methylation may influence the efficiency or accuracy of polyadenylation. Finally, the availability of a source of m^6A methyltransferase and an assay for methylation will make it possible to purify the enzyme for further functional studies.

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18. We are grateful to T. Nilsen and P. Maroney for their initial observation of adenosine methylation in nuclear extracts and discussion of these experiments, E. Goodwin for preparing the nuclear extracts used in this study, J. Bloom for technical assistance, and S. Carroll for providing the mutagenized prolactin clone. We thank J. Nilson, T. Nilsen, H.-J. Kung, and N. Maihle for comments on the manuscript and K. Fatica for preparation of the manuscript. This work was supported by grant CA 31810 from the National Cancer Institute and Cancer Center Grant P30 CA 43703.

16 June 1988; accepted 27 September 1988

A Continuous Cell-Free Translation System Capable of Producing Polypeptides in High Yield

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A cell-free translation system has been constructed that uses a continuous flow of the feeding buffer [including amino acids, adenosine triphosphate (ATP), and guanosine triphosphate (GTP)] through the reaction mixture and a continuous removal of a polypeptide product. Both prokaryotic (*Escherichia coli*) and eukaryotic (wheat embryos, *Triticum sp.*) versions of the system have been tested. In both cases the system has proven active for long times, synthesizing polypeptides at a high constant rate for tens of hours. With the use of MS2 phage RNA or brome mosaic virus RNA 4 as templates, 100 copies of viral coat proteins per RNA were synthesized for 20 hours in the prokaryotic or eukaryotic system, respectively. With synthetic calcitonin messenger RNA, 150 to 300 copies of calcitonin polypeptide were produced per messenger RNA in both types of continuous translation systems for 40 hours.

EXPRESSION OF ALIEN GENES IN LIVING cells is often subject to a number of limitations. The product polypeptide can be unstable in a given cell, and in some cases the product is toxic to the cell. These and other limitations could be avoided if translation were possible in cell-free systems. Unfortunately, however, different versions of cell-free translation systems that have been previously described have a general shortcoming, namely, a low yield of the polypeptide product. Typically only two to three polypeptide chains are produced per mRNA chain used in such systems (*1, 2*).

We report that a prolonged and effective functioning of a cell-free translation system can be achieved if the reaction products, including synthesized polypeptides, are continuously removed from the reaction mixture, and if the initial concentrations of consumable low molecular weight substrates (ATP, GTP, and amino acids) are continuously restored. This approach has been realized for cell-free translation systems both of prokaryotic (*Escherichia coli*) and eukaryotic

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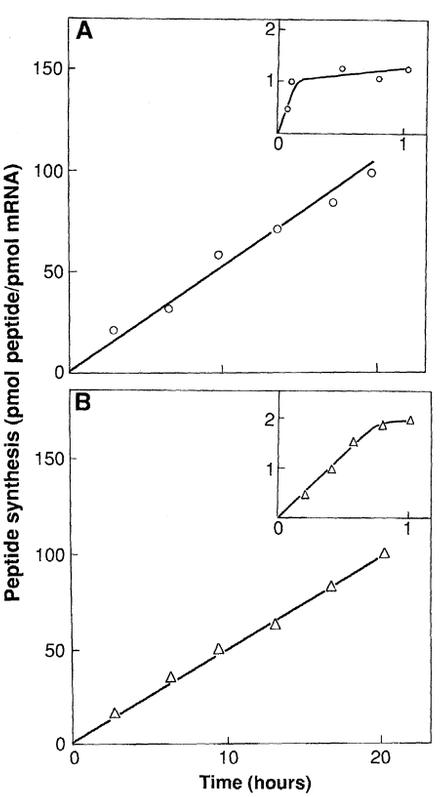

