MESSENGER RNA: AN EVALUATION[1,2]

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That RNA might serve as a template for the arrangement of amino acids in specific polypeptide chains has been discussed for many years (1). The recent elaboration and documentation of this concept has depended on advances in our understanding of the mechanism of protein synthesis and the concomitant appreciation of the variety of roles played by RNA in that process. The name messenger RNA was first used by Jacob & Monod (2) in their brilliant unifying interpretation of experiments concerning the synthesis of RNA after phage infection on the one hand, and the kinetics of enzyme induction and repression on the other. We shall begin our discussion with a definition of mRNA.[2] One important reason for so doing is that investigators in this field have used, either explicitly or implicitly, different definitions at different times. Another reason is to provide our readers with some framework for considering the supportive evidence. This appears especially important because work pertinent to the concept of mRNA has come from so many different disciplines. The definition that follows differs from the original description primarily in its generalization; this is demanded by the application of the mRNA concept to nonbacterial systems as well as by the experimental evidence that has accumulated since the original formulation (1).

[1] The literature survey for this article was completed in October 1965.

[2] The following abbreviated terminology will be used throughout: messenger RNA, mRNA; transfer (or soluble RNA), tRNA; ribosomal RNA, rRNA; the heavier and lighter subunits of ribosomal particles will be referred to as 50S and 30S, respectively, although it is recognized that in species other than *E. coli* the actual sedimentation values may vary considerably (3); A, C, G, and U refer to adenylic, cytidylic, guanylic, and uridylic acid, respectively, when present in polyribonucleotides; polyribonucleotides are written so that a small p to the left of the nucleotide abbreviation indicates a phosphate esterified to a C-5′-hydroxyl and a small p to the right of the nucleotide a phosphate esterified to a C-3′-hydroxyl (4), thus pApU is 5′-0-phosphoryladenylyl-(3′, 5′)-uridine; a small d indicates deoxyribonucleotides.

Messenger RNA is a polyribonucleotide which determines the sequence of amino acids in polypeptide chains. The mRNA is a carrier of the information of the genome. Therefore, when the genome is DNA, the base sequences of the mRNA chains duplicate the base sequences of portions of the pertinent DNA. The RNA of RNA-containing viruses may act as a genome and be formally analogous to DNA or may, at least in part, serve itself as messenger [(5), and article by Wittmann & Scholtissek in this volume]. In reconstructed cell-free systems, mRNA may be the RNA described in the previous sentences (natural mRNA) or any other polyribonucleotide capable of directing, with specificity, the incorporation of amino acids.

In our view, the experiments carried out with cell-free systems have provided the strongest evidence in support of the concept of mRNA. These studies have proven that polyribonucleotides can function as templates for the synthesis of specific polypeptides and further have yielded much information on how such templates may function and be synthesized. Such studies allow some assurance concerning the interpretation of experiments dealing with natural RNA. These latter experiments are generally either indirect or difficult to interpret given the current level of knowledge. Indeed, natural mRNA often seems to resemble the Scarlet Pimpernel (6):

> "We seek him here, we seek him there,
> Those Frenchies seek him everywhere.
> Is he in heaven?—Is he in hell?
> That demmed, elusive Pimpernel?"

The central role of mRNA in current hypotheses concerning the mechanism of protein synthesis [(7), and article by Schweet in this volume] is well known. In addition to its function in specifying the structural aspects of proteins, mRNA is believed to be involved in the regulation of protein synthesis (2). In the discussion that follows we shall outline certain questions relevant to these several roles and shall try to evaluate the pertinent available evidence. For references to most of the earlier work the reader is referred to previous reviews (1, 2, 3, 7–15). Our selections from the enormous volume of more recent publications reflect our own interests, and other relevant material may be found in this volume in the articles by Schweet and by Wittmann & Scholtissek.

We shall discuss first the problems that beset experiments designed to detect, isolate, and characterize mRNA. None of this work is completely satisfactory at this writing, and a critical review of the available techniques is desirable. The second section of this article is concerned with the mechanism by which the information of the genome is transcribed to an mRNA chain (transcription). Finally, the *in vitro* experiments which have led to a relatively detailed understanding of the means by which a sequence of nucleotides in mRNA specifies a sequence of amino acids in a polypeptide (translation) will be described.

Characterization and Isolation

The definitive characterization of an RNA fraction as mRNA would involve the isolation of a unique cellular RNA that had been made on a DNA template and the demonstration that that RNA could direct the synthesis of a specific protein. RNA isolated from RNA-containing bacteriophages directs the synthesis of the corresponding viral coat proteins in cell-free systems from *Escherichia coli* (16, 17, 18) and *Euglena gracilis* (19). However, similar definitive experiments with a unique *cellular* RNA have not been satisfactorily realized. Extraordinary technical difficulties exist both in the fractionation of polyribonucleotides and in the assay of mRNA. The difficulties are compounded by the fact that mRNA represents only a few per cent of total cellular RNA (2, 20, 21). Various criteria, based on the properties predicted for mRNA (2), have been used in identifying an RNA fraction as mRNA. Unfortunately, a major problem, common to all these criteria has become apparent over the last few years, namely, that no one of them is unique for mRNA. Therefore a re-evaluation of each of the criteria appears warranted. In this section we will attempt such a re-evaluation and then will describe a few examples of the application of the criteria to specific RNA preparations.

Base composition and base sequence.—The suggestion that the base composition of mRNA would reflect the base composition of the DNA of the organism (2, 21) was derived from the base-ratio similarities of T_2 phage DNA and the RNA produced on infection of *E. coli* with T_2 phage (22), and from the messenger notion itself (2). Although this criterion has been widely applied, the significance of such results is quite limited. Much of the published data was obtained by the estimation of radioactive nucleotides after labeling cells with inorganic ^{32}P. Randomization of the ^{32}P throughout the chains in the fraction studied is generally assumed, as is the equilibration of the precursor pools. However, it has been repeatedly demonstrated that such assumptions may be erroneous and should be documented for each case (23–26). Furthermore, interpretation of base ratios often depends on having a pure RNA, a standard that is currently difficult to meet. The fact that only a fraction of the DNA may be functional in any given system (27) poses a more fundamental limitation. Even with T_2 bacteriophage, different portions of the genome are transcribed into RNA at different times after infection (28, 29). In the differentiated tissues of higher organisms, overall base ratio equivalence of total DNA and mRNA may not occur (27, 30). In addition, if mRNA is a copy of only one strand of DNA (see section on transcription), there is no need to assume a base ratio equivalence between DNA and mRNA, unless the average base ratio of a single strand of DNA is the same as that of the whole.

Equivalence of base composition is a corollary of the idea that the base sequence of mRNA will reflect that of the DNA. It is possible at present to estimate equivalence of base sequence quite precisely. The method, known

as DNA-RNA hybridization, is based on the fact that single chains of polydeoxyribonucleotides and single chains of polyribonucleotides with complementary sequences of bases will, under suitable conditions, form base-paired complexes (hybrids). The technique and theory of hybridization have been critically reviewed (31). Early application of this method (31, 32) showed that the RNA produced after infection of *E. coli* with T_2 bacteriophage, forms a hybrid with T_2 DNA, whereas DNA from other bacterial and viral sources is not effective. However, the ability to hybridize with homologous DNA is not a unique characteristic of mRNA. In fact, all cellular RNA, including rRNA and tRNA, as well as material tentatively identified as mRNA, forms hybrids with homologous DNA and thus all cellular RNA may be made on a DNA template (32). Messenger RNA may be distinguished from other classes of RNA by quantitative hybridization experiments (29, 32, 33, 34). Such analysis depends on two facts: first, mRNA represents a small portion of the total RNA (2, 20, 21); and second, only a very small per cent of the DNA of a given organism can hybridize with rRNA or tRNA (32).

Molecular weight.—Although a unique mRNA would clearly be expected to have a unique molecular weight, heterogeneity of molecular weight is expected for the total mRNA of a cell (2). The history of experiments related to this point highlights the limited utility of this criterion. In general, sucrose density gradient centrifugation has been used for size determinations. Early work suggested that the sedimentation constants of bacterial mRNA range between 6S and 14S, but newer techniques, designed to avoid extensive degradation, give values of up to 30S (32). Similar results have been obtained with animal systems (35, 36, 37). Additional technical problems arise from the interaction of mRNA with rRNA in Mg^{++} (38–41), from the binding of mRNA to ribosomes (20, 21, 37), and from false sedimentation valves resulting from the contamination of RNA preparations with polysaccharides (42). Furthermore, although rRNA and tRNA are readily distinguished by their characteristic molecular weights (3), the same is not true of precursors of rRNA (37, 43–46).

Stimulation of amino acid incorporation.—The development of a stable, cell-free system capable of synthesizing polypeptides under the direction of polyribonucleotides (47, 48) permits a direct assay for mRNA activity. However, since almost any polyribonucleotide including random poly AGUC (49) and ribosomal RNA (48, 50) can, under suitable conditions, stimulate the incorporation of amino acids into protein, this characteristic is not discriminating unless the synthesis of a specific polypeptide is investigated. Indeed, rRNA appears to serve as a template for the synthesis of at least a portion of ribosomal protein (51, 52).

