24 were replotted in order to determine the inhibition constant at each mRNA concentration. The slope of each line is equal to the inhibition constant. This figure demonstrates that K_i is independent of the mRNA concentration, but is dependent on the type of mRNA. The higher slope obtained with Xenopus RNA indicates that protein synthesis in response to Xenopus RNA is less sensitive to inhibition by GDPCP than is protein synthesis in response to f2 RNA. GDPCP is less effective with Xenopus RNA, since, on the average, Xenopus codons take longer to be translated than f2 codons, and therefore require more time to complete steps other than GTP hydrolysis.

The second relationship is that the ratio of the overall time it takes to translate the codon in the presence of GDPCP, t + e, to the overall time it takes to translate the codon in the absence of GDPCP, t, is equal to the ratio of the rate of amino acid incorporation in the absence of GDPCP, V_o, to the rate of amino acid incorporation in the presence of GDPCP, V:

$$\frac{t + e}{t} = \frac{V_o}{V} \qquad \text{Equation 7}$$

For example, if the overall time of translation is three times as long in the presence of the inhibitor, then the uninhibited reaction rate will be three times as fast as the inhibited reaction rate. Equation 7 is algebraically identical to:

$$e = t\left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 8}$$

Setting the two expressions for e equal(equations 6 and 8):

$$\alpha h \frac{[GDPCP]}{[GTP]} = t\left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 9}$$

Equation 9 is algebraically identical to:

$$[GDPCP] = \left(\frac{[GTP]}{\alpha h}\right) t \left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 10}$$

Comparing Haldane's equation (equation 4) with equation 10, it can be seen that:

$$K_i = \left(\frac{[GTP]}{\alpha h}\right) t$$

Since [GTP], α, and h are constants, for a given set of experimental conditions, K_i is linearly proportional to t. This derivation is illustrated in Figure 26 on page 70.

This derivation is only applicable to the translation of one codon. However, mRNA's are composed of many codons, each of which has its own characteristic time of translation, under a given set of reaction conditions. Therefore, the derivation was revised to show that in the translation of a mRNA, K_i is linearly proportional to $\bar{t}$, which is the average time it takes to translate a codon of the mRNA. Figure 27, on page 71, illustrates the derivation of this mathematical relationship.

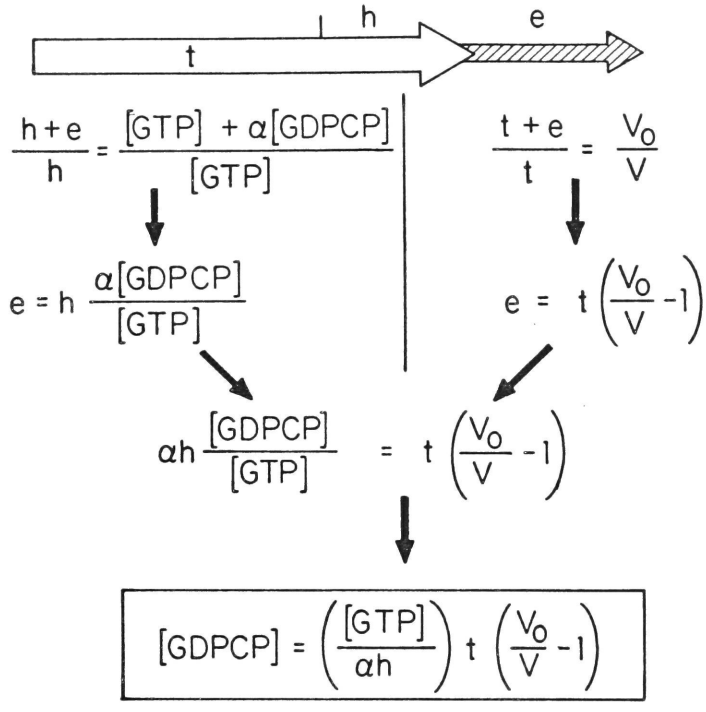

Figure 26. Derivation of an equation which describes the effect of GDPCP on the translation of a single codon. The white arrow represents t, the time it takes to translate the codon in the absence of GDPCP. h is the time it takes to hydrolyze a molecule of GTP in the absence of GDPCP. The crosshatched arrow represents e, the time of extension of GTP hydrolysis in the presence of GDPCP. α is a constant, probably close to 1, which represents the relative affinity of GDPCP, compared to GTP, for the site of GTP hydrolysis. V_o is the rate of translation in the absence of GDPCP, and V is the rate of translation in the presence of GDPCP.

The Haldane equation for non-competitive inhibition predicts that:

$$[GDPCP] = K_i \left(\frac{V_o}{V} - 1\right)$$

Therefore,

$$K_i = \left(\frac{[GTP]}{\alpha h}\right) t$$

Since [GTP], α, and h are constants, K_i is proportional to t.

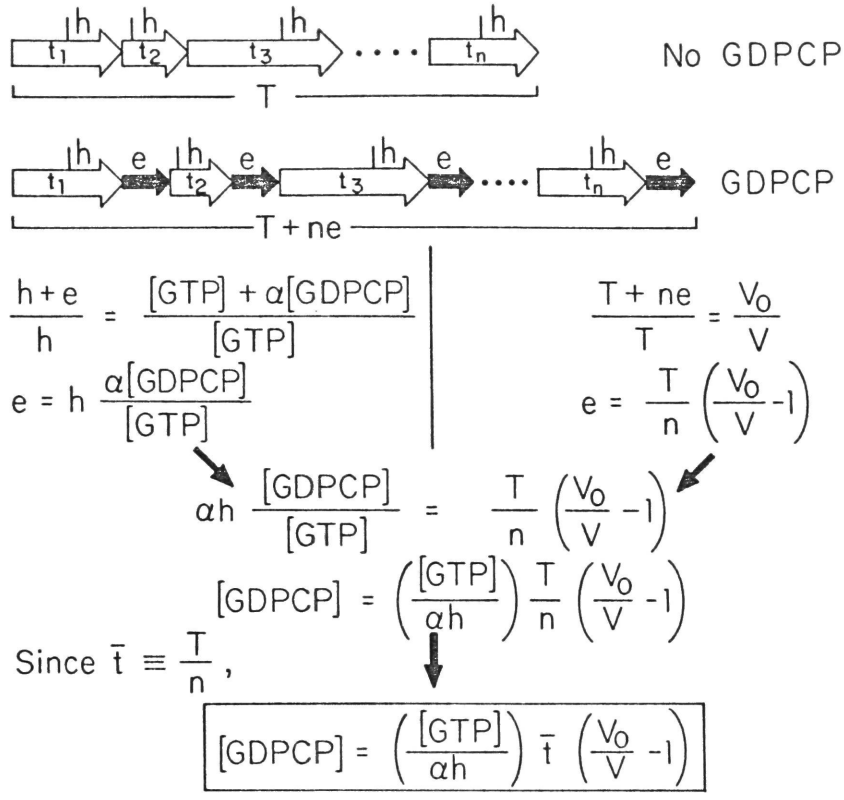

Figure 27. Derivation of an equation which describes the effect of GDPCP on the translation of many codons in an entire mRNA. The mRNA consists of n codons, each of which takes a different time to be translated (t_1, t_2, ..., t_n). h, the time it takes to hydrolyze a GTP molecule, is the same for each codon. Therefore, in the presence of GDPCP, the time it takes to translate each codon is extended by the same period of time, e. The time it takes to translate the entire mRNA in the absence of GDPCP is T, and the time it takes to translate the entire mRNA in the presence of GDPCP is T + ne. $\bar{t}$ is the average time it takes to translate each codon.

The final question is identical with the equation describing the effect of GDPCP on the translation of a single codon (see Figure 26), except that $\bar{t}$ replaces t. K_i is now proportional to $\bar{t}$. Therefore, when [GDPCP] is plotted against $\left(\frac{V_o}{V}\right)$, as in Figure 25, the slope of the resulting line is proportional to $\bar{t}$.

Assume that a mRNA is composed of n codons, each of which may take a different time to be translated. Let the total time for the translation of the entire mRNA be T. The time it takes to hydrolyze a molecule of GTP in the absence of GDPCP, h, is the same for each codon. In the presence of GDPCP, the time of GTP hydrolysis, for each codon, will be extended by a time, e. Therefore, in the presence of GDPCP, the time it takes to translate the mRNA will be T + ne.