Fig. 1. Kinetics of virus coat protein synthesis in the virus-RNA–directed continuous cell-free translation systems (20 hours). Inset (same axes units): kinetics of protein syntheses in standard cell-free systems of the same composition and volume. (**A**) Synthesis of MS2 coat protein in the *E. coli* system. A 1-ml reaction mixture contained 0.6 nmol of 70*S* ribosomes, 1 mg of S100 protein, 0.6 mg of total tRNA, 0.06 nmol of MS2 phage RNA, 7 μg of pyruvate kinase with a specific activity of 500 U/mg, 50 activity units of ribonuclease inhibitor from human placenta, and 0.1 μg each of aprotinin, leupeptin, and chymostatin in the feeding buffer A, which contained 20 m*M* tris-HCl, *p*H 7.4, 100 m*M* NH_4Cl, 10 m*M* $MgCl_2$, 1 m*M* ATP, 0.2 m*M* GTP, 5 m*M* phosphoenol pyruvate, 25 μ*M* [^{3}H]leucine (specific activity, 52 Ci/mol), and 25 μ*M* each of the other 19 amino acids. The incubation temperature was 37°C. In the continuous system, the feeding buffer A was passed through the Amicon diafiltration cell at a constant rate of 1 ml/hour; the ultrafiltration membrane PM-30 was used for continuously removing MS2 coat protein from the cell. (**B**) Synthesis of BMV coat protein in the wheat system. A 1-ml reaction mixture contained 17 A_{260} absorbance units of S30 extract from wheat (*Triticum* sp.) embryos (Sigma), 0.1 nmol of BMV RNA 4, 64 μg of creatinine phosphokinase with a specific activity of 350 U/mg, 50 activity units of ribonuclease inhibitor from human placenta (Amersham), and 0.1 μg each of aprotinin, pepstatin, and leupeptin in the feeding buffer B, which contained 40 m*M* Hepes, *p*H 7.6, 112 m*M* potassium acetate, 1.9 m*M* magnesium acetate, 0.25 m*M* spermidine, 6 m*M* dithiothreitol, 1.5% glycerol, 2 m*M* ATP, 50 μ*M* GTP, 8 m*M* creatine phosphate, 25 μ*M* [^{3}H]leucine (specific activity, 50 Ci/mol), and 25 μ*M* each of the other 19 amino acids. The incubation temperature was 27°C. In the continuous system, the feeding buffer B was passed through the Amicon cell at a constant rate of 1 ml/hour; the ultrafiltration membrane XM-50 was used for continuously removing BMV coat protein from the cell.

(wheat embryos) origin. Such a continuous cell-free translation system can function in a long-term regime for tens of hours, with a high constant rate, thus producing hundreds of copies of a synthesized polypeptide per mRNA molecule. The system can be used for preparative syntheses of polypeptides.

The following RNAs were used as templates: (i) MS2 phage RNA isolated from the phage by phenol deproteinization in the presence of 1% SDS; (ii) brome mosaic virus (BMV) RNA 4 prepared by phenol deproteinization of the virus with subsequent isolation of the RNA 4 fraction by sucrose gradient centrifugation; and (iii) mRNA for Val[8]-calcitonin (human) obtained by in vitro SP6 phage polymerase transcription (*3, 4*) of the synthetic calcitonin gene, with or without the preceding Shine-Dalgarno sequence, inserted into a plasmid under SP6 promoter.