The stability of mRNA.—The kinetics of induction and repression of bacterial enzymes indicate that the corresponding mRNAs are unstable, relative to the life of a cell (2). On the other hand, mammalian reticulocytes synthesize globin in the absence of RNA synthesis, suggesting that stable mRNA also exists (7, 8). Recent experiments which are discussed in this

section, support the view that all types of cells may produce both stable and unstable mRNA, and further, that other types of RNA may also turn over rapidly. Therefore, instability is neither necessary nor sufficient for the characterization of an RNA fraction as mRNA.

Two experimental approaches to the determination of mRNA stability have been widely used. Each of these will be discussed here with reference to work with both bacteria and more highly differentiated organisms. The first type involves the so-called pulse-labeling of RNA. Operationally, a rapid rate of turnover implies that at any given time mRNA will represent a high proportion of newly synthesized RNA, although it is a small proportion of total cellular RNA. Thus, a brief pulse of a radioactive precursor is expected to label mRNA preferentially (20, 21). However, two problems arise in the interpretation of such experiments; (*a*) relatively stable mRNA will not be labeled during a brief pulse and may escape detection; and (*b*) all pulse-labeled RNA is not mRNA. In bacteria (33, 34, 45, 46, 53–56), in animal tissues (37, 44, 57–61), and in yeast (23) a large portion of pulse-labeled RNA is relatively stable and appears to represent precursors of rRNA. The portion of the pulse-labeled RNA that is rapidly degraded when labeling is stopped, is often considered to be mRNA, but here, too, difficulties arise. Although bacterial rRNA may be stable under certain experimental conditions (62, 63), it may be unstable under others (26) and be degraded relatively rapidly. Similarly, liver rRNA has a half life of only about five days (41, 64), which is not a long time in a relatively long-lived cell (64). Precursors of ribosomal RNA certainly appear to be unstable (33, 58, 65–68). For example, the total pulse-labeled RNA of *Bacillus megaterium*, which includes rRNA precursor, decays rapidly with a half life of at most 1 min after RNA synthesis is inhibited by treatment with actinomycin D (33).[3]

The second type of experiment that has been used to measure mRNA half life involves the kinetics of specific enzyme induction and repression. Cessation of enzyme induction either by removal of inducer (69–72), or addition of actinomycin D[3] (73, 74, 75) to halt RNA synthesis, results in a decay of ability to synthesize the induced enzyme. The rate of decay of enzyme-forming ability is used as a measure of the instability of mRNA (69–75). In bacteria, the rate of decay is apparently independent of the presence or absence of inducer (73, 74), and of protein synthesis (70, 71, 76–79). It should be pointed out that the interpretation of such experiments rests on the assumption that mRNA is the rate-limiting component for protein synthesis under the given experimental conditions. Furthermore, the removal of inducer is often effected by dilution, although a recent report suggests that such procedures may not be as efficient as is generally believed (80).

Having described the experimental difficulties, we can now summarize the results of experiments dealing with mRNA stability. The half life of pulse-labeled RNA is of the order of 1 to 2 min in *Bacillus subtilis* (73, 81)

[3] The problems raised by the use of actinomycin D as a specific inhibitor of RNA synthesis are discussed in the section on transcription.

and *E. coli* (46). In these experiments, conducted between 33° and 37° C, actinomycin D was used to halt additional RNA synthesis. In the *E. coli* experiments, the half life correlated well with loss of ability to support amino acid incorporation *in vivo* (46). Using less specific reagents to stop RNA synthesis, namely, proflavine and dinitrophenol, half lives of 3 to 4 min and 6 to 8 min, respectively, were obtained for pulse-labeled, unstable RNA in *E. coli* (82, 83). Differential hybridization experiments have revealed a special class of pulse-labeled RNA that is associated with sporulation in *B. subtilis* and *Bacillus cereus* (84, 85, 86); apparently a stable mRNA, it is synthesized prior to spore formation and remains present throughout sporulation (85).

The half life of the mRNA for the β-galactosidase of *E. coli* has been estimated at about 2 min at 30° using both actinomycin D (74) and removal of inducer (71) to stop induction. Similarly, for the histidase of *B. subtilis* it is 2.4 min at 37° (73), using actinomycin D. Both the kinetics of enzyme synthesis and the stability of pulse-labeled RNA indicate that the mRNA for catechol oxygenase enzymes is stable for up to 8 min (72). Experiments with actinomycin D suggest that the mRNA for the penicillinase of *B. cereus* is also relatively stable (87, 88).

The two general approaches to mRNA stability have also been applied to animal systems. In mammalian liver (35, 36, 37, 61, 89, 90), in HeLa cells (44), and in a mouse adenocarcinoma (91) an RNA that can be partly distinguished from ribosomal RNA is indeed labeled rapidly and is therefore tentatively designated mRNA. The values reported for the sedimentation constants of this material vary from less than 10S (90, 91) to more than 28S (37, 44). In mouse liver, the time required to reach a steady state level of label in material between 5 and 20S indicates a half life of two hours for mRNA (90). Longer half lives for liver mRNA are obtained if actinomycin D is used to stop RNA synthesis and mRNA is then assayed by the loss of polysomes, the degradation of pulse-labeled polysomal RNA, or the ability to support amino acid incorporation (89, 92); values of from 12 hr (89) to three days (92) have been reported. Similar discrepancies between the rate of labeling of mRNA and actinomycin D experiments were found with a mouse mammary adenocarcinoma (91). Estimates of the half lives of the corresponding mRNAs have been obtained from studies of the rate of synthesis of specific proteins after treatment of animals with actinomycin D;[3] values ranging from several hours (75, 92, 93, 94) to a day or more (75, 95–99) have been reported. Evidence for the existence of a stable and perhaps inactive mRNA in unfertilized sea urchin and toad eggs has been reviewed by others (100).

The presence of mRNA in polysomes.—As a result of current concepts of protein synthesis [(7), and article by Schweet in this volume], mRNA should be found bound to ribosomes, at least under certain circumstances (2). With the recognition that protein synthesis occurs on multiribosomal structures called polysomes (or polyribosomes, or ergosomes) (3, 7, 101) the presence of mRNA in these structures was generally assumed. In this section the extensive evidence supporting this assumption is described. Wherever the distinc-

tion is possible, the word ribosome will indicate a single particle carrying no mRNA; a single ribosome bound to mRNA will be called a monosome (102).

Electron microscope studies support the idea that polysomes contain mRNA. Pictures of polysomes from animals and bacteria reveal a linear array of ribosomes. Thin threads are seen between the ribosomes and the diameter of the threads is consistent with their being RNA (101, 103–106).

Polysomes are readily degraded to ribosomes (or, perhaps, monosomes) by pancreatic ribonuclease (36, 101, 104, 106–110). This finding is generally interpreted to indicate that the mRNA holds the polysome together. Technically, this fragility demands that great care be used in the isolation of polysomes. In particular, RNase activity must be kept to a minimum (33, 110, 111). Other factors may also be involved in the stabilization of polysomes. For example, phospholipase (112) and trypsin (113, 114) cause deaggregation of polysomes, and deaggregation of reticulocyte polysomes occurs when iron is limiting (115, 116). The latter observation may reflect a change in the rate of ribosome attachment to or detachment from mRNA (see Schweet in this volume). Ribonuclease-resistant ribosomes are present in developing chick down feathers. After 13 days of development, these structures become sensitive to ribonuclease and functional for amino acid incorporation (117, 118).

The identification of mRNA as a component of polysomes is also supported by pulse-labeling experiments. Brief labeling of yeast (119), animal (36, 120) and bacterial (33, 121) cells with radioactive precursors of nucleic acids, results in the labeling of polysomes. For example, 30 min after injection of rats with ^{3}H-orotic acid, the labeled RNA of liver is associated primarily with the polysomes while the ribosomes have a low specific radioactivity (36). Actinomycin D,[3] which inhibits RNA synthesis and thereby decreases the amount of available mRNA, decreases the proportion of ribosomes found in the polysome region (33, 36, 89, 91, 120, 122, 123). The rate of breakdown of polysomes after actinomycin D treatment correlates well with both the rate of decay of pulse-labeled RNA sedimenting between 4.5 and 20S, and the decay of capacity to incorporate ^{14}C-leucine in a mouse mammary adenocarcinoma (91).

Hybridization experiments have also been used to characterize polysomal RNA. The pulse-labeled RNA associated with polysomes in *B. cereus* hybridizes with homologous DNA (33). As expected for mRNA, the percentage hybridized was not influenced by the ratio of RNA to DNA or by the addition of unlabeled rRNA (33). After actinomycin-D treatment, the rate of decay of pulse-labeled polysomal RNA was the same as the rate of decay of hybridizable material and somewhat faster than the rate of decay of the polysomes themselves (33).