Since h is the same for each codon, the first expression for e (equation 6), derived for the effect of GDPCP on the translation of a single codon, is still applicable. Thus,

$$e = \alpha h \frac{[GDPCP]}{[GTP]} \qquad \text{Equation 11}$$

However, the second expression (equation 7) can be revised to relate the time it takes to translate the entire mRNA in the presence of GDPCP, T + ne, to the time it takes to translate the entire mRNA in the absence of GDPCP, T. Thus,

$$\frac{T + ne}{T} = \frac{V_o}{V} \qquad \text{Equation 12}$$

Equation 12 is algebraically identical to:

$$e = \frac{T}{n}\left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 13}$$

Setting the two expressions for e equal (equations 11 and 13):

$$\alpha h \frac{[GDPCP]}{[GTP]} = \frac{T}{n}\left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 14}$$

Equation 14 is algebraically identical to:

$$[GDPCP] = \left(\frac{[GTP]}{\alpha h}\right)\frac{T}{n}\left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 15}$$

The term $\left(\frac{T}{n}\right)$ is the total time it takes to translate the entire mRNA divided by the number of codons composing the mRNA. This is, by definition, equal to the average time it takes to translate a codon, $\bar{t}$. Therefore,

$$\frac{T}{n} = \bar{t}$$

Substituting $\bar{t}$ for $\left(\frac{T}{n}\right)$ in equation 15:

$$[GDPCP] = \left(\frac{[GTP]}{\alpha h}\right) \bar{t} \left(\frac{V_o}{V} - 1\right) \qquad \text{Equation 16}$$

Again, comparing equation 16 with equation 4, it can be seen that K_i is linearly proportional to $\bar{t}$. Significantly, $\bar{t}$ is independent of n, the number of codons in the mRNA.

Thus, the inhibition constant is a measure of the average time it takes to translate a codon in a mRNA. For example, in Figure 25, the slope of the line obtained with Xenopus RNA is about five times as great as the slope of the lines obtained with f2 RNA. Thus, Xenopus codons took, on the average, five times as long to be translated as did f2 codons. This is not surprising, since f2 phage normally infect E. coli, and there would be a selective pressure in favor of a viral codon composition which is translated efficiently in the E. coli protein synthesis system.

In summary, the sensitivity of mRNA-dependent protein synthesis to inhibition by GDPCP is a direct measure of the average time it takes to complete one turn of the polypeptide chain elongation cycle. This average cycle time is different for different mRNA's. Thus, the rate of protein synthesis depends on the codon composition of the mRNA being translated.

Possibility of GTP Hydrolysis Occurring between Binding and Bonding

GTP is hydrolyzed during translocation. Recent evidence indicates that GTP may also be hydrolyzed during a reaction which occurs after the binding of amino-acyl tRNA, but before peptide bond formation. It was observed that, if GTP is replaced by GDPCP, T-dependent binding occurs, but the formation of the next peptide bond does not (Skogerson and Moldave, 1968a; Haenni and Lucas-Lenard, 1968). Moreover, the amino-acyl tRNA, bound to the acceptor site in the presence of GDPCP, does not interfere with the puromycin release of a peptidyl-tRNA analogue from the donor site (Haenni and Lucas-Lenard, 1968). This result suggested that GTP hydrolysis is required to align the amino-acyl end of the amino-acyl tRNA with the active site of peptidyl transferase. At present, this role for GTP is speculative, and experiments are in progress to find out more about this specific requirement for GTP (Lipmann, 1969).

However, the occurrence of a second GTP hydrolysis during each chain elongation cycle would not alter the relationship between K_i and $\bar{t}$, provided that the time it takes for this hydrolysis is approximately the same for each codon. It can be shown that even if two independent GTP hydrolyses occur, K_i will be linearly proportional to $\bar{t}$. Figure 28, on page 75, illustrates this derivation for the translation of a single codon. The final equation was obtained in the same manner as the equation for the effect of GDPCP on the translation of a single codon in which only one GTP hydrolysis occurs (see Figure 26), except that the extension of the time of translation in the presence of GDPCP is composed of two periods, e and e', instead of one. Comparing the final equation in Figure 28 with Haldane's equation (equation 4), and assuming that the times it takes to hydrolyze each GTP molecule in the absence of GDPCP, h and h', are constants, it can be seen that K_i is proportional to t. Moreover, in a manner analogous with the derivation illustrated in Figure 27, it can be shown that K_i is proportional to $\bar{t}$, for the translation of an entire mRNA, in which two GTP hydrolyses occur during each chain elongation cycle. Therefore, if a second GTP hydrolysis does occur, and if it takes the same amount of time for each codon, the effectiveness of GDPCP inhibition will still be a measure of the average time it takes to translate a codon in a mRNA.

GTP Hydrolysis during Chain Initiation

Protein synthesis occurs on polysomes, each of which consists of a mRNA molecule with a number of ribosomes attached to it (Warner et al., 1963a). The ribosomes move along the mRNA as the translation proceeds (Goodman and Rich, 1963). When translation is completed, the finished protein is enzymatically released from the ribosome (Ganoza, 1966; Capecchi, 1967; Caskey et al., 1968a; Scolnick et al., 1968). The ribosome is then released from the mRNA (Webster and Zinder, 1969), and exists as a free "run-off" ribosome (Kohler et al., 1968). The ribosome may then be enzymatically dissociated into its larger and smaller subunits (Subramanian et al., 1968). Thus, both large and small ribosomal subunits are found free in a cellular subunit "pool" (Mangiarotti and Schlessinger, 1966). Ribosomes are re-formed on a mRNA molecule by the joining together of a larger and a smaller subunit, selected at random from the subunit pool (Schlessinger et al., 1967; Kaempfer et al., 1968). Thus, the initiation of translation involves the joining together of

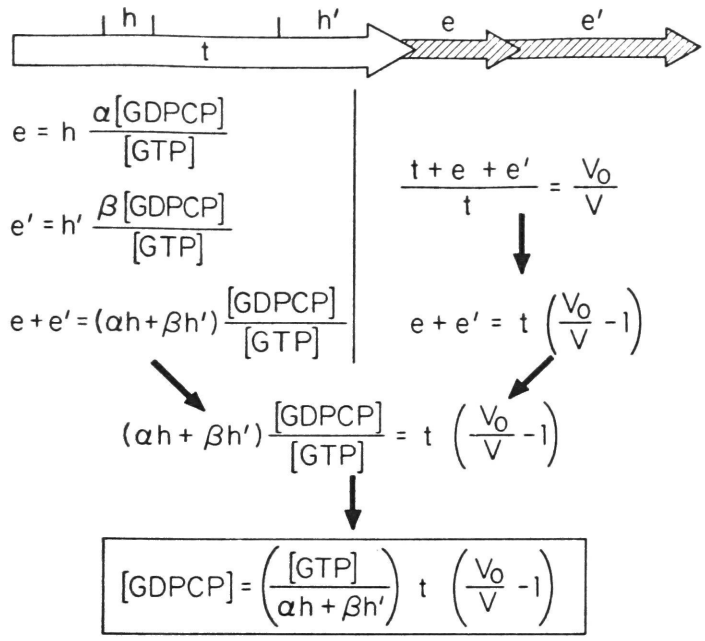

Figure 28. Derivation of an equation which describes the effect of GDPCP on the translation of a single codon, in which two independent periods of GTP hydrolysis occur. The two periods of GTP hydrolysis, h and h', need not be of the same duration, but each is assumed to take the same amount of time for all codons. In the presence of GDPCP, these periods are extended by the times e and e', respectively. α and β are constants which represent the affinity of GDPCP, compared to GTP, for the respective sites of GTP hydrolysis. The final equation is identical with the equation describing the effect of GDPCP on the translation of a codon in which only one GTP hydrolysis occurs (see Figure 26), except that $\alpha h + \beta h'$ replaces αh. Since [GTP], α, β, h, and h' are constants, K_i is still proportional to t. It can also be shown that for the translation of an entire mRNA:

$$[GDPCP] = \left(\frac{[GTP]}{\alpha h + \beta h'}\right) \bar{t} \left(\frac{V_o}{V} - 1\right)$$

Therefore, even if two periods of GTP hydrolysis occur during each chain elongation cycle, K_i would be linearly proportional to $\bar{t}$.