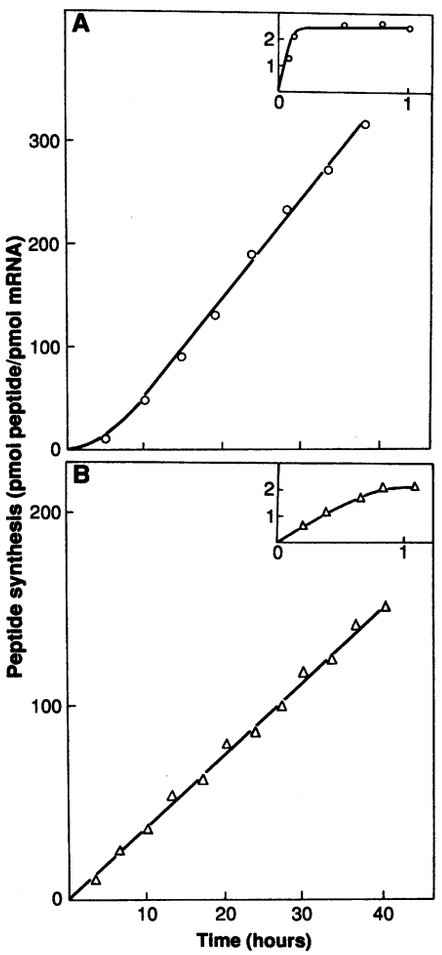

Fig. 2. Kinetics of the Val[8]-calcitonin synthesis in the continuous cell-free translation systems (40 hours). Inset (same axes units): kinetics of the polypeptide synthesis in the standard cell-free systems of the same composition and volume. (**A**) Synthesis of calcitonin in the *E. coli* system. All reaction conditions were the same as in Fig. 1A, except that 0.06 nmol of synthetic calcitonin mRNA was added instead of viral RNA, and the PM-10 membrane was used instead of the PM-30 for removing the product from the ultrafiltration cell. (**B**) Synthesis of calcitonin in the wheat system. All reaction conditions were the same as in Fig. 1B, except that 0.06 nmol of synthetic calcitonin mRNA was added instead of viral RNA, 75 m*M* potassium acetate was found to be optimal for this synthesis, and the PM-10 membrane was used instead of the XM-50 for removing the product from the ultrafiltration cell.

The prokaryotic cell-free translation system was composed of *E. coli* MRE-600 ribosomes, S100 extract freed of endogenous DNA and RNA, total transfer RNA, and a corresponding mRNA. The eukaryotic cell-free translation system was based directly on wheat embryo S30 extract to which a corresponding mRNA was added. The ribonuclease inhibitor from human placenta and several protease inhibitors were introduced into the reaction mixtures.

The Amicon 8 MC micro-ultrafiltration system was used as the simplest device for realization of the continuous action. A 1-ml incubation mixture in the working chamber of the instrument was supplied with a constant flow (1.0 ml/hour) of the feeding solution [consisting of the set of amino acids (one of them being radioactively labeled), as well as ATP and GTP] while the reaction products were continuously removed with the same rate through the ultrafiltration membrane. The amount of the polypeptide synthesized was determined from the radioactivity of hot trichloroacetic acid–insoluble material in the filtrate. The products were identified and their purity was checked by one-dimensional SDS-gel electrophoresis with Coomassie staining.

The kinetics of the polypeptide syntheses in the continuous prokaryotic and eukaryotic translation systems, as compared with those in the standard translation systems (without flowing the solution), are shown in Figs. 1 and 2. Although the synthesis in the standard systems (insets in Figs. 1 and 2) ceases in 1 hour or less, yielding one to two polypeptide molecules per mRNA molecule, the synthesis rate in the continuous action systems is constant for at least 20 or 40 hours, and the amount of the product reaches a hundred or hundreds of polypeptide molecules per mRNA molecule. The experiments presented in Figs. 1 and 2 were reproduced no less than ten times each, with incubation periods for 15 to 40 hours and yield variations from 5 to 20 nmol of polypeptide per 1 ml of incubation mixture. In a recent experiment the calcitonin-synthesizing system produced a constant synthesis rate for 100 hours. The results of electrophoretic analyses of the translation products from the continuous systems are presented in Fig.3.

