

Construction of a Synthetic Messenger RNA Encoding a Membrane Protein

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ABSTRACT We have synthesized microgram quantities of a functional eucaryotic mRNA by in vitro transcription. For this purpose, we constructed a plasmid in which the *Escherichia coli* lactose promoter was 5' to the vesicular stomatitis virus (VSV) G protein gene (Rose, J. K., and C. J. Gallione, 1981, *J. Virol.*, 39:519-528). This DNA served as the template in an in vitro transcription reaction utilizing *E. coli* RNA polymerase. The RNA product was capped using the vaccinia guanylyltransferase. A typical preparation of the synthetic G mRNA was equivalent to the amount of G mRNA that can be isolated from $\sim 10^8$ VSV-infected cells. This synthetic mRNA was translated by a wheat germ extract in the presence of microsomes, producing a polypeptide that was indistinguishable from G protein in its size, antigenicity, degree of glycosylation, and its membrane insertion. This technique should aid in identifying features needed by proteins for insertion into membranes.

An approach to identifying signals that direct the intracellular transport of a protein is finding mutations that interfere with the protein's proper cellular location. In bacteria, this approach has shown that single amino acid changes in the signal sequence can interfere with or totally prevent a protein's insertion into the cytoplasmic membrane (1, 2). It has also been shown that deletions or insertions in the carboxyterminus can affect the transport of proteins across membranes (3-5). The ability to clone genes and specifically alter them in vitro greatly extends our ability to identify functionally important domains of proteins. Recently, the development of eucaryotic viral and plasmid gene expression vectors has enabled investigators to express new genes in animal cells (6-8). Because viral vectors introduce their DNA into every nucleus in an experiment and can amplify their genomes by replication, they are an efficient means of producing large quantities of a protein in a cell, as exemplified by the production of the influenza hemmagglutinin (9, 10). Virus vectors, however, are limited because some DNA sequences can not be stably propagated, and are deleted from the viral genome (Paul Berg, personal communication). Their usefulness is also limited by the host range of the virus. While plasmid vectors can circumvent these problems, they no longer possess the virus vector's advantages of efficient infection and replication. To increase the expression from transfected plasmid vectors, Gluzman (11) developed a cell line that can replicate DNA molecules containing the SV40 origin of replication. The disadvantage of this approach is that it limits the experiments to one cell type, a transformed monkey fibroblast. Finally, both plasmid and viral vectors rely upon cellular

machinery for transcription and processing of RNA. There is no guarantee that the nucleus will produce large quantities of a properly processed mRNA from a new gene.

A more direct approach to producing a protein from an in vitro constructed gene is the in vitro synthesis of the mRNA, and the translation of the mRNA either in vitro or in vivo. This paper describes the construction of an mRNA coding for the VSV G protein, and the translation of the synthetic mRNA by a cell-free system.

MATERIALS AND METHODS

Isolation of DNA from *E. coli*: DNA was isolated on a preparative scale from 1 liter of chloramphenicol-amplified cultures using CsCl-ethidium bromide gradients (12). DNA was isolated on an analytical scale from 1-ml cultures using the method of Birnboim and Doly (13).

Agarose Gel Electrophoresis—Preparation of DNA Fragments: DNA fragments were separated for analytical and preparative purposes by electrophoresis through horizontal agarose gels (14). DNA was isolated from the gels by the NaI-glass bead technique (15).

DNA Modification Procedures: Restriction enzymes were used according to the manufacturer's directions. *TaqI*, *PvuII*, *PstI*, *HindIII*, *BglII*, and *StuI* were obtained from New England Biolabs (Beverly, MA); *ClaI* was obtained from Boehringer Mannheim Biochemicals (Indianapolis, IN); *SaII* was a gift from Ken Burtis (Stanford University, Stanford, CA); and *EcoRI* was a gift from John Carlson (Stanford University).

E. coli DNA polymerase I (DNA polI) (gift of Jon Widom, Stanford University) reactions were performed in 50 mM Tris HCl (pH 7.5), 10 mM MgCl₂, 70 mM KCl, and 0.2 mM of *d*ATP, *d*GTP, *d*-cytidine triphosphate (*d*CTP) and *d*-thymidine triphosphate (*d*TTP), at 12°C for 30 min.

T4 DNA ligase (gift of Stewart Scherer, Stanford University) reactions were performed in 50 mM Tris HCl (pH 7.3), 10 mM MgCl₂, 10 mM EDTA, and 0.1

mM ATP, at 12°C for 12 h.

Synthetic oligonucleotide linkers were purchased from Collaborative Research Inc. (Waltham, MA) and were ligated onto blunt-end DNA fragments according to the manufacturer's directions. In cases where restriction digests generated staggered ends, *E. coli* polymerase I was used to create blunt ends before the linker ligations were performed. After the ligations, excess linkers were removed and "sticky" ends were generated by digestion with the appropriate restriction enzyme.

Construction of the pG Lac Vector: Two plasmids were used as starting materials in this construction. One plasmid, pTV3 (gift of M. Gilman, University of California, Berkeley CA), contained the 95-base-pair *Lac* UV5 promoter and ribosomal binding site (16, 17) between the *Eco*RI and *Hind*III sites of pBR322 (pBR). The other plasmid was pSV2G (the construction of which is described in the following paragraph). The vector pG *Lac* was constructed from these two precursors as outlined in Fig. 1.