The identification of mRNA as a component of polysomes may be supported by the ability of polysomes to incorporate amino acids into polypeptide both *in vivo* and *in vitro*. Nascent protein, detected by brief labeling of intact cells or organisms with radioactive amino acids, is attached to polysomes from reticulocytes (101, 124–127), from *E. coli* (110, 111, 121), from

HeLa cells (120), from yeast (119), and from fertilized (107, 128, 129) and parthenogenetically activated (130) sea urchin eggs. Upon induction of β-galactosidase in *E. coli*, enzyme appears on polysomes before it appears free, and after treatment with RNase it is recovered in the monosome fraction (111). If polysomes are first isolated (usually by sucrose gradient centrifugation), and then tested in an *in vitro* amino acid incorporating system, they support the incorporation of amino acids in the absence of added polyribonucleotide (36, 104, 109, 119, 131). Polysomes are 10 to 20 times more active than isolated ribosomes from yeast (119), from muscle (109), from rat liver (36), and from *E. coli* (110). Isolated 80S reticulocyte ribosomes (132–134) and rat liver ribosomes (36) do incorporate amino acids independently and therefore appear to be, at least in part, monosomes. The reticulocyte monosomes form polysomes concomitant with the formation of polypeptide (132, 133). When isolated ribosomes are used in a cell-free incorporating system, the stimulation by added polyribonucleotides is assumed to indicate the absence of endogenous mRNA (132, 135, 136).

Several other lines of evidence are also consistent with the identification of mRNA as a component of polysomes. Multiribosomal complexes are formed *in vitro* when polyribonucleotides are mixed with ribosomes (see section on translation). The formation of polysomes depends on RNA synthesis in a cell-free preparation depleted of mRNA (137). In cells infected with viruses, viral RNA has been identified as a component of polysomes (121, 138–142).

It has been suggested that the number of ribosomes on a polysome is related to the size of the mRNA chain (35, 101, 119, 120, 143). The sedimentation rates of fractions of pulse-labeled RNA extracted from polysomes are proportional to the size of the polysomes from which they were isolated (35). A high proportion of reticulocyte polysomes contain from four to six ribosomes (101, 105) that are so spaced as to fill an mRNA chain of suitable size for globin synthesis (101). Recently, larger polysomes have been detected in reticulocyte lysates (105), and brief labeling of reticulocytes with ^{14}C-amino acid indicates that globin is synthesized on polysomes containing up to seven to ten ribosomes (26). The large polysomes are relatively rare and fragile: they may represent a sizable proportion of polysomes in the intact reticulocyte (105). Polysome size, customarily measured by sucrose density gradient centrifugation, depends on the conditions of isolation; both breakdown (33, 35, 111, 144) and aggregation (35, 36, 133, 145, 146) may occur. In addition, the number of ribosomes per unit length of mRNA may vary (see article by Schweet in this volume), and the array of ribosomes can be nonlinear (126). In general, then, the sedimentation rate of polysomes cannot be used to measure the length of mRNA chains directly.

The isolation of mRNA from bacterial sources.—The RNA produced in *E. coli* upon infection with T-bacteriophage has been characterized by all of the criteria. The base ratios of the RNA are those of the DNA (22, 147) and the frequency of dinucleotide pairs in T_4-mRNA is similar to that of T_4-DNA (148) although T_4-mRNA is essentially single stranded (147,

149). The RNA is unstable (22, 121, 150, 151), is found associated with ribosomes (20, 121), stimulates the incorporation of amino acids into polypeptide in a cell-free system, and appears to be heterogeneous in size (147). Of the RNA produced 5 to 8 min after T_4 infection, the highest molecular weight material is unable to promote amino acid incorporation (147). The RNA forms hybrids with DNA isolated from the infecting phage (32), and T_4-mRNA has been purified by hybridization (152). The T_2-genome produces different mRNAs at various times after infection (28, 29).

An RNA fraction characterized as the mRNA corresponding to the cyclic decapeptide Gramicidin S has been isolated from *Bacillus brevis* (153, 154). The molecular weight of the fraction is about 11,000 and it contains 36 or 37 nucleotides (154). This preparation stimulates the incorporation of those amino acids found in Gramicidin S. It binds to ribosomes in an unorthodox manner in that it binds to both 30S and 50S ribosomal subunits but not to 70S ribosomes (155). In view of other experiments (156–159) which indicate that Gramicidin S and related polypeptide antibiotics are not synthesized by a mechanism analogous with protein synthesis, the relevancy of these exciting results to the mechanism of general protein synthesis is not clear.

Induction and derepression of specific bacterial enzymes is expected to lead to the production of the corresponding mRNA (2). Specific peaks of pulse-labeled RNA of sedimentation values 34S and 30S have been demonstrated in cells constitutive for the histidine operon (160) and induced for β-galactosidase (161), respectively. The sensitivity and reliability of this general approach is increased enormously by characterizing the rapidly labeled RNA with specific hybridization tests (32, 162). For example, mRNA corresponding to the enzymes of the galactose operon in *E. coli* should only form hybrids with DNA from *E. coli* containing that operon. Additional sensitivity is gained if the DNA containing the operon is obtained from transducing phage, or episomes (32, 162–165). Irrelevant hybridization can be eliminated in controls with DNA from homologous particles that lack the region under study. These procedures have permitted the detection and study of RNA corresponding to the mRNA for the lactose operon (32, 162, 163), the galactose operon (162), and the tryptophan operon (164, 165). The proteins of the lactose operon are synthesized at especially high differential rates during adaptation to lactose following the exhaustion of glucose (diauxic growth) (163). A large portion of the RNA pulse-labeled just prior to new growth on lactose hybridizes specifically with DNA containing the lactose region (163).

The isolation of mRNA from mammalian sources.—Several attempts to isolate the hypothetical mRNA of reticulocytes and reticulocyte polysomes have been made. Reticulocyte RNA stimulates amino acid incorporation in a typical cell-free system from *E. coli* but globin has not been an identifiable product (132, 166, 167). Reticulocyte RNA also stimulates amino acid incorporation in a cell-free system derived from reticulocytes (168–171), and globin, or globin-like material, is produced (168–171). There is little agree-

ment concerning the sedimentation constant of the active fraction (166, 167, 171, 172). Interactions with ribosomal RNA, or aggregation, may be reponsible for the differences. A relatively highly labeled RNA, sedimenting at 9S, has been isolated from reticulocytes 15 hours after ^{32}P injection (173). Treatment of reticulocyte ribosomes with 0.5 *M* KCl produces a soluble fraction which is then required for globin synthesis with the stripped ribosomes. The activity of the supernatant fraction is lost after phenol treatment, proteolytic digestion, or nuclease treatment [(174), and article by Schweet in this volume].

Attempts to purify mRNA from mammalian liver have also been made. Nuclei are generally chosen as a favorable source since they are the site of the bulk of RNA synthesis (175–177) and incorporate labeled RNA more rapidly than the cytoplasm (175–178). Nuclear RNA extracted from rat liver stimulates the incorporation of amino acids in a cell-free system from *E. coli* more efficiently than does cytoplasmic RNA (179, 180). The active material is heterogeneous in sucrose density gradient centrifugation as is the rapidly labeled RNA of liver nuclei (90, 179–181). The base ratios of the active fractions have some resemblance to DNA but the material most like DNA, which sediments at 10S, has the lowest template activity (180, 181). Variation in the temperature used for the extraction of RNA with phenol permits the separation of two rapidly labeled RNA fractions from mammalian nuclei. At lower temperatures rRNA is extracted while the RNA extracted at 60° is more like DNA in base composition (59, 60, 182). Variations in the pH used for phenol extraction achieve similar fractionations (180, 181). Cytoplasmic RNA with DNA-like base ratios and template activity has also been detected (90, 179, 180, 183, 184) in mammalian cells. The rate of sedimentation of the active material varies enormously with the temperature of extraction, the Mg^{++} concentration, and other conditions (90, 179, 180, 181, 183).

Transcription

Transcription is the enzymatic synthesis of RNA by a process in which the bases in a genome specify the sequence of bases in the RNA. This process provides for the synthesis of mRNA as well as for the synthesis of rRNA, tRNA (32, 185), viral RNA [(5), and article by Wittmann & Scholtissek in this volume], and perhaps certain regulatory sequences. Various enzymes capable of catalyzing transcription have been characterized. We shall use the term replicase (187) to denote such enzymes and refer the reader to several recent and extensive reviews of their properties [(5, 8, 11, 186), and article by Wittmann & Scholtissek in this volume]. The synthesis of infectious viral RNA by a purified replicase is striking proof that these enzymes can copy their respective templates faithfully (187).

The chemical mechanism of transcription must account for the specificity of the replicases and for the initiation, polarity, and asymmetry (the copying of only one strand of a double-stranded template) of transcription. It must also provide for the termination and release of the product and, finally, it must define control mechanisms which regulate transcription. We shall at-

tempt to evaluate the putative solutions to these problems in reference to the available data.