Preliminary experiments suggest that GDPCP has a very low affinity for the T enzyme (unpublished observations of Dr. Jean Lucas-Lenard). Thus β might be very small, and the observed inhibition would almost entirely be due to the effect of GDPCP on the time it takes to hydrolyze GTP during translocation.

two ribosomal subunits on a mRNA molecule (Nomura and Lowry, 1967). A number of distinct steps occur during this initiation process, and one of them requires the hydrolysis of a GTP molecule (Ohta et al., 1967; Anderson et al., 1967b; Takeda and Webster, 1968). This section discusses the effect of GDPCP on this initiation process.

At present, the following sequence of events is thought to occur during the initiation of protein synthesis in bacterial systems: The smaller ribosomal subunit (30s) attaches to the mRNA (Nomura and Lowry, 1967; Guthrie and Nomura, 1968) at the site of a particular initiator codon (Ghosh and Khorana, 1967). Then, a distinct tRNA, bearing an N-formyl-methionyl moiety (Marcker and Sanger, 1964; Marcker, 1965), binds to the 30s subunit (Nomura and Lowry, 1967; Hille et al., 1967; Ghosh and Khorana, 1967; Mukundan et al., 1968), in response to the specific initiator codon (Clark and Marcker, 1966a; Sundararajan and Thach, 1966; Salas et al., 1967; Ghosh et al., 1967). The larger ribosomal subunit (50s) then joins this initiation complex (Nomura et al., 1967; Mukundan et al., 1968; Kolakofsky et al., 1968a). At this stage, the formyl-methionyl tRNA is located at the acceptor site on the ribosome (Sarker and Thach, 1968; Ohta and Thach, 1968). The next step involves the translocation of the formyl-methionyl tRNA from the acceptor to the donor site (Takeda and Webster, 1968; Kolakofsky et al., 1968b). After this translocation has occurred, the next amino-acyl tRNA can bind to the acceptor site (Ohta et al., 1967; Ohta and Thach, 1968) and the first peptide bond can be formed. The translocation of the formyl-methionyl tRNA is thus the last step in the initiation sequence, and occurs prior to the repetitive steps of the polypeptide chain elongation cycle.

As a result of the initiation process, formyl methionine becomes the N-terminal amino acid of the growing polypeptide chain (Adams and Capecchi, 1966; Webster et al., 1966; Clark and Marcker, 1966a,b; Capecchi, 1966a). The formyl methionine is later cleaved from the polypeptide chain by a specific deformylase and an aminopeptidase (Fry and Lamborg, 1967; Adams, 1968; Takeda and Webster, 1968).

The reactions involved in the formation of the initiation complex are catalyzed by specific enzymes (Stanley et al., 1966; Eisenstadt and Brawerman, 1966; Revel and Gros, 1966), three of which have been isolated and termed F_1, F_2, and F_3 (Revel et al., 1968a; Iwasaki et al., 1968). F_3 binds to the

30s subunit and catalyzes the attachment of the subunit to mRNA (Eisenstadt and Brawerman, 1967; Brown and Doty, 1968; Revel et al., 1968a; Iwasaki et al., 1968). F_1 and F_2 catalyze the attachment of formyl-methionyl tRNA to the initiation complex (Salas et al., 1967; Allende and Weissbach, 1967; Leder and Nau, 1967; Revel et al., 1968b), and F_2 catalyzes the translocation of formyl-methionyl tRNA from the acceptor to the donor site (Kolakofsky et al., 1968b). The initiation enzymes, F_1 and F_2, are distinct from the chain elongation enzymes, T and G (Lucas-Lenard and Lipmann, 1967). The T enzyme catalyzes the binding of amino-acyl tRNA, after the translocation of formyl-methionyl tRNA has occurred (Ohta and Thach, 1968), and the G enzyme is not required until after the formation of the first peptide bond (Erbe and Leder, 1968). Thus, the translocation of formyl-methionyl tRNA is catalyzed by the F_2 enzyme and the translocation of peptidyl tRNA is catalyzed by the G enzyme.

GTP is required for the enzymatic binding of formyl-methionyl tRNA to the initiation complex (Allende and Weissbach, 1967; Leder and Nau, 1967; Hille et al., 1967), though it is not required for the enzymatic binding of the 30s subunit to mRNA (Anderson et al., 1967b). Since the binding of formyl-methionyl tRNA to the initiation complex is stimulated by GDPCP, as well as GTP (Anderson et al., 1967a; Ohta et al., 1967), GTP serves as a cofactor in the binding reaction. Formyl methionine bound in the presence of GDPCP can not be released by puromycin (Hershey and Thach, 1967; Anderson et al., 1967b; Takeda and Webster, 1968), indicating that the formyl-methionyl tRNA is bound at the acceptor site (Bretscher and Marcker, 1966). However, if GTP is used, instead of GDPCP, formyl methionine can be released by puromycin (Hershey and Thach, 1967; Anderson et al., 1967b; Mukundan et al., 1968; Takeda and Webster, 1968), indicating that the formyl-methionyl tRNA is now bound at the donor site. Thus, a GTP hydrolysis occurs after the binding of the formyl-methionyl tRNA, but before the formation of the first peptide bond (Thach et al., 1967). Since the 50s subunit can bind to the initiation complex in the presence of GDPCP (Kolakofsky et al., 1968a), GTP is hydrolyzed during the subsequent translocation step. Moreover, since GTP binds to the F_2 enzyme (Allende and Weissbach, 1967), and since the F_2 enzyme displays ribosome-dependent GTPase activity (Kolakofsky et al., 1968b), the F_2 enzyme is responsible for translocation of formyl-methionyl tRNA from the acceptor to the donor site. GTP hydrolysis, therefore, occurs during the last step in the initiation sequence, after both ribosomal subunits have been bound to the mRNA.

In general, the addition of GDPCP to an in vitro protein synthesis system, containing GTP, increases the time it takes to complete GTP hydrolysis. Therefore, GDPCP should act as a competitive inhibitor, increasing the time it takes to hydrolyze GTP during the F_2-catalyzed translocation which occurs during initiation. Since this translocation occurs after the binding of the ribosomal subunits, it does not seem likely that GDPCP would cause the subunits to dissociate from the mRNA. Thus, GDPCP should only affect the time of GTP hydrolysis.

GDPCP extends the time of every GTP hydrolysis which occurs. Thus, GDPCP probably increases the time it takes to hydrolyze GTP during initiation, as well as during each chain elongation cycle. The effect of GDPCP on the GTP hydrolysis which occurs during initiation could be greater or lesser than its effect on each GTP hydrolysis which occurs during chain elongation. However, there is only one GTP hydrolysis during chain initiation, while there are many GTP hydrolyses during chain elongation. Therefore, in the presence of GDPCP, the time of extension of the first GTP hydrolysis will be very small, when compared to the sum of the times of extension of the many GTP hydrolyses which occur during chain elongation. Thus, the inhibition of the one GTP hydrolysis associated with chain initiation should have only a negligible effect on the relationship between K_i and $\bar{t}$; and the effect of GDPCP on protein synthesis should be due, almost entirely, to an increase in the time of chain elongation.

Effect of Mg^{++} Concentration on Inhibition by GDPCP

When [GDPCP] is plotted against $\left(\frac{V_o}{V}\right)$, as in Figure 25, the result is a straight line. This indicates that GDPCP is only affecting the rate of chain elongation. The line would have been curved, if GDPCP had also caused the ribosomal subunits to dissociate during initiation, since this would have resulted in a reduction in the number of chains elongated, as well as in the rate of chain elongation. Figure 29, on page 79, illustrates an experiment in which the effect of GDPCP on the template activity of f2 RNA was determined at three concentrations of magnesium. At 12 and 16 mM Mg^{++}, the plot relating [GDPCP] to $\left(\frac{V_o}{V}\right)$ is a straight line, indicating that GDPCP is not affecting the number of chains initiated. Thus, the effect of GDPCP on protein synthesis at 12 and 16 mM Mg^{++} is confined to the inhibition of the rate of chain elongation.

