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MEDICAL PROGRESS

THE INVOLVEMENT OF RNA IN PROTEIN SYNTHESIS (Concluded)*

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TRANSLATION OF mRNA

Direction of Translation

Messenger RNA possesses a chemical polarity in its structure. The phosphodiester linkage runs from 5′ to 3′ on the ribose moiety of adjacent nucleotides. When the message is translated into protein, in which direction is it read? Direct in vitro experiments bearing on this subject suggest that the reading is from the 5′ to the 3′ end.

It is known[55] that in the synthesis of a protein, the NH_2 terminal end is synthesized first. Accordingly, if a polynucleotide messenger of known base composition is used to direct in vitro protein synthesis, the NH_2 terminal amino acid may be correlated with the codon known to be present on the 5′ or 3′ end of the message. The polynucleotide ApApAp . . . $(AP)_{3N}$ . . . ApApC directs the synthesis of peptides whose NH_2 terminal residue is lysine and whose COOH terminal residue is asparagine.[105] Similar results have been obtained with the hexanucleotide ApApApUpUpU, resulting in the synthesis of lysyl-phenylalanine, again with the 5′ triplet represented at the NH_2 terminus.

In vivo studies involving the alteration of a peptide chain by the use of proflavine as a mutagen support the conclusion that mRNA is read from 5′ to 3′. This mutagen works by inserting an extra base into the DNA chain. The messenger RNA subsequently made must also have an extra base and therefore, since reading of only contiguous triplets occurs, the extra base must alter the reading of succeeding triplets. Analysis of a short chain of altered amino acids caused in this manner shows it to be consistent only with 5′ to 3′ reading of the message.[106]

Initiation of Translation

Just as natural mRNA must have a specific direction of reading, the initiation and the reading frame of translation must be controlled. Although short synthetic polynucleotides apparently have no specific starting triplet, natural mRNA appears to be restricted as to where reading starts. The initiation at the 5′ end of the message may be dictated on the one hand by restrictions involving secondary structure or by the existence there of a specific starting triplet or sequence of triplets that directs its binding to the ribosome. If binding could occur at any 5′ end of the message the number of molecules bound should vary linearly with the number of 5′ ends available. Partial random degradation of viral RNA does not increase its number of ribosome binding sites.[107] This points to base specificity as the mechanism for proper alignment of the message on the ribosome. Two species of tRNA specific for methionine exist. One is involved in the formylation of the amino group of the methionine. This latter tRNA binds to ribosomes specifically under the aegis of the triplet AUG, the former in response to AUG, CUG, GUG and AUA.[82,83,108,109] Experiments employing AUG in known positions in a synthetic polynucleotide indicate that this codon is a

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"phaser" – that is, it promotes the reading of the adjacent 3′ triplet by selecting the reading frame.[110] This kind of evidence strongly implicates AUG as at least part of the initiation signal. The adjacent triplets, in combination with the location of AUG at the 5′ position, may therefore be involved in the mechanism of initiation on natural mRNA.

Termination of Translation

Although it is apparent that ordered initiation of translation is a necessity, a signal for chain termination is less so. When ribosomes reach the physical end of a message, release may occur as an uncomplicated event. In the formation of a peptide bond, the bond between tRNA and the amino acid is broken. Once this bond is broken the absence of an oncoming amino-acyl tRNA may result in the release of the finished protein. Release of a finished protein made from a polycistronic* message suggests the requirement of a special release mechanism. Also, in vitro studies with polyuridylic acid as a messenger suggest the necessity of a release triplet (or triplets) as the product polyphenylalanine is not released from the ribosome. Genetic experiments reveal that certain known mutations (termed "amber" and "ochre") result in nonsense codons. Synthesis of protein is arrested at the point in the message that the nonsense triplet occurs, and the incompleted protein is released.[111] This indicates that nonsense triplets do not code for any amino acid and suggests the possibility that these codons (UAA and UAG) serve as natural "release" triplets. The problem of protein release seems critical mainly in relation to polycistronic (polygenic) messenger RNA (one in which the message for several proteins is strung together). In this case, however, if the initiating triplet of each cistron always involved an N-formylated amino acid, peptide condensation at this point would be prevented, and release of the completed protein may occur without any special mechanism.

Fidelity of Translation

Fidelity in Vivo

Given a gene designating a specific amino acid sequence, what is the frequency with which an incorrect amino acid is inserted in a position in the protein? In a careful measurement of the frequency with which leucine, isoleucine, and valine substituted for one another at particular positions in hen ovalbumin, Loftfield[112] concluded that a maximum estimate of the error frequency was 1 part in 3000. Although there are a number of reasons why this estimate could be off in either direction, it is clear that the system has a high degree of precision.