Replicase specificity.—DNA-dependent replicases from bacteria are able to transcribe a wide variety of naturally occurring (188, 189) and synthetic (190–192) DNA and RNA (193–199) templates. Quantitative differences in template efficiency have, however, been noted (188, 195, 198). Despite the lack of specificity for template exhibited by various bacterial replicases *in vitro*, other experiments suggest that many replicases may show a high degree of template specificity *in vivo*. Infection of mammalian or bacterial cells with RNA-containing viruses results in the induction of new, RNA-dependent replicases (200–204). A DNA-dependent replicase differing from that usually present in the intact rat liver appears to be induced by partial hepatectomy (205). An inhibitor of DNA-dependent replicase has been detected in *E. coli* after infection with T_4-bacteriophage, suggesting that a new, inhibitor-insensitive replicase, may be required for phage maturation (206). The strongest evidence favoring the highly specific nature of replicases comes from the elegant studies of Spiegelman and co-workers (187, 201, 207, 208). The RNA-dependent replicases which arise following infection of *E. coli* with either of two different RNA phages have been purified extensively from a bacterial strain devoid of *E. coli* ribonuclease I and polynucleotide phosphorylase. Under suitable conditions, each of the enzymes exhibits a striking template specificity for homologous phage RNA; heterologous RNA is inactive as template. A similar enzyme, purified from a normal strain of *E. coli* infected with a third RNA phage, utilizes a variety of RNAs as template (202, 209); these findings may be complicated by the presence of contaminating ribonuclease. Thus, specificity may be shown by replicases, and it will be interesting to attempt to extend this idea to the DNA-dependent replicases.

Initiation and the transcriptive complex.—The mRNA concept itself, as well as the existence of operons (12) suggest that transcription commences at particular points of initiation on the genome and proceeds to specified termini. Chemically, an initiation site implies the interaction of the replicase with a specific site on the DNA and subsequent synthesis of RNA from that point only. DNA-dependent replicases interact strongly with DNA. Great difficulty is experienced in trying to separate mammalian enzymes from DNA (175, 200, 210–212), although solubilization of mammalian enzymes has recently been achieved (213–215). Analogous complexes have been reported for bacterial DNA-dependent replicases (216, 217) and viral RNA-dependent replicases (202, 218, 219). The irreversible formation of complexes between DNA and DNA-dependent replicase has been demonstrated *in vitro* (220–222). Newly synthesized RNA is also bound to the DNA-replicase complex *in vitro* and can be released by treatment with sodium dodecyl sulfate (220) or polyamines (222). The binding of the RNA product to the DNA-replicase complex occurs early in the course of synthesis. The binding is irreversible, resulting in an inhibition of enzyme activity and thereby explaining the very limited RNA synthesis generally found in such reactions (220, 221). Direct evidence for the formation of template-enzyme complex

has been provided by electronmicrographs (223, 224). In contrast to these results, the interaction between replicase and template appears to be readily reversible when synthetic, single-stranded oligodeoxyribonucleotides are employed as templates (192, 225).

The chemical nature of the initiation sites on DNA or RNA templates, or even of the binding sites for replicase, is unknown. However, relevant experiments utilizing *E. coli* DNA-dependent replicase have been published. The 3′-OH ends of the template may be involved in the binding of replicase because in the DNA-replicase complex they are unavailable to other enzymes (221). However, the chain ends themselves are probably not required for initiation since the ring form of ϕX 174 DNA is a template for the replicase (226–228). Denatured DNA contains more binding sites for replicase than an equivalent amount of native material (221, 229, 230). It has been suggested that unpaired regions in double-stranded templates may act as initiator sites for transcription (221, 230). Ends of chains might well be associated with such regions. The binding of replicase to homopolymers (222) suggests that a specific sequence is not required for binding. But the lack of this requirement does not rule out the possibility that a specific sequence is involved in initiation. Indeed, recent evidence indicates that RNA synthesis with *E. coli* replicase starts preferentially with purine residues (230, 231).

Polarity.—In bacteria, genetic information can be expressed in a polarized fashion commencing at the operator end of an operon (2, 12, 232, 233). Evidence obtained with the tryptophan operon suggests that transcription also starts with the operator end and is polarized (165). The chemical polarity of transcription with *E. coli* replicase is such that chain synthesis starts at the 5′-OH end of the new RNA and proceeds, stepwise, to the 3′-OH end (i.e., left to right in the conventional expression) (230, 231).[3] Assuming that copying proceeds by antiparallel, Watson-Crick type base pairing, chain synthesis starts by copying the 3′-OH end of the template.

Asymmetry.—Until recently an apparent paradox existed in which it appeared that transcription *in vivo* was asymmetric while purified replicases copied both strands of the template *in vitro* (11, 234). Asymmetric synthesis has now been demonstrated *in vitro*. If the circular, replicating form of phage ϕX 174 DNA is used as template for purified *E. coli* replicase, only one strand of the DNA is transcribed (226). Both strands are copied if the DNA is first fragmented by sonication. With crude extracts of *Bacillus megaterium* as a source of replicase, phage α DNA directs asymmetric transcription even if the DNA is sonicated (235). It is important to point out that the fragments obtained by sonication of phage α DNA are much larger than those similarly obtained from phage ϕX 174 DNA (226, 235). The asymmetric copying of phage α DNA does depend on the native conformation of the DNA. Similar results are obtained when crude extracts from a variety of bacteria are used and with DNA from other bacteriophage (236). The general conclusion that purified DNA-dependent replicases carry out asymmetric transcription if the DNA template is in a native conformation and if its length is not excessively short, has been confirmed (237, 238). The apparent requirement for

circularity (226) with ϕX 174 DNA may reflect specific characteristics of this DNA, or may be related to the small size of the fragments obtained when the circles were disrupted by sonication. Restriction of copying to a single chain of a double-stranded template is demonstrable in model systems. A double-stranded polydeoxyribonucleotide containing, for example, alternating T and C residues in one chain and alternating A and G residues in the other (poly dTC:AG) has been used as template for the *E. coli* DNA-dependent replicase. If two ribonucleoside triphosphates, appropriate for the copying of only one of the chains, are supplied in the reaction mixture, single-stranded products containing those two nucleotides in alternating sequence are synthesized (239).

It is well to note that except for one case (240), all the *in vivo* and *in vitro* work indicating asymmetric or so-called single-stranded transcription has involved the use of viruses. If the genetic information is always on only one strand of the DNA, and if all the replicases of a cell show the same chemical polarity, then all the operons should be oriented in the same direction. However, in *Salmonella typhimurium* the histidine and tryptophan operons are oriented in one direction on the genome, but the leucine operon has the opposite polarity (241–243). Furthermore, transformation studies in *B. subtilis* show that two linked markers (histidine and tryptophan) are expressed together even if they are present on opposite strands of the transforming DNA (244). It is important that no net synthesis of DNA occurred prior to gene expression under the conditions used (11, 244). It may be that in different regions of a bacterial chromosome, different strands serve as the template for transcription, but other explanations are also possible (241–245). These results emphasize the rudimentary nature of our understanding of transcription and of the factors controlling genetic expression.

Size of transcriptive unit.—The coordinated regulation of groups of related cistrons may be accomplished by transcribing entire operons as single mRNA molecules (2, 12). The detection of a few such polycistronic messengers was discussed in the first section of this article. Deletion mutants in the tryptophan operon of *E. coli* produce mRNA that is smaller than that derived from strains possessing the intact operon (164, 165), thus confirming the notion that the size of the mRNA produced is determined by the size of the corresponding DNA unit. It has been suggested that in some cases transcription might occur in units that encompass several contiguous operons having the same polarity (243).

Release and termination.—The copying of DNA in units of a particular size implies a specific termination mechanism as well as a mechanism for initiation. The release of completed mRNA from the template-replicase-RNA complex must also be explained, and termination and release may be coupled processes. It is possible that the entire length of the mRNA may be involved in the ternary complex until transcription is complete, at which point the whole molecule would be released. On the other hand, the association with the complex may involve only a few bases close to the growing end of the mRNA chain (246) leaving the completed portion of the mRNA free for

other interactions. This latter alternative is supported by the fact that in the complex between newly synthesized RNA and double-stranded phage ϕX 174 DNA, the ribonuclease-resistant portion of the RNA corresponds to the growing end of the chain (247). It is also supported by the formation, *in vitro*, of a complex containing DNA and ribosomes, a reaction that is dependent on concomitant RNA synthesis (248). This latter alternative permits the simultaneous synthesis and utilization of mRNA (234). One popular hypothesis envisages the ribosome as an active participant in the separation of mRNA from the DNA template (229, 231, 234, 248, 249).