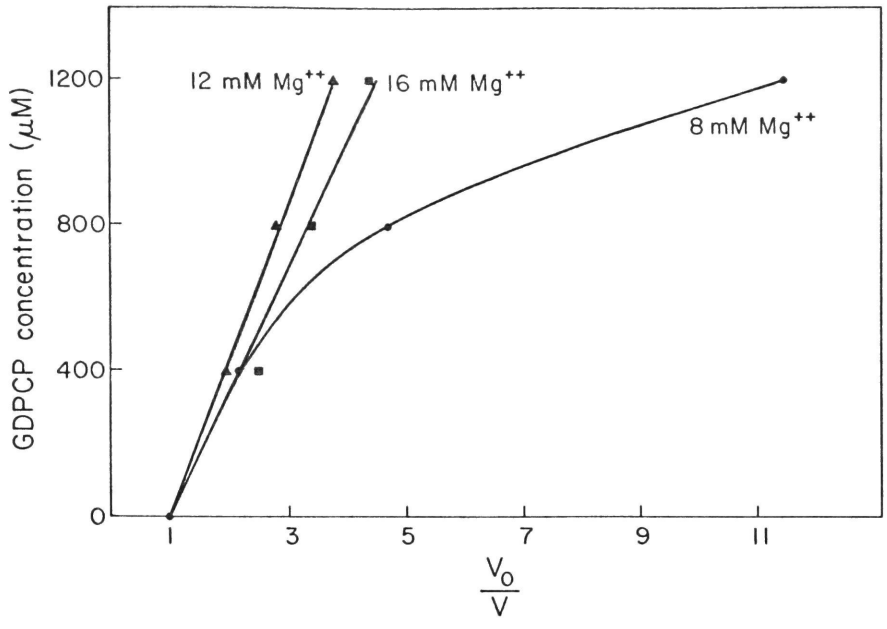

Figure 29. Effect of Mg^{++} concentration on inhibition by GDPCP. Reaction mixtures contained 50 μg/ml f2 RNA and the GTP concentration was 200 μM. The straight lines obtained at 12 and 16 mM Mg^{++} indicate that the effect of GDPCP was confined to decreasing the rate of chain elongation. The curve obtained at 8 mM Mg^{++} was caused by the additional inhibition of chain initiation, as the GDPCP concentration was increased. Since the other GDPCP experiments described in this dissertation were performed at 13 mM Mg^{++}, the inhibitions observed were probably due only to the effect of GDPCP on chain elongation.

At 8 mM Mg^{++} a curve is observed, suggesting that at low Mg^{++} concentrations GDPCP may have affected the number of chains initiated. This effect might be due to the greater tendency of ribosomes to dissociate at Mg^{++} concentrations below 10 mM (Ohta et al., 1967). It has been demonstrated that ribosomes bearing a peptidyl tRNA in the donor site are more stable at low Mg^{++} concentrations than ribosomes which lack a peptidyl tRNA in the donor site (Schlessinger et al., 1967; Ron et al., 1968). Moreover, 50s subunits derived from polysomes (thus bearing a peptidyl tRNA) will spontaneously reassociate with a 30s subunit at 10 mM Mg^{++} (Schlessinger et al., 1967), but 50s subunits derived from "run-off" ribosomes (thus lacking a peptidyl tRNA) will not reassociate with a 30s subunit at 10 mM Mg^{++} (Mangiarotti and Schlessinger, 1966). Thus, below 10 mM Mg^{++}, ribosomes are intrinsically unstable, unless a peptidyl tRNA acts as a clasp to hold the subunits together. Formyl-methionyl tRNA is an analogue of peptidyl tRNA (Nakamoto and Kolakofsky, 1966). During initiation at low Mg^{++} concentrations, the formyl-methionyl tRNA, bound at the donor site, probably serves to hold the two ribosomal subunits together. The binding of the formyl-methionyl tRNA to the donor site requires translocation. In the presence of GDPCP, it takes a longer period of time for this translocation to occur. Therefore, at low Mg^{++} concentrations, a delay in the time of translocation would increase the probability that the unstable initiation complex would dissociate. Thus, at magnesium concentrations at which ribosomal subunits tend to dissociate, the GDPCP-induced delay in the translocation of formyl-methionyl tRNA to the donor site could decrease the number of chains initiated. The experiments to be described in the remainder of this dissertation were performed at 13 mM Mg^{++}. Under these conditions, the plot relating [GDPCP] to $\left(\frac{V_o}{V}\right)$ is a straight line. Therefore, at 13 mM Mg^{++}, GDPCP would not significantly affect chain initiation. Thus, the effectiveness of GDPCP inhibition is essentially a measure of the rate of polypeptide chain elongation.

Discussion

This chapter has explored the effect of GDPCP on protein synthesis. It has been shown that GDPCP increases the time it takes to hydrolyze a GTP molecule. As a consequence of this increase in the time of GTP hydrolysis, the overall time of translation is increased. The effect of GDPCP as an inhibitor of protein synthesis is a measure of the proportion of the overall

time of translation devoted to GTP hydrolysis. Since the time required for GTP hydrolysis is probably the same for all codons, the effect of GDPCP on the overall time it takes to translate a codon will depend on the time required to carry out the other steps which do not require GTP hydrolysis. GDPCP will be an effective inhibitor if these other steps are short, and will be a less effective inhibitor if these other steps are long. Therefore, the effectiveness of GDPCP inhibition is a measure of the average time it takes to translate a codon in a mRNA. The next chapter will describe experiments designed to demonstrate that the differences observed in the effectiveness of GDPCP inhibition on the translation of different mRNA's are due to differences in the codon composition of each mRNA; and it will also be shown that GDPCP has a differential effect on the translation rate of individual codons.

CHAPTER FIVE: TRANSLATION RATE AS A FUNCTION OF CODON COMPOSITION

GDPCP has a differential effect on the translation of mRNA's obtained from different sources. Presumably, the significant difference between two heterologous mRNA's is their dissimilar codon composition. However, other factors might be responsible for the observed differences in the effect of GDPCP. This chapter describes experiments which demonstrate that the differential effect of GDPCP is a function of the type of codons being translated. The influence of factors other than codon composition was controlled by observing the effect of GDPCP on the synthesis of different polypeptides whose sequences were encoded by the same mRNA. It will be shown that GDPCP differentially affects the synthesis of two different proteins encoded by different regions of one polycistronic mRNA molecule; and that GDPCP differentially affects the synthesis of two different homopolypeptides encoded by the same regions of a synthetic ribopolynucleotide containing a repeating trinucleotide sequence.

Differential Effect of GDPCP on the Translation of Two Cistrons in the Same mRNA

f2 is one of a closely related group of small bacteriophages which consist of a single RNA strand of approximately 1,000,000 Daltons (Strauss and Sinsheimer, 1963; Gesteland and Boedtker, 1964), surrounded by a protein shell constructed from a large number of identical subunits. The genome of these viruses is "polycistronic" (Ohtaka and Spiegelman, 1963), in the sense that it codes for three different phage-specific proteins (Horiuchi *et al.*, 1966; Gussin *et al.*, 1966; Lodish, 1968). These proteins are: a coat protein (Nathans *et al.*, 1962), a viral RNA-dependent RNA polymerase (Haruna *et al.*, 1963), and a "maturation" protein (Lodish and Robertson, 1969), which is a minor component of the protein shell (Steitz, 1968). The synthesis of each protein may be initiated independently (Lodish, 1968; Webster and Zinder, 1969). Both *in vivo* and *in vitro*, the three proteins are not synthesized in equal amounts. The coat protein is the predominant product (Ohtaka and Spiegelman, 1963; Nathans *et al.*, 1965), accounting for approximately 74% of the proteins synthesized *in vitro* (Lodish, 1968). The RNA polymerase and the

maturation protein account for approximately 22% and 4%, respectively, of the proteins synthesized *in vitro* (Lodish, 1968). Thus, the two main *in vitro* products are coat protein and RNA polymerase.