There are four possible sources for the errors observed by Loftfield: Somatic mutations arising from incorrect replication of the DNA; errors in transcription of the mRNA; errors in the formation of the amino-acyl-tRNA complex; or errors arising from incorrect interaction between the amino acyl-tRNA and the mRNA-ribosome complex. In most of these cases the incorrect hydrogen bonding of one base pair would produce an error. However, from thermodynamic arguments, the hydrogen bonding will not provide adequate discrimination. It is now considered that the base pairing is supplemented by conformational requirements of the synthetase enzymes – that is, the hydrogen bonding acts only as the key that causes a lock to close. Thus, streptomycin probably produces translational errors by acting on the ribosome to change the conformation of the tRNA site so that it will accept tRNA's that do not bond perfectly with the messenger RNA.

It should be pointed out that, precise as it is, the protein synthetic system is by no means perfect. Given the 20 amino acids and the measurement that 1 amino acid substitutes for another 1 time in 3000, the mean number of errors in a polypeptide of 150 amino acids may be $\frac{1}{3000} \times 20 \times 150 = 1$. Since there are no available measurements, we have no idea whether this is a correct analysis of the situation. However, one estimate, based on the analysis of various classes of mutants[113] indicates that more than 95 per cent of proteins with a single, random amino acid replacement retain at least some enzymatic activity.

Ambiguity in Vivo

It has recently been found that in certain cases, 1 gene can give rise to more than 1 polypeptide chain – in fact, to a whole family of closely related peptide chains. The observation is that certain positions of the alpha chain of rabbit hemoglobin can be occupied by more than 1 amino acid.[101] It is unlikely that these results are due to a high error frequency, as the other positions of the chain have only a single amino acid. The amino acid multiplicities are attributed to ambiguous codons – that is, codons that interact with either of 2 or more tRNA molecules. Although such an ambiguity has been observed in cell-free studies using polynucleotides as messengers, this is the first evidence that ambiguity is a natural phenomenon. Its evolutionary significance remains to be assessed.

Fidelity of Translation in Vitro

Although in vitro studies have revealed a considerable ambiguity in the reading of codons, this phenomenon has been manifested only under conditions of environmental duress involving temperature,[114] presence of organic solvents[100] or excesses of magnesium ions.[114] Extensive ambiguity in vivo would probably be lethal to an organism. Excitement has recently been generated by the finding that certain drugs – notably, the aminoglycoside antibiotics – produce extensive misreading of mRNA both in vivo[115] and in vitro.[116] The original

*Cistron indicates a genetic material representing a single gene or single polypeptide chain.

discovery was that certain mutants of *Esch. coli* were able to grow only in the presence of low concentrations of streptomycin. These mutants, whose growth required the presence of arginine, were sensitive to streptomycin but were able to grow in the absence of arginine when the antibiotic was present at sublethal concentrations. It was then found that in vitro, streptomycin caused the misreading of polyuridylic acid, resulting in the incorporation of leucine and isoleucine in the place of phenylalanine. These results were obtained only when ribosomes from streptomycin-sensitive bacteria were used. The ribosomes of bacteria highly resistant to the antibiotic failed to show this effect (or did so at a very low level). The interpretation given to these results was the following:

> The initial mutation to the requirement for arginine resulted in an altered triplet in the mRNA so that either an incomplete or an abnormal protein was made.
>
> The presence of streptomycin causes misreading of the code, with the result that occasionally the abnormal triplet was read as normal (for example, UUU read as CUU), resulting in an active enzyme for arginine synthesis.
>
> Since the ability to allow this action of streptomycin was associated with that part of the ribosome (30S) that binds the message it was concluded that the ribosome takes an active role in the translation of the message, and that the antibiotic, by binding to the 30S subunit and interfering with this function, altered the reading of mutant codon. The mutation to resistance apparently alters the 30S component of the ribosome in a way that although streptomycin still binds to the ribosome, it does so in an innocuous fashion.

Analysis of the specific amino acid substitutions induced by streptomycin reveals a pattern of the misreading of the bases.[117] Streptomycin causes very specific amino acid substitutions. Apparently, only the 2 pyrimidine bases are misread, the pyrimidines in the 5′ position of the triplet being misread for the opposite pyrimidine (for example, U for C) and the internal pyrimidines of a triplet being read as a pyrimidine or as a purine depending upon the neighboring base. In this way it is seen that the misreading induced by streptomycin is specific. Several classes of the mutants termed conditionally streptomycin dependent have now been found. The pattern of misreading can be shown to differ for some of these classes. It is hoped these mutants will allow further study of the chemical aspects of miscoding. This, coupled with analysis of the genetics, which is now possible, of the ribosomes may open up this cloudy area of protein synthesis.