In vitro studies have shown that when the template is native DNA, RNA synthesis by DNA-dependent replicase involves a conservative mechanism, thereby preserving the double helix of the template (186, 190). When single-stranded DNA is used as the template, the product appears initially in a DNA-RNA hybrid (227, 228, 246, 250). The hybrid product may also serve as template and appears to function by a mixture of conservative and semiconservative replication (228, 246). During conservative replication on a double-stranded template, the strands of DNA may be separated over consecutive short regions to allow RNA synthesis by base pairing (246, 247, 251, 252). The chemical problem posed by the subsequent release of single-stranded RNA involves the competition between two polynucleotide strands, differing from one another only in their sugar moiety, for complementary interaction with a third (251). DNA-RNA hybrids formed from natural nucleic acids are generally less stable than homologous, double-stranded DNA. However, DNA-RNA hybrids formed from homopolymer pairs may be more or less stable than the homologous DNA-DNA pairs (251). Such DNA-DNA homopolymer pairs have been used as templates for the replicase-catalyzed synthesis of corresponding polyribonucleotides. Copying was restricted to one strand by supplying only one nucleoside triphosphate at a time. In each case the choice between conservative and semiconservative replication is directed by the relative stabilities of the possible products. For example, when the thermal stability of the possible hybrid product is greater than that of the DNA-DNA template, semiconservative copying occurs, and the products are the hybrid and a free polydeoxyribonucleotide (251). These findings do not eliminate the possibility that some more active process may also be involved in RNA release (194, 229, 231, 234, 246, 248, 249, 253). The mechanism required for specific termination of RNA synthesis appears to be absent from purified, synthetic systems, thus permitting reiterative copying of template sequences (186, 191, 192, 225, 254).

Actinomycin D.—It is well known that at suitably low concentrations, actinomycin D specifically inhibits DNA-dependent replicase both *in vivo* and *in vitro* (186, 255–257). The recent literature contains hundreds of experiments in which actinomycin D is used to study the role of mRNA transcription in biological processes. The scope of such studies includes induction and repression of bacterial and animal enzyme synthesis (255, 258), development and differentiation in higher organisms (255), the replication of viruses (5, 255), and the mechanism of hormone action [(255, 259), and article by

Pastan in this volume]. Most of these studies have a common rationale. The influence of actinomycin on some aspect of protein synthesis is studied. If actinomycin D inhibits the primary effect, then mRNA synthesis is considered to be involved. The converse is also assumed to be true. We would like to review recent work which suggests that such experiments cannot, in fact, be interpreted in such an unambiguous manner. These difficulties reflect three basic problems, given the assumption that replicase makes mRNA: (*a*) the precise nature of the interaction between actinomycin D and DNA is not known (255, 256); (*b*) the precise mechanism of the copying of particular DNA sequences by replicase is not known; and (*c*) the assumption that the availability of mRNA is always the rate-limiting component in protein synthesis is not necessarily true (12, 53, 92, 135, 260, 261), especially in view of the more general, lethal effect of actinomycin D (255).

Although earlier evidence showed that the inhibition of the DNA-dependent replicase by actinomycin D is restricted to reactions in which the template is a double-stranded, polydeoxyribonucleotide containing guanine residues (186, 255), inhibition also occurs when reovirus RNA, a double-stranded RNA, is used as a template (198). Hybridization experiments suggest that all cellular RNA is made on a DNA-template (32), and in fact, actinomycin D can inhibit synthesis of all types of RNA. Thus, while experiments with this drug suggested that 80 per cent of pulse-labeled bacterial RNA is mRNA (8 per cent of total RNA) (78, 81), hybridization experiments showed that only 30 per cent of the pulse-labeled RNA is mRNA (1 to 2 per cent of total RNA) (53, 262). Differential effects of actinomycin D on the synthesis of various types of RNA have also been observed (60, 184, 255, 263) and indeed, preferential inhibition of rRNA synthesis has been reported (60, 184, 264–266). The labeling of tRNA in cells treated with actinomycin D, which is usually ascribed to labeling of the -CCA terminus, may also involve some synthesis of internal diester bonds (267, 268).

It is generally recognized that bacteria may be impermeable to this drug (269–272) but the permeability of target organs in higher organisms is rarely considered although it is known that the drug is rapidly concentrated in liver (255). In view of the different types of DNA-actinomycin D interaction at different drug concentrations (256), dosage and distribution are important problems in experiments with higher organisms and effects may well vary with dosage (84, 273). In animals, high doses (about 5 mg/kg) inhibit hepatic RNA and protein synthesis (92, 122, 274) but are also lethal. Loss of hepatic amino acid-incorporating ability has been correlated with a decrease in the percentage of ribosomes found as polysomes after actinomycin D treatment (89, 91, 122, 274); however, it has been reported that these observations depend on homogenization of the tissue. Neither effect is demonstrable with slices (274). Thus, the decrease in amino acid incorporation may not be directly related to inhibition of RNA synthesis. Lower doses of actinomycin D, about 1.5 mg per kg, also inhibit the labeling of rat liver RNA but have little effect on amino acid incorporation into protein either *in vivo* or *in vitro* (92, 274). Other unrelated effects of actinomycin D on protein and

RNA synthesis have also been observed (275, 276). When *B. megaterium* cells are treated with actinomycin D, inhibition of *in vivo* amino acid incorporation results. Contrarily, estimation of the template activity of isolated RNA suggested an increase in mRNA (276). This might reflect the template activity of degradation products of mRNA (276).

If the bulk of RNA synthesis is inhibited by actinomycin D, synthesis of some proteins proceeds normally and the synthesis of others is stimulated. These effects have been noted both with mammalian (98, 277–279) and bacterial organisms (87, 280, 281), and have, in general, been explained by involving a stable mRNA (87, 98), or the actinomycin D-sensitive synthesis of a repressor (87, 277, 279, 280). An alternative explanation is the relative lack of susceptibility of the gene in question to actinomycin D, presumably because of peculiarities of base sequence (87, 280, 281).

Another anomalous effect of actinomycin is seen in its inhibition of the formation of T_4 progeny in infected *E. coli* (282). This inhibition occurs in the absence of any detectable effect on RNA synthesis, and indeed occurs even if the drug is added after cessation of viral RNA synthesis. Actinomycin D also inhibits the rate of methylation of DNA (283). Surprisingly, this inhibition is greater the lower the G+C content of the DNA. It has also been reported that in animal cells, actinomycin D may inhibit the transport of nuclear RNA to the cytoplasm (176, 255, 284, 285).

One of the most troublesome factors to be considered in evaluating experiments with actinomycin D is the possibility that the drug actually stimulates the breakdown of RNA (65, 276, 286). Although experiments arguing against such a stimulation have been reported (62, 73, 287, 288), the situation is complicated and the data not unequivocal. Our primitive understanding of the mechanisms and control of nuclease activity *in vivo* adds to the uncertainties in evaluating these experiments.

Translation

Having described the extant problems related to the isolation and synthesis of mRNA, we turn now to the question of how mRNA performs its presumed function. Quite apart from any studies with natural mRNA, the availability of a great variety of well-defined polyribonucleotides, synthesized primarily with polynucleotide phosphorylase, has permitted extraordinarily rapid progress in this area. Translation refers to that process in the synthesis of proteins by which a particular sequence of bases in mRNA determines a specific sequence of amino acids in a polypeptide chain. Translation is accomplished not by direct interaction of an amino acid with a specific nucleotide sequence (codon) on the mRNA, but through an intermediate adaptor molecule, tRNA, which is linked covalently to the amino acid (289-294). It is believed that a specific sequence of nucleotides (anticodon) in a tRNA molecule interacts by complementary, hydrogen-bonded, base pairing with the codon on the mRNA chain. The specificity with which a tRNA molecule is aminoacylated by its corresponding amino acid is therefore a

crucial event. The mechanism of this specificity is not well understood [(7), and review by Schweet in this volume].

The binding of mRNA and tRNA to ribosomes.—Translation requires an interaction of mRNA with ribosomes and with tRNA. A large number of *in vitro* studies, using polyribonucleotides and isolated ribosomes, have focused on the nature of the binding reactions. If, as discussed in the section on transcription, the initial interaction of mRNA with ribosomes *in vivo* is an active part of the transcription process, then serious reservations exist concerning the significance of the model studies. The model systems present several other problems, all of which are related to the purity of the ribosomal preparations. For example, *E. coli* ribosomes, usually the material of choice, contain a bound ribonuclease (295) and are generally contaminated with an RNA-specific phosphodiesterase (296, 297). Attempts to study the binding of mRNA to ribosomes in the absence of tRNA, or the binding of tRNA in the absence of mRNA, are further limited by the presence of both types of RNA in the usual ribosome preparations [(131, 298), and section on characterization and isolation]. With these reservations in mind we shall discuss first the binding of polyribonucleotides to ribosomes in the absence of added tRNA.

E. coli (76, 299) and reticulocyte (132, 300) ribosomes form complexes with, for example, poly U, poly UC, and poly UG. With limiting concentrations of polymer, several ribosomes are bound to a poly U chain (132, 299–302) resulting in complexes which are analogous to polysomes. These complexes form at low temperatures in the presence of 10 m*M* Mg^{++}, and in the absence of other factors required for polypeptide synthesis (e.g., energy source, GTP, amino acids). The binding is specifically with the 30S ribosomal subunit of *E. coli* ribosomes: 50S particles are inactive (301). With bacteriophage f_2 RNA (303), with turnip-yellow mosaic virus (TYMV) RNA (102), and with an excess of poly U (299–301), monosomes are formed with *E. coli* ribosomes. With somewhat higher Mg^{++} concentrations, more than one ribosome appears to associate with TYMV RNA (304). Some of the fragments obtained by brief alkaline degradation of TYMV RNA are also able to bind to ribosomes (305). When complexes of ribosomes with poly U (306) or bacteriophage f_2 RNA (303) are treated with pancreatic ribonuclease, the bulk of the polymers are degraded, but a small portion is resistant and remains bound to the ribosomes. The resistant fragments have chain lengths of from 22 to 28 residues in the case of poly U and about 35 residues in the case of f_2. The base composition of the f_2 fragment differs markedly from that of total f_2 RNA.