Since the coat protein has a molecular weight of approximately 14,000 Daltons and the viral RNA polymerase has a molecular weight of approximately 50,000 Daltons (Capecchi, 1966b), these two major viral products can be separated with the aid of acrylamide gel electrophoresis (Viñuela *et al.*, 1967; Sugiyama and Nakada, 1968). Figure 30, on page 84, illustrates the electrophoretic fractionation of the proteins synthesized *in vitro*, in response to f2 RNA. The predominant product is the relatively low molecular weight coat protein.

Since the major product of the *in vitro* system is coat protein, the incorporation of any amino acid into protein will reflect the synthesis of coat. Histidine is the one exception, since it is the only amino acid not found in the coat protein (Nathans *et al.*, 1962; Notani *et al.*, 1965). However, histidine is incorporated into other proteins, in response to viral RNA (Ohtaka and Spiegelman, 1963; Nathans *et al.*, 1965; Engelhardt *et al.*, 1967), and is found in the viral RNA polymerase (Capecchi, 1966b). Since RNA polymerase accounts for 85% of the non-coat protein synthesized, the incorporation of histidine will reflect the synthesis of RNA polymerase.

Figure 31, on page 85, illustrates an experiment with f2 RNA, which was designed to compare the effect of GDPCP on the translation of the polymerase cistron to its effect on the translation of the coat cistron. Histidine incorporation was used to monitor polymerase synthesis, and both threonine and proline incorporation were used to monitor coat synthesis. At each GDPCP concentration, the percent inhibition of histidine incorporation was greater than the percent inhibition of threonine or proline incorporation. Thus, GDPCP had a differential effect on the translation of the polymerase cistron, compared to the translation of the coat cistron.

The difference between two cistrons is structural, in the sense that the sequence of nucleotides composing them is different. The reason for the differential effect of GDPCP on the translation of different cistrons should be related to this structure. In view of the discussions in the previous chapters, it is likely that the critical structural difference between two cistrons is

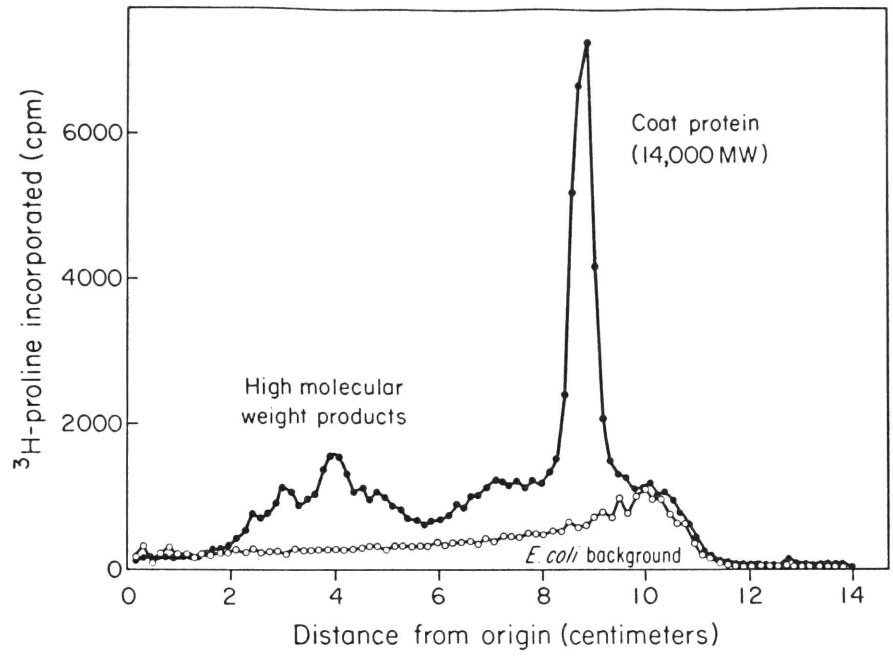

Figure 30. Acrylamide gel electrophoresis of f2 proteins synthesized in vitro. 251 μg/ml f2 RNA was added to the cell-free system and incubated for 10 minutes. A second reaction mixture, containing water, in place of f2 RNA, was also prepared and incubated. The reactions were stopped by the addition of 1/10 volume of glacial acetic acid. Sodium dodecyl sulphate salts of the reaction mixture proteins were prepared according to the method of Summers and his co-workers (1965), and dialyzed against a 0.01 M Na phosphate buffer, pH 7.4, containing 1/10% sodium dodecyl sulphate, 1/10% 2-mercaptoethanol, and 0.5 M urea. The proteins were then electrophoresed on 14 cm long, 10% acrylamide gels (Davis, 1964) in a 0.1 M Na phosphate buffer, pH 7.2, containing 1/10% sodium dodecyl sulphate, for 15 hours at a constant current of 5 mA/gel. Each gel was then mechanically pulverized and divided into approximately 90 fractions (Maizel, 1966). The fractions were counted in scintillation vials containing 0.15 ml water, 1.5 ml NCS (Amersham/Searle, Des Plaines, Ill.), and 15 ml of a toluene-based scintillation fluid containing 4 g/liter 2,5-diphenyloxazole and 50 mg/liter p-bis-[2-(5-phenyl-oxazolyl)]-benzene. Only the proteins synthesized during the incubation were labelled. Coat protein was the predominant product.

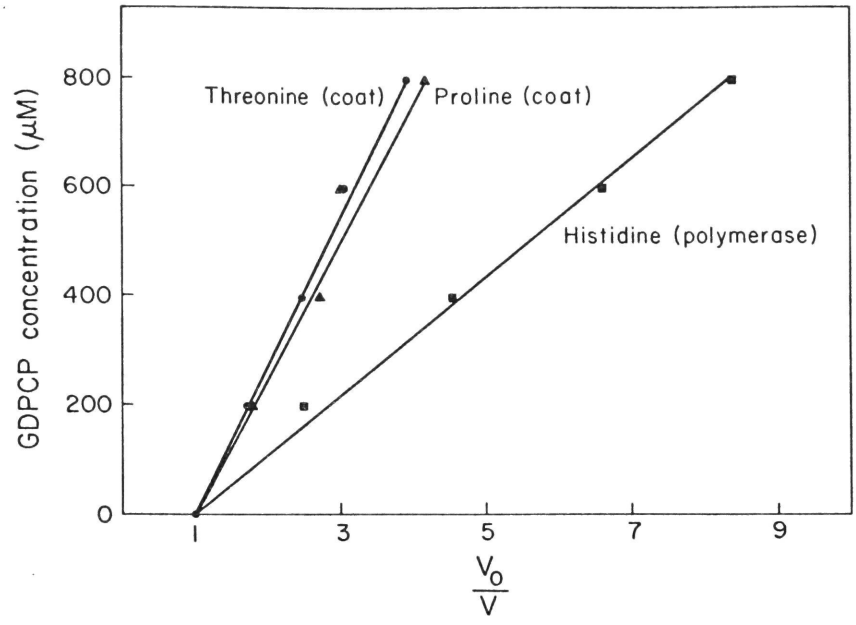

Figure 31. Differential effect of GDPCP on the synthesis of f2 RNA polymerase and f2 coat protein. Each reaction tube contained 78 μg/ml f2 RNA. Three parallel experiments were performed, differing only in respect to the identity of the labelled amino acid. Histidine incorporation reflects the synthesis of RNA polymerase; and threonine and proline incorporation reflect the synthesis of coat protein. Threonine incorporation is probably more indicative of coat synthesis than is proline incorporation, since each coat molecule contains nine threonine residues, as compared to six proline residues (Weber and Konigsberg, 1967). GDPCP has a greater inhibitory effect on the synthesis of polymerase than it does on the synthesis of coat. The higher slope of the lines associated with coat synthesis indicates that the average time it takes to add an amino acid to a growing coat protein molecule is longer than the average time it takes to add an amino acid to a growing RNA polymerase molecule. Thus, under these experimental conditions, polymerase is synthesized faster than coat. The difference in the rate of synthesis of these two proteins is probably due to the different codon compositions of their respective cistrons.

their differing codon composition. However, the experiment described in this section did not rule out the possibility that the secondary structure of the RNA, rather than the primary structure, was responsible for the differential effect of GDPCP. It is known that bacteriophage RNA's do possess considerable secondary structure (Strauss and Sinsheimer, 1963; Gesteland and Boedtker, 1964), and it is reasonable that this structure would differ from one cistron to another. In order to rule out the influence of secondary structure, it was necessary to study a mRNA in which different proteins are synthesized from the same part of the molecule. The next section describes an experiment performed with such a mRNA. The results of that experiment rule out the influence of secondary structure, and allow the conclusion to be drawn that codon composition is the critical factor influencing the effect GDPCP has on the translation of mRNA.