More extensive evaluation of codon misreading reveals that related antibiotics — notably, neomycin[48,115,116] — also behave in this manner. Neomycin may even induce single-stranded DNA to act as a message for protein synthesis.

Whether the sole lethal mechanism of these antibiotics resides in induced code misreadings is doubtful,[117] but the phenomenon has elucidated the role of the ribosome in the translation process and opened the possibility of its further investigation.

Experiments designed to investigate the translational fidelity of mammalian,[118] avian[119] and euglena[120] ribosomes show that these are inherently resistant to the action of aminoglycoside antibiotics. Our inability to understand differences such as these between bacterial and nonbacterial ribosomes resides in our almost total ignorance of how the ribosome partakes in the process of protein synthesis.

The phenomenon of streptomycin-induced misreading has its natural counterpart. Certain mutations appear to have their effects reversed or "suppressed" by second mutations in the same organism. These second or "suppressor" mutations may be mapped (located genetically) within or distant from the cistron in which the initial mutation occurred. When it occurs within the same cistron a second faulty amino acid is inserted into the protein that, by further altering the configuration of the already distorted protein, restores some activity. Some "suppressors" that map away from the first mutation are now known to cause misreadings of the code, similar to those induced by streptomycin, but by a different mechanism. In some cases a faulty tRNA is produced such that the accuracy of adapting its proper amino acid has been altered. This abnormal RNA with a certain low probability misreads the nonsense (UAG) codon as though it were sense (serine, UCG). Some nonsense mutants of bacteriophage R17 have been shown to make coat protein in vitro only in the presence of tRNA from a species of *Esch. coli* carrying a "suppressor" mutation.[121] Suppressors may thus salvage otherwise lethal mutations. All mutations, however, cannot be corrected by a single suppressor. This shows that misreading caused in this manner, like that of streptomycin, is not general, but quite specific — that is, the mistake frequency is enhanced only for a certain codon, with the result that a specific amino acid substitution occurs.

Control of Protein Synthesis at the Level of Translation

The induction or repression of certain enzymes take place very rapidly in relation to the generation time of the cell. The control of this function has for some time been believed to exist mainly at the level of transcription (that is, the cellular response to an inducer or repressor molecule is mediated through the turning on or off of the synthesis of mRNA specific for a given enzyme). The ability to identify messages only during induction, and the disappearance of these messages during repression strengthened this idea. Little attention was paid to the possibility of the existence of control at the level of translation.

The theory of the *operon*[32] was predicated at least in part on the finding of 0° (for operator-off) mutants.* These mutants failed to synthesize beta-galactosidase in the presence of an inducer molecule, and genetic analysis led to their being considered incapable of synthesizing mRNA specific for this enzyme. However, Beckwith[122] discovered that certain 0° mutants could be suppressed. Since the phenomenon of suppression is associated only with translation, this meant that under certain circumstances of 0° mutants, the mRNA of 0° mutants must be made, but that at the step of translation into protein, it was impaired. This implied the possibility that certain control steps could be mediated after transcription, and that translational control elements must be considered.

Although the operon concept does not require the synthesis of a polycistronic messenger, the findings of Ames and Hartman[123] suggested that this was true. In this case a single species of mRNA was identified whose chain length was consistent with the ability to code for the whole series of enzymes necessary for the synthesis of histidine. Suggestive evidence indicates that polycistronic messages are also translated as a single unit.[124] It is conceivable that ribosomes might attach at the beginning of each *cistron* or only at a single initiation point. The evidence presented above suggested that only single points of initiation exist. If only 1 chain of mRNA coded for several enzymes, and if the ribosomes could only attach at 1 end, as the ribosomes moved over the mRNA, the number of protein molecules synthesized for each cistron should be the same. Extensive characterization of the enzymes involved in both the his and lac operons allowed the enumeration of the actual protein molecules synthesized under the direction of these messages. It was found that[125] the number of protein molecules synthesized from the beginning (operator end) of the message might be thirty to thirty-five times greater than that of proteins synthesized from the distal end. A mechanism for this paradoxical situation was suggested by analysis of mutants that produced an analogous condition. "Polarity" mutants, those in which a mutation to the absence of 1 enzyme proximal to the operator resulted in the lowering of the number of enzyme molecules made from the distal end of a polycistronic message, were described for both the lactose[126] and the histidine operons. Recently, technics permitting the classification of polarity[127] mutants into the nonsense or missense variety† have become available. It is quite clear that only nonsense mutants may be polar. Beckwith's suppressible 0° mutants are now interpreted as extreme polar mutants. The most comprehensive view of polar mutants is summarized as follows: there appears to be only 1 normal point of binding of the message to the ribosome, and this is at the operator end. The hypothesis is that a mutation causing a nonsense triplet in the message results in the disalignment of the ribosome from the proper reading frame; the ribosome then slips along the message out of phase with the triplet codons with a certain probability of disassociating from the message; if the ribosome remains attached to the message until the beginning of the next cistron, an initiating type of codon once again establishes the reading frame, and protein synthesis resumes; in this theory, the degree of polarity depends both upon the physical distance the ribosome slips, the probability of disassociation being proportional to this distance, and upon the efficiency of frame realignment at the beginning of the next cistron.