Polyribonucleotides that contain significant secondary structure bind less readily to ribosomes or 30S subunits (102, 301, 305, 307) than do polymers, such as poly U, which have little or no such structure (308). Destruction of the secondary structure permits interaction. For example, the binding of tobacco mosaic virus RNA to *E. coli* ribosomes requires incubation at 35° (299); poly C is bound only after deamination (307); the binding of poly A

and poly AU requires preliminary treatment with formaldehyde (307). Furthermore, polyribonucleotides can compete with one another for binding to ribosomes and the ability to compete effectively can be related to decreased secondary structure (305). Secondary structure also limits the mRNA activity of polyribonucleotides in a cell-free system for amino acid incorporation (48, 309, 310), and if that observation reflects the restrictions of the binding reaction, the latter may be limiting in *in vitro* polypeptide synthesis (102, 305). If mRNA is free *in vivo* prior to the formation of an mRNA-ribosome complex then areas of decreased secondary structure may direct the binding of ribosomes and consequently the starting point for reading of the message (305). On the other hand, if, as seems likely, the attachment of ribosomes to mRNA plays an active part in the release of mRNA from DNA (see section on transcription), the secondary structure of the mRNA would be irrelevant. It is noteworthy that the viral mRNA produced after infection of HeLa cells with vaccinia virus is free in the cytoplasm briefly and then forms a complex with ribosomes (139).

Although tRNA binds to ribosomes and in particular to 50S subunits in the absence of added mRNA (298), in the presence of added polyribonucleotides, tRNA is bound with specificity. The binding of any particular amino acid-specific tRNA is dependent on the presence of a polyribonucleotide containing the trinucleotide sequence corresponding to the codon for that aminoacyl tRNA (311–317). For example, poly U specifically directs the binding of phenylalanyl-tRNA and poly A the binding of lysyl-tRNA (314–316); similarly, GpUpU directs the binding of valyl-tRNA, but not leucyl- or cysteinyl-tRNA, both of which also have codons containing two uracils and one guanine (318). (The use of this binding reaction for the determination of the base sequences of triplet codons for each of the 20 common amino acids is described further below.) This specific association of tRNA and ribosomes has been studied both by sucrose density gradient centrifugation (311–315) and by taking advantage of the fact that the resulting complexes are retained by cellulose nitrate filters while tRNA itself is not (316). With *E. coli* (313–318) or liver ribosomes (319) the reaction is rapid and appears to be temperature-dependent. High concentrations of Mg^{++} ion are required (e.g., 10 m*M* or more (314–319) and K^+ or NH_4^+ ions are also necessary (313) with *E. coli* ribosomes. The amount of tRNA bound depends on the concentrations of both the ribosomes and the specific polyribonucleotide (239, 316, 320) and indeed, the latter also seems to be bound to the ribosomes in a manner that is interdependent with the binding of tRNA (321). In contradistinction to earlier studies which showed that unspecific binding of tRNA is to the 50S subunit (298), both the tRNA and the polyribonucleotide appear to bind to the 30S ribosomal subunit when they are bound with specificity (322, 323). Neither GTP, transfer enzymes, or polypeptide synthesis are required for binding with *E. coli* (313, 316) or liver ribosomes (319). Even N-acetyl phenylalanyl-tRNA binds to *E. coli* ribosomes in the presence of poly U (324) and indeed, tRNA need not be acylated with amino acid to be bound with specificity (314, 325). With reticulocyte ribosomes, GTP and an

enzyme fraction are required for binding [(311, 312), and article by Schweet in this volume].

Complete understanding of the binding reaction will require considerably greater understanding of the structure of the ribosome than is now possible (3). A newly proposed model of ribosome structure, which envisages an essentially nonhelical RNA with its phosphates exposed to the medium and its bases facing the protein, is of particular interest (326). Pretreatment of ribosomes with proteolytic enzymes or pancreatic ribonuclease destroys their ability to support the binding of either poly U or tRNA (327). The most compelling indication of the importance of ribosomal structure to complex formation comes from experiments with streptomycin. This antibiotic changes the specificities normally observed in both the polyribonucleotide-dependent binding of tRNA to ribosomes, and the polyribonucleotide-dependent incorporation of amino acids into polypeptide in cell-free systems. These effects are observed only if the 30S subunits of the ribosomes used are obtained from organisms that are sensitive to streptomycin. For example, under such conditions streptomycin inhibits the poly U-directed synthesis of polyphenylalanine (328–330), and stimulates both the poly U-directed incorporation of isoleucine into polypeptide and the poly U-directed binding of isoleucyl-tRNA to ribosomes (331–333). The binding of phenylalanyl-tRNA to sensitive ribosomes directed by UpUpU and UpUpC is also inhibited by streptomycin while the analogous binding of lysyl-tRNA in the presence of ApApA is stimulated (332). In addition to being dependent on 30S ribosomal subunits from streptomycin-sensitive organisms, many of these effects are highly dependent on the Mg^{++} concentration (330–332, 334).

The coding experiments.—The brilliant success of recent experiments that have permitted a general description of the genetic code has tended to obscure the fact that they also presented the first direct evidence that polyribonucleotides can serve in the manner predicted by the mRNA hypothesis. The discussion that follows is not meant to provide a review of the present status of the genetic code, but rather to point out how the coding experiments support the mRNA hypothesis and what they reveal about the mechanism of mRNA translation.

Nirenberg & Matthaei (47, 48) described a cell-free system from *E. coli* (containing ribosomes and a soluble fraction) which was depleted of DNA by treatment with DNase and of mRNA by preincubation thereby losing most of the endogenous capacity to synthesize protein. The addition of polyribonucleotides to such a system permits amino acid incorporation into polypeptide and the specific amino acids incorporated depend on the particular nucleotide content of the added polymer. This work, which has been reviewed extensively (13, 14, 49, 335), led to the description of the base composition of RNA codons for the 20 amino acids, assuming a triplet code, and suggested certain properties of the code: (*a*) the code is degenerate—a given amino acid may be coded by more than one codon; and (*b*) codons may be ambiguous—the specificity of codons for amino acids is not absolute. The recent further elucidation of the code has depended on the

polyribonucleotide-directed, specific binding of tRNA to ribosomes. Using trinucleoside diphosphates of known sequence, the base sequence of triplet codons for each of the 20 common amino acids has been determined for *E. coli* (Table I) (317, 318, 335–340).

TABLE I

NUCLEOTIDE SEQUENCES OF RNA CODONS IN *E. COLI*

5′-OH Terminal Base	Middle Base				3′-OH Terminal Base
	U	C	A	G	
U	*Phe*	*Ser*	Tyr	*Cys*	U
	Phe	Ser	Tyr	Cys	C
		Ser			A
	Leu	Ser		Trp	G
C	Leu	Pro	His	Arg	U
	Leu	*Pro*	*His*	Arg	C
		Pro	Gln	Arg	A
	Leu	Pro	Gln	Arg	G
A	Ileu	Thr	Asn	Ser*	U
	Ileu	Thr	Asn	Ser*	C
		Thr	*Lys*	Arg	A
	Met	Thr	*Lys*		G
G	Val	Ala	Asp	Gly	U
	Val	Ala	Asp	Gly	C
	Val	Ala	*Glu*	Gly	A
	Val	Ala	*Glu*	Gly	G

These results are compiled from the work of Nirenberg and co-workers and Khorana and co-workers. The references are given in the text. There is no disagreement concerning the assignment of any codon but certain codons are either unassigned or only predicted by one or the other group. The italicized codons are those derived from both polypeptide synthesis and binding experiments.

* Uncertainties exist in these assignments (317, 340).

Work with long chain polymers of known sequence (239, 320, 341) supports the validity of the assigned codons and also proves several important facts relevant to the mechanism of mRNA translation. Two examples of such experiments will, therefore, be described in some detail. A polymer containing a strictly alternating sequence of adenylic and cytidylic acid residues and therefore a sequence of two strictly alternating, trinucleotide sequences, namely, ApCpA and CpApC was prepared. This polymer directs the syn-

thesis of a polypeptide containing alternating threonine and histidine residues. The incorporation of each amino acid depends on the incorporation of the other and the two are incorporated in equimolar amounts (239, 341). This confirms the assignment of ApCpA and CpApC as codons for threonine and histidine, respectively (Table I). The polypeptides produced in the presence of polyribonucleotides containing repeating trinucleotide sequences, as for example, . . . ApApGpApApG . . . ApApG . . . , have also been identified (320, 341). With this polymer three, and only three, different homopolypeptides are formed, namely, polylysine, polyarginine, and polyglutamic acid, again confirming the codon assignments derived from trinucleoside diphosphate binding studies (Table I).