Differential Effect of GDPCP on the Synthesis of Different Homopolypeptides in Response to a Ribopolynucleotide Containing a Repeating Trinucleotide Sequence

"Poly r-UUG" is a high molecular weight ribopolynucleotide, containing the repeating trinucleotide sequence, uridylyl-uridylyl-guanylyl. This ribopolynucleotide can serve as a mRNA in the bacterial cell-free system. Three different homopolypeptide products are synthesized in response to this mRNA, depending on the "phase frame" in which translation occurs (Morgan et al., 1966). Thus, poly r-UUG may be translated as a long string of UUG codons, or as a long string of UGU codons, or as a long string of GUU codons. In general, mRNA's which possess the bacterial initiator codon, AUG, are only translated in the phase frame in which AUG is contained (Sundararajan and Thach, 1966). However, poly r-UUG does not code for an initiator codon, and the presence of formyl-methionyl tRNA and initiation enzymes has no effect on the translation of this mRNA (Ghosh et al., 1967). Thus, poly r-UUG may be translated in all three phase frames. Since UUG, UGU, and GUU are the codons for leucine, cysteine, and valine, respectively (Nirenberg et al., 1965; Söll et al., 1965), poly r-UUG stimulates the synthesis of homopolyleucine, homopolycysteine, and homopolyvaline. The key point here is that all three homopolypeptide products are synthesized from the same mRNA. Physically, the difference in the mRNA employed in the synthesis of each product is negligible. Functionally, however, the difference is very great, since a different subset of the protein synthesis

machinery is required to translate each of the three "homopolycodons." Thus, the significant difference in the synthesis of each homopolypeptide is the functional nature of the type of codon which specifies each product.

The effect of GDPCP on the synthesis of each of the homopolypeptides should reflect differences in the nature of the codons. Since each of the three homopolycodons possesses essentially the same secondary structure, a differential effect of GDPCP on the synthesis of each of the three homopolypeptides would not be due to the influence of secondary structure. Moreover, the effect of GDPCP on the synthesis of any one of the homopolypeptides (as measured by K_i) would be independent of the frequency with which that particular phase frame was selected, since the effectiveness of GDPCP inhibition is independent of mRNA concentration. Thus, a differential effect of GDPCP on the synthesis of the three homopolypeptides would indicate that this inhibition is a codon-specific effect; and, in particular, it would support the view that the effectiveness of GDPCP inhibition is a function of the time it takes to translate a codon.

The synthesis of poly r-UUG, as well as the synthesis of other defined mRNA's, is accomplished through a number of chemical and enzymatic steps (Khorana, 1965). The first steps entail the chemical synthesis of a double-stranded, oligodeoxyribonucleotide, whose nucleotide sequence is completely defined (Khorana et al., 1965). This is then used as a template for the enzymatic synthesis of a high molecular weight polydeoxyribonucleotide by E. coli DNA-dependent DNA polymerase (Byrd et al., 1965; Wells et al., 1965). If the chemically-synthesized, short-chain template contains a repeating trinucleotide sequence, the product of this enzymatic reaction will be a long-chain, double-stranded DNA, containing the same repeating trinucleotide sequence (Wells et al., 1967). These high molecular weight DNA's can be isolated, and may then be used as templates for the enzymatic synthesis of defined mRNA's by E. coli DNA-dependent RNA polymerase (Nishimura et al., 1965b). Thus, poly r-UUG may be enzymatically synthesized from the double-stranded DNA template: poly d-TTG:CAA (in which one strand contains the repeating deoxyribotrinucleotide sequence: thymidylyl-thymidylyl-deoxyguanylyl; and the other strand contains the repeating sequence: deoxycytidylyl-deoxyadenylyl-deoxyadenylyl).

Figure 32, on page 89, illustrates the design of a two-stage experiment, in which poly r-UUG was synthesized from a DNA template, and then used to stimulate the synthesis of three homopolypeptides in the S-30 system, in the presence of different concentrations of GDPCP. This experiment was based on a two-stage procedure which was described by Wood and Berg (1962), and later modified for use with synthetic DNA's (Nishimura et al., 1965b; Jones et al., 1966; Morgan et al., 1966). In the first stage of the experiment, the only nucleotides present were uridine triphosphate and guanosine triphosphate. Therefore, only the poly d-CAA strand could be used as a template in the synthesis of mRNA. In the second stage of the experiment, aliquots of the reaction mixture from Stage I, containing the newly-synthesized poly r-UUG, were added to the components of the S-30 system, and the effect of GDPCP on the resulting incorporation of leucine, cysteine, and valine was determined.

Poly r-UUG was selected as the template in this experiment for three reasons. Firstly, cytidine triphosphate would not be present during either stage of the experiment, and the transcription of the other DNA strand requires cytidine triphosphate; therefore, there was no possibility that the complementary polyribotrinucleotide (poly r-CAA) could be synthesized. Secondly, poly r-UUG does not contain a bacterial initiator or terminator codon. And lastly, all three homopolypeptide products are insoluble under the reaction conditions (Berger et al., 1956; Katchalski and Sela, 1958), and therefore would not be destroyed by proteases.

Figure 33, on page 90, illustrates the time course of the synthesis of poly r-UUG during Stage I. The amount of uridine incorporated was twice the amount of guanosine. In three hours, the amount of poly r-UUG synthesized was 8½ times the amount of DNA which served as template. As a control, a second reaction mixture was incubated during Stage I, in which the DNA template was omitted. No significant RNA synthesis occurred in the control. This preparation was used, in place of the poly r-UUG-containing preparation, to determine the background incorporation during Stage II.

In the second stage, three parallel experiments were performed, differing only in respect to the identity of the labelled amino acid. Unfortunately, cysteine incorporation could not be observed, as a result of the high background caused by the formation of disulfide bonds between labelled cysteine and the free sulfhydryl groups of the S-30 proteins. Figure 34, on page 91, illustrates a model reaction, in which it was shown that a 15-fold