In the normal situation described above for the lactose operon, it is assumed that a nonsense triplet exists between the end of 1 cistron and the beginning of the next. The evolutionary appearance of the operon suggests that it is expedient for the cell to control co-ordinately the synthesis of mRNA for several enzymes concerned with 1 function. However, the efficiency of the individual enzymes within a given operon may be such that unequal numbers of molecules are needed. The findings illustrated with polarity mutants propose a scheme within which transcription of a polycistronic message ensures that a group of enzymes are co-ordinated, but control at the level of translation allows different numbers of the actual proteins to be produced.

Control of Transcription at the Level of Translation

Recently, more attention has been paid to the inter-relatedness of protein and RNA synthesis. The inhibition of protein synthesis by the removal of an essential amino acid results in the immediate cessation of net RNA synthesis in normal bacterial cells. Recent experimentation suggests that the role of the amino acid in allowing transcription is quite complicated and may involve the translation step itself.

The report by Byrne et al.[128] that an apparently natural complex of DNA-RNA and ribosomes could be found in *Esch. coli* prompted new speculations. It is now proposed that mRNA is removed from DNA by the binding of mRNA to active ribosomes. The relative movement of the message past the ribosomes assists in some manner in the separation of mRNA and DNA.

A summary of several facts concerned with macromolecule synthesis may be of help in an understanding of the basis of this formulation: proteins are synthesized beginning with the amino and ending with the carboxyl terminal ends; RNA is synthesized beginning with the 5′ end, and ending with the 3′ end; and mRNA is translated from the 5′ end – that is, the NH_2 terminal amino acid of a protein is coded for by the triplet at the 5′ end.

Thus, it is possible that since the 5′ end of a

*Operon is defined as a genetic unit consisting of a cluster of genes all under the control of an adjacent chromosomal locus, termed an operator region.

†Frame-shift mutants also exhibit polarity, but this is probably mediated through the reading of a nonsense triplet.

message is made and presumably ready to engage in protein synthesis, its attachment to a ribosome and its subsequent translation begin even before the completion of the 3′ end of the message. Indeed, the finding that the direction of transcription and translation are the same supports the hypothesis that ribosomes assist in the removal of the message. Viewed from this perspective, the apparent control at the level of translation of the transcriptive process may be considered in more mechanical terms.

Some indirect evidence to support this idea now exists. Bacteria may be depleted of ribosomes by magnesium starvation.[129] The rate of mRNA synthesis in these ribosome-depleted bacteria is very slow. When magnesium ion is restored the capacity for mRNA formation appears to increase in proportion to the increase in cellular ribosomes. Also, the in vitro synthesis of RNA on a DNA template does not require the presence of ribosomes. However, the addition of ribosomes and protein-synthesis machinery stimulates RNA synthesis markedly.[130]

Thus, although ribosomes and translation appear to control the rate of RNA synthesis, some inconsistencies must be noted. Chloramphenicol, which in bacteria inhibits the formation of peptide bonds, does not appear to prevent or even to delay mRNA synthesis.[129] Since there is no direct evidence that ribosomes may move over mRNA without the concomitant formation of peptide bonds, some reservations must be maintained.

Many reports now appearing suggest another form of control at the level of translation. Such diverse systems as the normal liver, the mitotic HeLa cell, the unfertilized egg, serum-deprived cultured chick-embryo cells and hormone deprived tissue all evince the same general phenomenon. Under these circumstances, mRNA appears to be complexed to ribosomes in an inactive or repressed manner. Although the rate of translation rather than the absence of mRNA, appears to be the limiting step, we are totally ignorant of the mechanism (or mechanisms) by which inactive or repressed mRNA occurs.