Two unexpected points emerged from the experiments on the prolonged translation in the flow system. First, the mRNAs in the cell-free systems were used in limiting amounts, so that the action of nucleases should lead to a decrease in the synthesis rate. This was not the case, however. Since nuclease activities in the extracts used cannot be excluded, even despite the initial presence of the placentar inhibitor, we believe that the permanent engagement of mRNA with ribosomes and other translation components safely protects it during prolonged incubation at a physiological temperature. Second, the ultrafiltration membranes PM-30 or XM-50 used have the pore size large enough for the penetration of small proteins, in particular some initiation factors; the washing-off of the factors from the incubation mixture should also decrease the synthesis rate. Again this was not the case for at least 40 hours. It is possible that the translation components both in the eukaryotic and in the prokaryotic cell-free translation systems are continuously involved in interactions with each other, thus creating dynamic multiprotein units that are incapable of passing through the pores.

We believe that the realization of the continuous action principle in cell-free translation systems opens new fields for

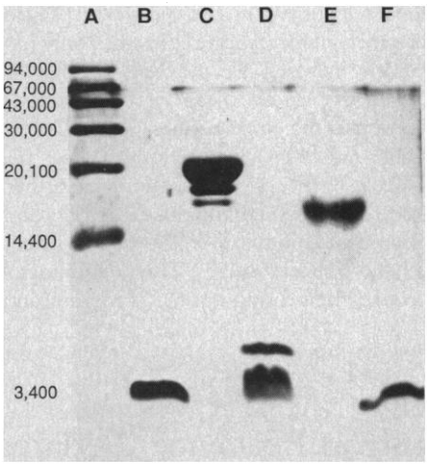

Fig. 3. Electrophoretic patterns of the translation products. Electrophoretic analysis was done in dodecyl sulfate–urea–polyacrylamide gel according to Schagger and von Jagow (*5*). The gels were stained with Coomassie brilliant blue G-250. (**A**) Standard polypeptide set. (**B**) Standard salmon calcitonin. (**C**) Product of the BMV RNA 4 translation in the wheat cell-free system (see Fig. 1B). In addition to the main band, some amounts (about one-third) of abortive translation products (*6*) are revealed. (**D**) Product of the Val[8]-calcitonin mRNA translation in the wheat cell-free system (see Fig. 2B). In addition to the main band, some amount (about 20%) of the S–S dimer is revealed. (**E**) Product of the MS2 RNA translation in the *E. coli* cell-free system (see Fig. 1A). (**F**) Product of the Val[8]-calcitonin mRNA translation in the *E. coli* cell-free system (see Fig. 2A).

their scientific use. The cell-free expression of genetic information avoids the many limitations imposed by the cell, so that the preparative biosynthesis of a number of nonstructured polypeptides, as well as unstable and cytotoxic proteins, becomes possible. In particular, this method may provide researchers with the intermediates of protein folding and modification for their biochemical and physical studies. The possibilities of protein engineering can be also significantly extended. We also anticipate new developments in biotechnology based on preparative cell-free translation systems of continuous action.

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7. We thank J.-P. Ebel and B. Ehresmann at the Institut de Biologie moleculaire et cellulaire, Strasbourg, and M. Grunberg-Manago and her colleagues at the Institut de Biologie physico-chimique, Paris, for fruitful discussions and suggestions. We also thank A. N. Fedorov, S. N. Proshkina, and V. Ya. Pankratova at the Institute of Protein Research for technical assistance and K. G. Skryabin and his colleagues at the Institute of Molecular Biology, Moscow, for the plasmids with the calcitonin gene.

13 June 1988; accepted 17 October 1988

Intron Existence Predated the Divergence of Eukaryotes and Prokaryotes

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Nucleotide sequences for the nuclear genes encoding chloroplast (GapA and GapB) and cytosolic (GapC) glyceraldehyde-3-phosphate dehydrogenases (GAPDHs) from *Arabidopsis thaliana* were determined. Comparison of nucleotide sequences indicates that the divergence of chloroplast and cytosolic GAPDH genes preceded the divergence of prokaryotes and eukaryotes. In addition, some intron-exon junctions are conserved among GapB, GapC, and chicken GAPDH genes. These results provide evidence at the molecular level to support the idea that introns existed before the divergence of prokaryotes and eukaryotes.