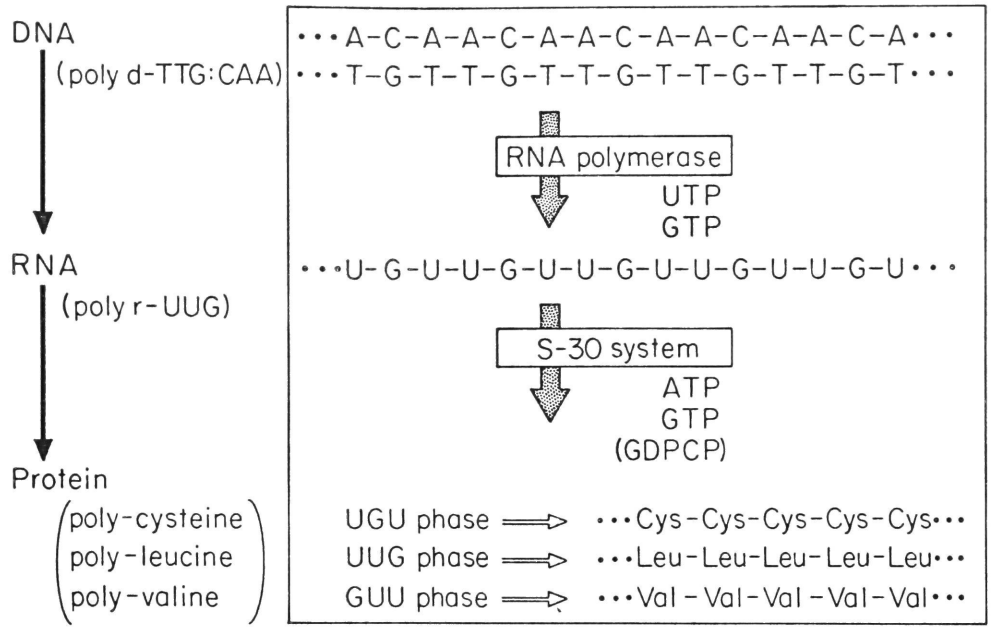

Figure 32. Two-stage synthesis of homopolypeptides. In the first stage of the experiment, poly r-UUG was synthesized from uridine triphosphate and guanosine triphosphate on a poly d-TTG:CAA template by E. coli DNA-dependent RNA polymerase. Since only two types of nucleotide were present, only one of the two DNA strands served as a template. In the second stage of the experiment, a portion of the Stage-I reaction mixture was added to the components of the E. coli cell-free system. The poly r-UUG synthesized in Stage I served as a template for the synthesis of homopolypeptides in Stage II. Poly r-UUG can be translated in three phase frames, each of which results in a different homopolypeptide product. Thus, each of the homopolypeptides was specified by one of three "homopolycodons," which are all located on the same mRNA. The effect of GDPCP on the synthesis of each of these three products was determined by performing parallel experiments, differing only in respect to the identity of the labelled amino acid. The effect of GDPCP was independent of the frequency with which each phase frame was selected, since the effectiveness of GDPCP inhibition is independent of mRNA concentration. Therefore, this experiment provided a means of observing the effect of GDPCP on protein synthesis, independent of influences other than the functional nature of the codons which specify each polypeptide product.

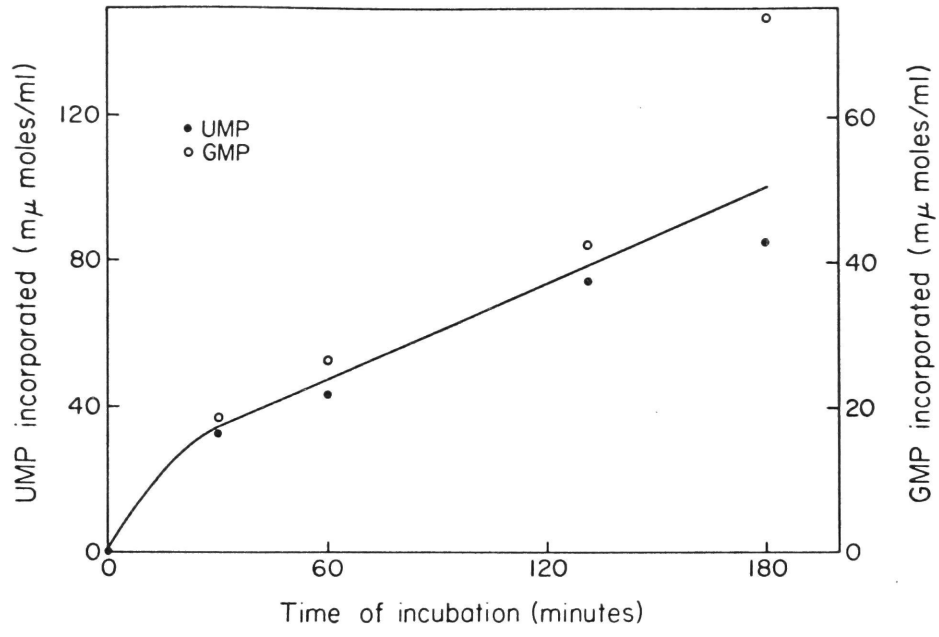

Figure 33. Synthesis of poly r-UUG by RNA polymerase in response to poly d-TTG:CAA. The reaction mixture contained 0.46 OD_{260} units/ml poly d-TTG:CAA, which was the gift of Dr. R. D. Wells. (This is equivalent to 18 mμmoles/ml of nucleotide residues located on the poly d-CAA strand of the DNA.) E. coli DNA-dependent RNA polymerase was the gift of Dr. A. R. Morgan, who isolated the enzyme complex by the method of Chamberlin and Berg (1962), and further purified it on Agarose 5M columns (Gallard-Schlesinger, Carle Place, N.Y.). The enzyme complex was then fractionated on a phosphocellulose column into two components: the "PC" enzyme and the "σ" subunit (Burgess et al., 1969; Travers and Burgess, 1969). The reaction mixture contained 250 μg/ml PC enzyme and 125 μg/ml σ subunit. The reaction was carried out in a 42.5 mM Tris-HCl buffer, pH 8.0, which contained 4 mM $MgCl_2$, 1 mM $MnCl_2$, 12 mM 2-mercaptoethanol, 10% glycerol, 426 μM uridine triphosphate, and 212 μM guanosine triphosphate. The reaction mixture was incubated at 37°C for 3 hours. Two parallel, small-scale reactions were also carried out, in which one of the two nucleotides was labelled with carbon-14. At various time intervals, aliquots of each reaction mixture were absorbed onto Whatman 3MM filter paper discs and washed free of precursors with 5% TCA at 0°C. The discs were dehydrated in a 1:1 (v:v) mixture of ethyl ether and ethanol, immersed in ethyl ether, dried, and counted in a Packard TriCarb scintillation counter. The amount of uridine incorporated was twice the amount of guanosine incorporated.

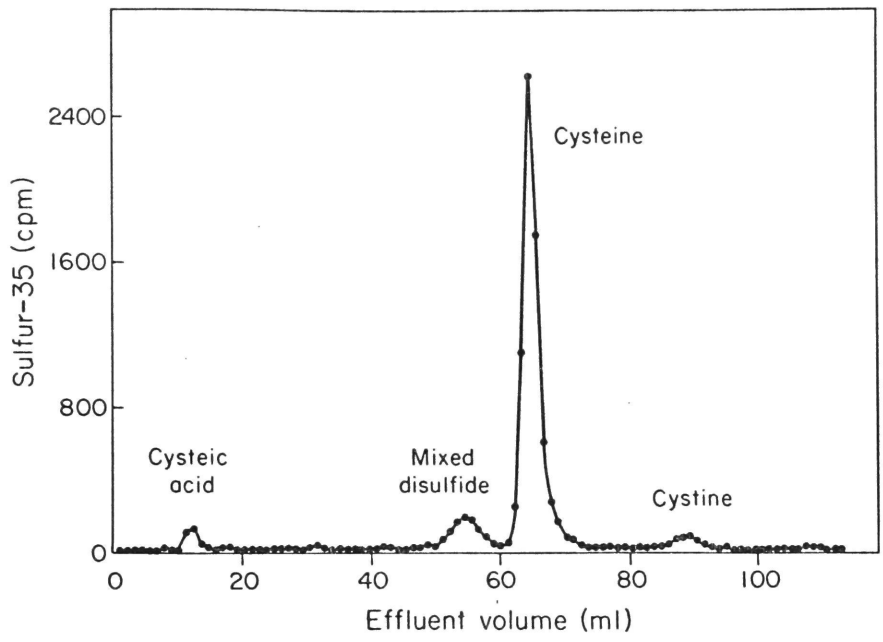

Figure 34. Chromatographic analysis of the reaction products of the reduction of ^{35}S-cystine by glutathione. A pH 7.8 reaction mixture, containing 2 mM ^{35}S-cystine and 30 mM reduced glutathione, was allowed to react for 10 minutes at 23°C. An aliquot of the reaction mixture was analyzed by ion exchange chromatography at pH 3.28 (Moore et al., 1958) on a Beckman 120B amino acid analyzer (Spackman et al., 1958). The radioactivity of each peak was determined by passing the effluent from the analyzer column through a solid-state flow cell in a Nuclear Chicago scintillation counter. The amino acid peaks were identified by their elution sequence and by their characteristic spectra after reaction with ninhydrin (Moore and Stein, 1948). One of the reaction products was identified as a mixed disulfide of cysteine and reduced glutathione (Spackman et al., 1960). The reduction did not go to completion and a substantial amount of cystine and mixed disulfide was observed. The occurrence of these disulfides could be predicted from the known equilibrium constants of the reaction of glutathione with cystine (Kolthoff et al., 1955). When ^{14}C-cysteine is used as a label in an in vitro protein synthesis system, a very high endogenous background is observed (Khorana et al., 1966). Had this high background been due to the metabolic conversion of cysteine to serine and other ^{14}C-amino acids (via pyruvate), the use of ^{35}S-cysteine would have resulted in a much lower background. However, ^{35}S-cysteine was used in the poly r-UUG experiment, and the background was very high. In light of the model reaction illustrated above, it seems reasonable that this high background was due to the formation of disulfide bonds between the labelled cysteine and the free sulfhydryl groups of the S-30 proteins.

excess of reduced glutahione over cystine (which is similar to the reducing conditions of Stage II) was not sufficient to decrease the proportion of disulfide compounds in the reaction mixture to an acceptable minimum. Fortunately, no problems were encountered with the incorporation of leucine or valine.