A triplet code.—An oligonucleotide of chain length 3 represents both a minimum and sufficient length to induce the binding of a specific tRNA; dinucleotides are ineffective (316, 318). The experiments with polyribonucleotides of known base sequence such as . . . .ApCpApCpApC. . . . ApC. . . . and . . . .ApApGpApApG. . . .ApApG. . . . confirm unequivocally that the RNA code is a triplet code (341). Earlier genetic experiments suggested that the DNA code is a triplet code (342). In this characteristic RNA functions as a faithful messenger for DNA.

Degeneracy and the recognition process.—The list of codons shown in Table I is highly degenerate and several patterns of degeneracy are seen (317, 339): (*a*) for a given amino acid, the first two bases of degenerate codons are the same while the third base (3′-hydroxyl terminal) may be either of the two purines, or, similarly, either of the two pyrimidines; (*b*) for a given amino acid, the first two bases of degenerate codons are the same while the third base may be any one of the four common bases; and (*c*) the first base (the 5′-hydroxyl terminal) may vary while the second two remain constant. Degeneracy in the RNA code is consistent with conclusions from genetic experiments (343, 344). It is possible that more than one of the degenerate codons for a given amino acid may be recognized by the same tRNA, or else that a different tRNA may exist for each of the degenerate codons. Both alternatives may in fact occur. The existence of more than one tRNA for each of several amino acids has been demonstrated (7) and it is clear, both from amino acid incorporation studies and binding experiments, that degenerate codons may indeed be recognized by different tRNA molecules. One of the four different leucine tRNA species that have been detected is specific for poly UG (345–347) in incorporation studies, and the trinucleoside diphosphate, UpUpG, binds to ribosomes specifically with this fraction (317, 336). The codon CpUpG is probably specific for another one of the four known leucine tRNA fractions (317). Furthermore, a functional distinction between two of the leucine tRNA species has been demonstrated by labeling one with ^{3}H-leucine and the other with ^{14}C-leucine. The two fractions were then used simultaneously as sources of leucine in the cell-free synthesis of hemoglobin. Degradation of the globin product yielded characteristic leucine-containing peptides which were labeled either with ^{3}H or with ^{14}C (348). Other experiments suggest that a given tRNA may recognize more

than one degenerate codon. Although 100 per cent of phenylalanine tRNA binds to ribosomes in the presence of poly U, 60 per cent can also bind to ribosomes in the presence of UpUpC (337). Binding experiments have been carried out (349) with highly purified alanine tRNA which represents an essentially homogeneous collection of molecules (350), except that in 50 per cent of the molecules one dihydrouracil moiety is replaced by uridine. Three trinucleoside diphosphates representing degenerate codons for alanine (GpCpU, GpCpC, and GpCpA) supported the binding equally well, while a fourth (GpCpG) was less efficient. Similarly, one species of serine tRNA binds to ribosomes in the presence of either UpCpU or UpCpC, but not with UpCpA or UpCpG (317, 347). Another species of serine tRNA shows the reverse specificity (317). However, binding experiments with trinucleoside diphosphates can be misleading. For example, although poly A and poly $(AAG)_n$ each directed the binding of a different lysine tRNA fraction, no such specificity was demonstrable with ApApG and ApApA (317, 320). Thus, it seems likely that both alternatives may occur, i.e., multiple and single tRNA molecules may exist for sets of degenerate codons (337, 339, 351).

Some insight into the function of degeneracy may come from recent experiments with *E. coli* methionyl-tRNA. A portion of the methionine amino groups of total methionyl-tRNA can be formylated enzymatically (352, 353) and the resulting formylmethionyl-tRNA appears to respond, in binding experiments to UpUpG (353). More generally, it has been proposed that the degenerate codons may be used for the same amino acid in different positions in mRNA, for example, either as internal or external (terminal) codons (337, 340).[4]

It is widely believed that recognition occurs by means of Watson-Crick type, hydrogen-bonded base pairing between the codon on the mRNA and a complementary, trinucleotide anticodon on the tRNA. The base sequence of only one tRNA, namely, the yeast alanine tRNA, is known at this writing (350) and within the molecule there are several triplet sequences complementary to alanine codons. The experiments described above, however, suggest that the specificity of recognition may, in many cases, reside primarily in the 5′-terminal doublet of the codon. The anticodon might then be only a doublet, or the anticodon base corresponding to the nonspecific base in the codon may be an unusual one, allowing nonclassical base pairing with any of the possible corresponding bases on the codon (339, 351). It has, in fact, been suggested that the inosine residue in yeast alanyl-tRNA plays this role, and that the anticodon of yeast alanine tRNA is IpGpC (350, 351). If this is true, then codon-anticodon interaction would be antiparallel (Table I). Many investigators have pointed out that the instability of hydrogen-bonded complexes between trinucleotides and polymers at physiological temperatures makes it unlikely that the proposed codon-anticodon inter-

[4] Very recent work (397, 398) in fact suggests that the codon for N-formylmethionyl-tRNA is the signal for initiation of polypeptide synthesis.

action occurs. The hydrogen-bonded complex between pApApA and poly U is not stable above 18° and for the dinucleotide pApA the corresponding temperature is 10° (354). This argument, however, overlooks the role of the ribosome since the binding of both mRNA and tRNA to ribosomes may afford spatial restrictions which add stability to recognition. A direct influence of ribosomal structure on recognition is suggested by the experiments with streptomycin (described in the section on binding, p. 211).

Nonsense codons and chain termination.—The list of codons in Table I contains several triplets which are not assigned to amino acids (nonsense codon). Since known codons may fail to stimulate the binding of appropriate amino acyl-tRNA (317), negative results may simply represent experimental difficulties. Recent genetic experiments indicate, however, that at least two of the nonsense triplets do not, in fact, correspond to any amino acid but rather serve to code for the termination of a polypeptide chain. Under normal conditions, certain known mutations prevent the complete synthesis of the polypeptide specified by the mutated gene. Synthesis is blocked at the point of mutation (355) and the mutated codons appear incapable of coding for any amino acid (i.e., are nonsense codons) [for an excellent review, see (356)]. These mutations are partially reversed when they are present in an organism containing a second type of mutation, known as a suppressor (356). It has been demonstrated directly that a suppressor gene can correspond to a tRNA species which permits the recognition of what is normally a nonsense codon (18). The particular suppressor gene studied is known to insert serine at the point of nonsense (357–359), and serine tRNA from the organism containing the suppressor permits the translation of RNA from a suppressible mutant of R_{17} bacteriophage *in vitro*, while tRNA from a normal organism does not (18). Studies of amino acid substitutions in revertants from nonsense mutants for *E. coli* alkaline phosphatase (360), interpreted in the light of the known codons, indicate that UpApG is the nonsense codon for one group of nonsense mutants. Studies of reversion mechanisms have led to the same conclusion as well as indicating that UpApA is the nonsense codon in another group (361). These results support the conclusions of the coding experiments (Table I) and suggest that the information for polypeptide chain termination is present on the mRNA molecule in the form of so-called nonsense codons. The details of the mechanism of chain termination are unknown, and indeed the evidence summarized here comes from experiments with only one organism, *E. coli*.

Ambiguity.—The results of binding experiments confirm those of incorporation studies in demonstrating ambiguities (320). Ambiguities are dependent on Mg^{++} concentration, temperature, and tRNA concentrations (10, 317, 320, 362, 363), and are enhanced by streptomycin as discussed earlier. The replacement of phenylalanine by leucine, a well-documented *in vitro* ambiguity, may also occur *in vivo* (364).

Characteristics of the RNA code.—Genetic experiments indicated that the information of the DNA genome is colinear with the protein produced (343, 355, 365). That is, the order of the codons in the gene is the same as the order

of the amino acids in the corresponding polypeptide. The experiments with polymers containing alternating trinucleotide sequences that are described above show that the information in mRNA is also colinear with the polypeptide. Furthermore, these experiments as well as those with the polymers containing regularly repeating trinucleotide sequences demonstrate that the code is not overlapping. This is also consistent with predictions about the DNA code obtained from genetic experiments (13, 344, 366). The experiments with polyribonucleotides containing alternating triplets show that contiguous triplets are read sequentially. Once polypeptide synthesis is initiated, it continues in a phased manner. Nucleotides are not skipped in the reading and no commas are required; the DNA code is also comma-less (13). The sequential and consecutive reading of codons is supported by *in vitro* experiments utilizing phage f_2 RNA as a messenger. In this system, incorporation of an amino acid relatively distal from the amino terminal end of the polypeptide being synthesized depends on the prior incorporation of an amino acid more proximal to that end (367). The *in vitro* experiments, using synthetic polyribonucleotides as mRNA, have clearly demonstrated that RNA can serve as a functional replica of the DNA genome. The characteristics of the DNA code deduced from genetic experiments are duplicated by the RNA code.