THREE ALTERNATIVE MODELS CAN BE proposed concerning the time of intron appearance during evolution. First, introns existed in the progenitor genes but were lost from prokaryotes (*1*), and differential intron loss may occur among members of the same gene family of eukaryotes (*2*). Second, introns existed in the progenitor genes, but intron insertion or deletion may occur later during evolution. Third, prokaryotic genes resemble the progenitor genes, and the addition of introns occurred at or after the emergence of eukaryotes (*3*). Current available evidence is more consistent with the first two models (*2*). However, there is no direct evidence as to whether introns existed before the divergence of prokaryotes and eukaryotes, information crucial to distinguishing these models. Current data can only trace the existence of introns up to the divergence of animals and plants (*4–6*).

We have shown previously that the nuclear genes (GapA and GapB) encoding chloroplast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of higher plants are the direct descendants of the symbiont genes that gave rise to the chloroplast and were subsequently transferred from the chloroplast to the nuclear genome during evolution (*7*). In addition, our data showed that the time of divergence between GapA/B and cytosolic GAPDH genes (~1700 million years) is much earlier than the divergence between plants and animals (~1000 million years). Therefore, comparison of the exon-intron junctions between GapA/B and cytosolic GAPDH genes should provide information on the age of the intron. If introns existed in the progenitor GAPDH gene, conservation between some exon-intron junctions of GapA/B and cytosolic GAPDH genes would be expected.

Genomic DNA blot analyses indicate that there is one copy each of the GapA, GapB, and GapC (cytosolic GAPDH) genes in *Arabidopsis thaliana* (*8*). The complete nucleotide sequences for these three genes and the cDNAs encoded by them have been determined (*8*). When compared to the corresponding cDNA sequences GapA has three, GapB has seven and GapC has eight introns. In contrast, there are 11 introns in the chicken GAPDH gene (*9*). The introns for all three Gap genes are generally very short, consistent with observations on other *Arabidopsis* genes that have been sequenced (*10, 11*).

On the basis of the sequences of the coding regions of the three Gap genes and the information published previously (*7*), an evolutionary tree for the GAPDH genes (Fig. 1), with the number of nonsynonymous substitutions (*12, 13*) used as a measure of the evolutionary distance, was constructed according to the Neighbor Joining method (*14*). This tree topology is consistent with our previous tree that took into account the evolutionary rates of GAPDH and with the biochemical evidence obtained from a comparison of the amino acid sequences of the S loop regions among different GAPDHs (*7*). GAPDHs can be divided into two groups: thermophilic, which includes two bacteria and chloroplast GAPDHs; and mesophilic, which includes *Escherichia coli* and all cytosolic GAPDHs. There is 100% similarity of the S loop sequences within the same group, while there is less than 50% similarity between the two groups. The tree topology indicates that the divergence of GapA/B and cytosolic GAPDH genes occurred before the divergence of prokaryotes and eukaryotes.

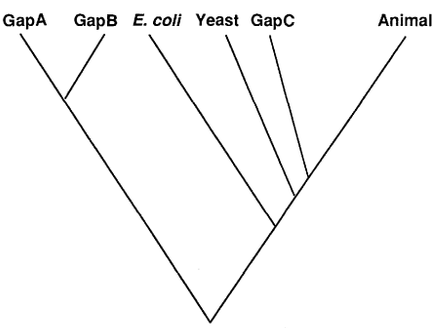

Fig. 1. Evolutionary tree of GAPDH genes. The number of nonsynonymous substitutions between each pairwise combination of GAPDH genes was calculated according to the method described by Li *et al.* (*13*) and used as the measure of the evolutionary distance in constructing the evolution tree. The tree presented here is constructed by the Neighbor Joining method of Saitou and Nei (*14*). Two additional methods, described by Li (*21*) and Dickerson and Geis (*22*), were used in constructing trees. All three trees give identical topology. The *E. coli* data are from Branlant and Branlant (*23*) and the *Arabidopsis* data are from this manuscript. All other data are from Shih *et al.* (*7*).

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