Discussion

Universality of Protein Synthesis

The basic process of protein synthesis is the same in every living thing on this planet. All cells have ribosomes, made of 2 dissociable pieces of ribonucleoprotein; all cells have transfer RNA and messenger RNA representing protein with the same genetic code. Although these statements are, of course, infinite extrapolations from finite data, there is little likelihood that any exceptions will be found. The very fact that we understand protein synthesis through a combination of experiments using such diverse organism as *Esch. coli*, rabbit reticulocytes, HeLa cells and tobacco mosaic virus attests to the universality of the process. The proof lies in experiments in which *Esch. coli* tRNA can be used with the ribosome-mRNA complex of reticulocytes to make rabbit hemoglobin,[131] and in experiments in which an animal virus grows in a bacterial cell.[104]

From an evolutionary point of view these facts mean that the process of protein synthesis was highly developed before the occurrence of any of the cellular evolution that led to our kingdoms, orders and species. The way in which the process evolved is of fundamental importance, but, at present, there is no experimental approach to this problem. Surely, the development of 2 distinct chemical languages, inter-related functionally but not chemically, was a major event in the history of the universe.

Although the major facets of protein synthesis are universal, there are numerous differences in detail. The ribosomes of higher organisms are slightly larger than those of bacteria; the ribosomal and transfer RNA's of different species are different, although the synthetases of 1 species often recognize at least some of the tRNA's of another. The degeneracy of the code is probably responsible for permitting the wide divergence of DNA base composition among various species in spite of relatively small differences in total amino acid composition. Whereas all the tRNA sequences so far reported are from yeast tRNA, the numerous sequences from other species that will soon be worked out will provide grounds for speculation on the limits of the evolutionary divergence of protein synthesis.

Implications for Future Research

Although basic processes of protein synthesis appear to be similar for all species, it is not clear that microbial and animal cells possess similar control mechanisms. The ability to produce a variety of mutants, and to vary the growth conditions of micro-organisms, has allowed bacterial physiologists to explore the mechanisms by which bacteria control general and specific macromolecular synthesis. These means have not been available to those who study the animal cell. Furthermore, the added complexity of the potential interaction between cells of a given tissue, and between tissues within the body, makes facile extrapolation from the bacterial to the animal cell a hazardous one.

The great majority of the known human metabolic defects appear to fall into 2 classes: mutations that result in the synthesis of abnormal proteins; and mutations that result in either a deficiency or an excess of a specific enzymatic activity. Some of these results may be explained by a simple gene-dose relation — for example, mongolism. However, it is likely that faulty control mechanisms are involved in some cases, particularly in diseases characterized by intermittency such as porphyria. Furthermore, chronic physiologic stress may strain the homeostatic ability of a cell, resulting in a breakdown of controls so important to normal functioning. Whereas much of the popular reporting of the re-

sults of modern biology suggests that such defects will soon be cured by "genetic tinkering," in practice this appears a formidable task. However, it is now realistic to attack the problem of how mammalian cells control their functions. The methodology and information regarding animal cells is now advanced enough to permit analysis of control phenomena at the level of RNA or protein synthesis. It is also evident that the knowledge thus gained may shed light on hitherto unexplained pathologic processes and may open paths to their amelioration.

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MEDICAL INTELLIGENCE

CURRENT CONCEPTS

Biology of Tendon Repair*

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ALTHOUGH restorative hand surgery is correctly regarded as having been developed primarily in military centers, repair of flexor tendons, one of the most frequent and difficult problems in civilian practice, was not significantly influenced by war experience. The frequency of flexor-tendon lacerations, the relatively long amplitude of tendon motion required to flex a digit, and the nonselective nature of the healing process have been responsible for discouraging results and for the increased attention that flexor-tendon repair has received in the postwar period. Now, some twenty years since injuries of flexor tendons and digital nerves have become more important than the massive soft tissue and metacarpal restoration problems of the 1940's; it is realized that restoration of gliding function, although markedly influenced by attention to and perfection of technical details, is not a problem that can be solved completely by technical innovations. This is not to say that careful attention to technical details is not mandatory in successful repair of tendons; evidence is accumulating, however, that success in flexor tendon repair is quite probably dependent to a much greater extent than in other restorative problems upon rather complex biologic phenomena outside of technical control.

Successful restoration of gliding function represents a combination of technical skill within one's

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