The direction of translation.—Protein synthesis starts from the NH_2-terminal amino acid and proceeds stepwise towards the carboxy terminal amino acid (368–372). An mRNA chain also has a polarity derived from the direction of the internucleotide bonds. Thus, the message might be translated starting at the 3′-hydroxyl end (3′ to 5′ reading, i.e., right to left[2]) or starting at the 5′-hydroxyl end (5′ to 3′ reading, i.e., left to right[2]). In the most direct approach to this question a polyribonucleotide containing a specific triplet at one end of an otherwise homopolymer chain is used in an incorporation experiment; the location of the amino acid specified by the terminal triplet in the resulting polypeptide chain determines the direction of reading. Early and inconclusive experiments suggested that reading was 3′ to 5′. More recently a cell-free system low in nuclease activity and improved methods for the characterization of both the polynucleotide and polypeptide have been developed. Data obtained with the new system give the opposite result, namely, that reading is 5′ to 3′. The polynucleotide ApApAp. . . . . .pApApC (chain length between 21 and 23) directs the synthesis of a group of polypeptides containing lysine as the NH_2-terminal residue, a sequence of internal lysine residues, and a COOH-terminal asparagine (373). Similarly, the hexanucleoside ApApApUpUpU directs the synthesis of lysylphenylalanine (374), again showing that reading is 5′ to 3′.

Investigation of the effect of specific exonucleases on the synthesis of hemoglobin in a cell-free system has led to the opposite conclusion, namely, that reading proceeds in the 3′ to 5′ direction (375, 376). The authors of these reports consider and rule out the possibility that the results are attributable to endonucleases that contaminate the spleen and snake venom phosphodiesterases used, but such an explanation still seems plausible. Since the

exonucleases were present both during a preincubation of the reticulocyte polysomes and in the subsequent amino acid incorporation incubation, some component of the reaction other than the mRNA for globin may have been affected. One possibility is the ribosome itself.

Convincing results pertinent to the question of the direction of mRNA translation have come from a completely different type of experiment (377). Mutants of bacteriophage T_4 that lack lysozyme can be produced by proflavin. Two such mutants, each devoid of lysozyme, were used to obtain a recombinant organism having some lysozyme activity. Analysis of the lysozyme proteins showed that in the recombinant enzyme, each of the five amino acids in a particular polypeptide sequence is different from the five amino acids appearing in the same positions in the wild-type protein. By selecting triplet codons for each of these amino acids from the list of known codons (Table I), a possible structure for the mRNA denoting the wild-type pentapeptide was constructed. Assuming that the mechanism of mutagenesis by proflavin involves the deletion or the insertion of a single base pair on the DNA (378) and assuming further that one of the two original mutations was such a deletion and the other an insertion, various structures for the mRNA denoting the recombinant pentapeptide can be written. One such sequence is consistent with the known triplet codons (Table I), and that sequence depends on a translation of the code in a 5′ to 3′ direction. Several comments on this interpretation must be made. First, the mechanism of proflavin mutagenesis is not definitely proven (356). Indeed, this experiment itself provides the most direct substantiation of the proposed mechanism. Second, given the high level of codon degeneracy, a large number of alternative mRNA sequences may be written for both peptides. Using the known codons, we have tried to construct alternative mRNA sequences, in particular ones which would lead to 3′ to 5′ reading, but we were not successful. Therefore, the most satisfactory experiments available (373, 374, 377) at this writing all indicate that translation is in the 5′ to 3′ direction.

As pointed out by Stent (234), 5′ to 3′ translation means that mRNA transcription and translation take place in the same direction. The initiation of translation prior to the completion of transcription is then feasible (see section on transcription). Very little data relevant to the mechanism of the initiation of translation is available. In binding experiments, oligonucleotides with a 5′-terminal phosphate group are more active than those lacking a terminal phosphomonoester group (337, 339), and esterification of the 5′-phosphomonoester group of poly U causes a loss of template activity (379). Similarly while ApUpU may give only a small stimulation of the binding of isoleucyl-tRNA, ApApApApUpUpUpU, which contains ApUpU as an internal triplet, affords good stimulation (325). It has been suggested that a 5′-phosphomonoester group may be involved in the initial attachment of mRNA to the ribosomes (316, 325). In *in vitro* systems it is possible that initiation may occur anywhere along the mRNA chain since any trinucleotide sequence within a short mRNA chain can direct the binding of a specific tRNA regardless of whether or not the end of the chain has a 5′-phospho-

monoester group (325). The *in vitro* systems may lack certain components required for specific initiation. These factors might include a structural property of natural mRNA.

Polycistronic messengers.—The RNA of RNA viruses appears to be polycistronic (5, 12, 16, 17, 32, 380), and the existence of polycistronic mRNA in DNA-dependent systems was described earlier in this article. The genes of the lactose operon of *E. coli* (232) and the histidine operon of *S. typhimurium* (233), both of which produce polycistronic mRNA, can be expressed sequentially starting from the operator locus. Sequential expression may reflect either sequential translation or sequential transcription, or both. In a cell-free system, the translation of phage MS2 RNA appears to be sequential (380), although a criticism of this interpretation has been made (305). The translation of several viral-specific peptide chains in HeLa cells infected with polio virus is not synchronous (381). The binding of only one ribosome to f_2-bacteriophage (303) and turnip-yellow mosaic virus (102) RNA is consistent with a mechanism of sequential translation since independent translation might lead to multiple binding sites. It is, of course, quite possible that both sequential and independent translation are functional in different situations. If translation of a polycistronic mRNA involves the consecutive synthesis of polypeptides by a single group of ribosomes traveling the length of the chain, then mechanisms for the control of translation must exist. Such controls are demanded by the fact that separate genes of an operon can cause the production of different numbers of polypeptide molecules (12, 32, 381–383), and by the phenomenon of operon polarization (12). The modulation theory suggests that a particular tRNA may act to control translation rates (12). Other mechanisms have also been discussed (384).

The Degradation of mRNA

The mechanism responsible for mRNA degradation in bacteria is not clearly understood. Anaerobiosis protects the synthetic capacity of *E. coli* preinduced for β-galactosidase (385) and inhibits the decay of pulse-labeled RNA of *B. subtilis* (78). Polynucleotide phosphorylase (150, 151, 386–388) may be involved in mRNA breakdown but since pulse-labeled RNA can be degraded in the absence of inorganic phosphate (389), other mechanisms must also exist. The potassium-activated phosphodiesterase of *E. coli* (RNase II) (296, 297) has also been considered, but it, too, is probably not an exclusive mechanism (67). The specificity of both of these enzymes for single-stranded polyribonucleotides (297, 390) is consistent with a specific action on mRNA, and indeed, it has been demonstrated that formation of helical structures protects poly U against degradation in cell-free amino acid incorporation systems (391). The endonuclease that is found bound to *E. coli* ribosomes (*E. coli* RNase I) is probably not involved in mRNA breakdown (11, 392, 393).

Conclusion

We have attempted to prepare a critical evaluation of the messenger problem and to emphasize certain vague facets of the chemistry of mRNA structure and function. In general, the experiments performed since the proposal of the mRNA hypothesis are remarkable in their support of the overall scheme. Although some uncertainties exist, it is likely that the DNA-dependent RNA replicases represent the mechanism of transcription. The *in vitro* experiments on polypeptide synthesis similarly describe a detailed mechanism for translation. Studies with viruses demonstrate clearly that mRNA is both synthesized and translated.

Problems do, however, arise when one considers the lack of direct evidence for the synthesis and translation of mRNA in organisms other than infected organisms. The ideal experiment should include (*a*) the isolation of a unique molecular species of RNA having a base sequence identical to some portion of the DNA; and (*b*) the synthesis of a specific polypeptide under the direction of that RNA. Furthermore, the base sequence of the mRNA should be consistent with that predicted for the amino acid sequence of the polypeptide by the codon dictionary. The recent studies by Khorana and co-workers are prototypes for such an experiment since synthetic DNA templates were used to prepare the synthetic messengers of known sequence. Several attempts have been made to develop cell-free systems capable of the combined processes of transcription and translation, leading to the *de novo* synthesis of specific enzymes (394, 395). Although positive results have been reported, unambiguous interpretation thereof requires definitive evidence of lack of contamination by whole cells or protoplasts. Another question that may reasonably be posed concerns the generality of the scheme. Is it necessary that all protein synthesis be mediated by an RNA template? The demonstration that deoxyribopolynucleotides can serve directly as templates for amino acid incorporation *in vitro* is of interest in this connection (396).

The heuristic value of the mRNA hypothesis is strikingly evident in considering biological control mechanisms. Indeed, the initial statement of the hypothesis was interdependent with an hypothesis concerning control mechanisms for protein synthesis and focused on control at the level of transcription. Subsequent work has largely followed this lead. Clearly, the hypothesis is equally cogent because of the inherent possibilities for control of protein synthesis at the level of translation. A sophisticated appreciation of the variety of possible control mechanisms will depend on the clarification of still nebulous processes, in particular those of initiation and termination of transcription and translation, and of recognition of codons by tRNA.

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