Figure 35, on page 93, illustrates the effect of GDPCP on the incorporation of leucine and valine in response to poly r-UUG, during Stage II. As can be seen, GDPCP had a differential effect on the synthesis of homopolyleucine, compared to the synthesis of homopolyvaline. Since both homopolypeptide products were synthesized from homopolycodons occurring on the same physical template, and since GDPCP had a differential effect on the translation of each homopolycodon, it can be concluded that the inhibitory effect of GDPCP is codon-specific. Since GDPCP only inhibits those codon translation steps which require GTP hydrolysis, it is likely that the codon-specific property that determines the sensitivity to GDPCP inhibition is the length of time it takes to perform the codon translation steps which do not require GTP hydrolysis. Therefore, the observation that the effect of GDPCP is codon-specific supports the view that the time of translation of each codon varies, and supports the conclusion that the rate of translation of a mRNA depends on its codon composition.

Discussion

Two sets of observations have led to the conclusion that template activity is a function of codon composition. The first is that the relative specific template activity of different mRNA's is a function of the reaction conditions. The second is that GDPCP has a differential effect on the translation of different mRNA's, although it probably increases the translation time of all codons by the same amount of time. Moreover, GDPCP has a differential effect on the translation of different cistrons within the same mRNA, and it has a differential effect on the translation of different homopolycodons within the same "cistron." Therefore, it seems likely that each codon has a characteristic time of translation, under a given set of reaction conditions. Since different mRNA's are composed of different codons, the template activity of each mRNA is a function of its codon composition.

Most probably, the cause of the variation in codon translation times is the availability of the tRNA species which recognize each codon. It is well established that different tRNA species occur in the cell in widely varying

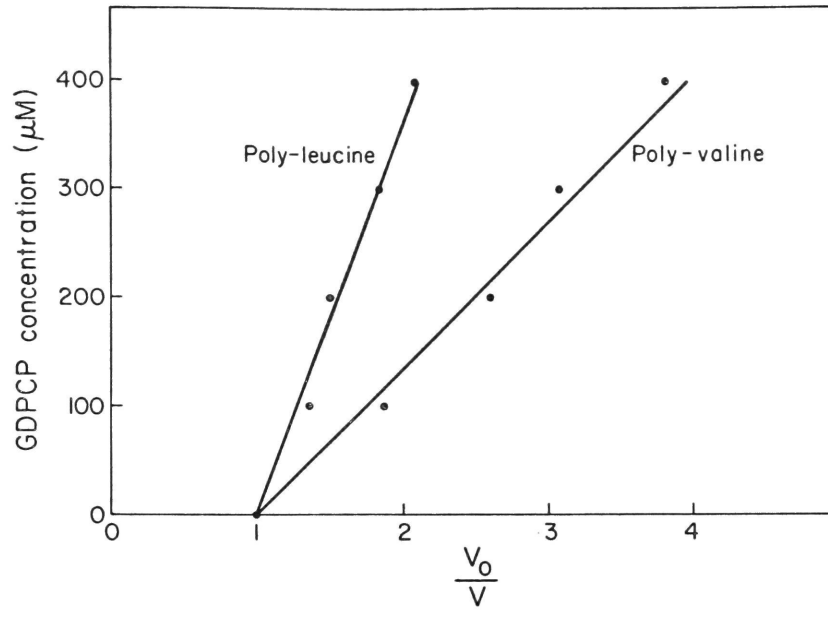

Figure 35. Differential effect of GDPCP on the synthesis of homopolypeptides in response to poly r-UUG. Each reaction tube contained 45 mμmoles/ml of nucleotide residues composing poly r-UUG. All three amino acids were present in each reaction tube, though only one was labelled. GDPCP had a greater inhibitory effect on the incorporation of valine than it did on the incorporation of leucine. Since both homopolypeptide products were synthesized from homopolycodons occurring on the same physical template, and since GDPCP had a differential effect on the translation of each homopolycodon, it can be concluded that the inhibitory effect of GDPCP is codon-specific. The codon-specific property that determines the sensitivity to GDPCP inhibition is probably the length of time it takes to translate a codon.

amounts (Söll et al., 1967; Muench and Safille, 1968; Caskey et al., 1968). Since amino-acyl tRNA's are the precursors of proteins, it would not be surprising if the rate of protein synthesis was dependent on the concentration of these precursors. Moreover, it seems reasonable that if there should be a short supply of the tRNA species which recognize a particular codon, the synthesis of the protein would be held up at that codon until an appropriate amino-acyl tRNA is found. This sort of reasoning has led many workers to postulate that the rate of protein synthesis is a function of tRNA concentration (Ames and Hartman, 1963; Stent, 1964; Boyer et al., 1964; Itano, 1965; Nirenberg et al., 1966). Direct proof of this hypothesis has recently been obtained by Anderson (1969), who showed that the rate of translation of synthetic mRNA's is proportional to the concentration of the appropriate tRNA species. Moreover, the range of tRNA concentrations he tested coincided with the concentration of these tRNA's in the intact cell. Further support for the view that translation rate varies comes from the work of Winslow and Ingram (1966). Using the pulse-labelling technique of Dintzis (1961), and the analytical procedure described by Englander and Page (1965), they were able to demonstrate that different portions of human hemoglobin chains are synthesized at different rates. The experiments described in this dissertation, taken together with the experiments of Anderson and of Winslow and Ingram, lend strong support to the conclusion that template activity is a function of codon composition.

It is interesting to speculate on the consequences of a variable codon translation rate. For example, the genes might influence the rate of synthesis of a protein, in addition to specifying the protein's amino acid sequence. This property would be a consequence of the degenerate nature of the genetic code. Most amino acids can be specified by more than one codon (Nirenberg et al., 1966). A set of codons which specifies the same amino acid may be termed "synonym" codons. In general, synonym codons should be translated at different rates. The presence of "slow" synonym codons in a mRNA, rather than "fast" synonym codons, would reduce the translation rate of the mRNA. Because synonym codons often differ from each other in the identity of only one of their three nucleotides, genetic mutations frequently occur in which a codon is converted to one of its synonyms. These mutations have no effect on the amino acid sequence of the protein that the gene specifies. However,

these so-called "silent" mutations, might well be "heard," in the sense that the substitution of one synonym codon for another might alter the rate at which the protein is synthesized. Significantly, this sort of mutation would be subject to natural selection, in the sense that a phenotype is determined by the amount of protein, as well as by the type of protein. Since 18 of the 20 amino acids are specified by synonym codons, there is ample opportunity for a codon composition to be selected which results in an optimal rate of synthesis for a particular protein. In summary, the degenerate nature of the genetic code, combined with a range in the availability of the different tRNA species, leads to the speculation that through the mechanism of natural selection, the genes might specify the amount, as well as the type, of each protein synthesized in the cell.

SUMMARY

1) mRNA's are stored in Xenopus oocytes for use later during embryogenesis.

2) Specific template activity varies from one mRNA to another.

3) GDPCP is a non-competitive inhibitor of protein synthesis.

4) The effectiveness of GDPCP inhibition, as measured by the inhibitor constant, is proportional to the average time it takes to translate a codon.

5) GDPCP has a differential inhibitory effect on the translation of heterologous mRNA's, on the translation of different cistrons within the same mRNA, and on the translation of different codons within the same cistron.

6) Therefore, the rate of translation (and consequently, the specific template activity) of any length of mRNA, from 1 to n codons, depends on its codon composition.

7) The presence of synonyms in the genetic code permits the evolutionary selection of a codon composition for each mRNA which optimizes its specific template activity so that each protein will be synthesized at an advantageous rate.

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