To construct pSV2G, the vector pSV2dhfr (18) was first modified by inserting a *Sa*I linker at its *Hind*III site and a *Cl*aI linker at its *Bg*III site. The G clone was then removed from pG1 (full-length cDNA of the VSV G protein cloned into the *Pst*I site of pBR322 by Dr. J. K. Rose, The Salk Institute San Diego, CA [19]) by a partial *Pst*I digest and recloned into the *Sa*I site of pBR322 after the addition of *Sa*I linkers. This molecule was partially digested with *Stu*I, followed by *Cl*aI linker ligation. The fragments were then digested with *Sa*I and the fragment containing the complete G coding sequence was isolated and cloned between the *Sa*I and *Cl*aI sites of the modified pSV2dhfr vector.

Preparation of the Synthetic mRNA: To prepare 5 µg of DNA template for the transcription reaction, 20 µg of pG *Lac* was digested to completion with *Cl*aI and *Eco*RI. The 1.75-kilobase (kb) fragment containing the *Lac* UV5 promoter and the G protein gene was purified by agarose gel electrophoresis.

In a typical transcription reaction, 3 µg of template DNA was added to 80 µl of 40 mM Tris HCl (pH 8.0), 60 mM NaCl, 10 mM MgCl₂, 1 mM dithiothreitol (DTT), 100 µg/ml gelatin, 0.4 mM ATP, α-³²P-GTP (100 cpm/pmol), uridine triphosphate (UTP) and CTP, 20 U of RNasin (Biotec, Madison, WI), and 4 µg *E. coli* RNA polymerase (gift of Dr. M. Chamberlain, University of California, Berkeley, CA), and incubated for 30 min at 37°C. Typically, 1 µg of RNA was produced from 1 µg of DNA template as judged by the incorporation of ³²P. The

reaction was stopped by extraction with 40 µl of phenol:chloroform. The aqueous phase was ethanol precipitated (12).

The precipitate was resuspended in 25 µl of H₂O, and 25 µl of 4 M LiCl was added to precipitate the RNA (DNA is soluble in 2 M LiCl [20]). The RNA was resuspended in 50 µl H₂O and ethanol precipitated. The RNA was resuspended in 60 µl of H₂O.

Next, the RNA was modified with the "capping" enzyme (21) in a 100-µl reaction. It contained ~3 µg of RNA (~4.5 pmol of the transcript), 25 mM Tris HCl (pH 7.8), 5 mM DTT, 2 mM GTP, 2 mM MgCl₂, 0.1 mM *S*-adenosylmethionine (stored as a 2.5-mM solution with 1 mM HCl, and obtained as a HSO₄⁻ salt from Boehringer Mannheim Biochemicals), 100 U of RNasin, and 7 U of vaccinia guanylyltransferase (Bethesda Research Laboratories, Gaithersburg, MD). The reaction was performed at 37°C for 30 min. It was stopped by extraction with phenol:chloroform. The aqueous phase was ether extracted, and the RNA was ethanol precipitated. A large precipitate was noted. The RNA was further purified by precipitating it from 2 M LiCl and then ethanol-precipitating the RNA using 0.4 M potassium acetate (pH 6.2) as the salt. The final precipitate was resuspended in 15 µl of H₂O and stored at -70°C.

Analysis of Transcription Products: The sizes of the radioactive RNA transcripts were determined by glyoxal treatment of the RNA (22), followed by electrophoresis of the molecules in agarose gels. The gels were dried and autoradiographed with Kodak XR5 film. ³²P-Labeled DNA restriction fragments were used as molecular weight standards.

In Vitro Translation: The synthetic RNA was analyzed for its capacity to direct the synthesis of G protein by adding it to a wheat germ extract. The wheat germ extract (gift of Dr. W. Braell, Stanford University) was prepared according to the method of Roberts and Patterson (23). The extract was in 20 mM potassium HEPES (pH 7.2), 120 mM KCl, 5 mM magnesium acetate, and 1 mM DTT. The translation reaction was prepared by mixing 0.3 vol of: 300 µCi/ml [³⁵S]methionine (1,000 Ci/mmol, Amersham Corp., Arlington Heights, IL), 52 mM potassium acetate, 20 mM potassium HEPES (pH 7.2), 17 mM creatine phosphate, 2.5 mM ATP, 0.3 mM GTP, 6 mM DTT, 1.5 mM magnesium acetate, 800 µM spermidine, 120 µM of each amino acid (except methionine), 60 µg/ml creatine phosphokinase (Boehringer Mannheim Biochemicals), 0.3 U/ml RNasin; with 0.3 vol of wheat germ extract and 0.4 vol of RNA solution (3.3 µg/ml).

Typically, 50 ng of synthetic RNA was added to a 25-µl reaction. The RNA was preheated to 65°C for 2 min or incubated with 2.5 mM CH₃HgOH (24) to denature the molecules (which increased the translation efficiency twofold).

The translation was performed at 25°C for 90 min. Incorporation of [³⁵S]-methionine into protein was assayed by treating a 1-µl aliquot of the reaction with 10 µl of 0.2 M KOH at 37°C for 30 min to hydrolyze the methionyl-tRNA molecules. 100 µl of 10% (wt/vol in H₂O) trichloroacetic acid was added, and the proteins were precipitated at 0°C for 5 min. The precipitates were collected on glass fiber filters. The filters were washed with 10 ml of 0.1 M HCl and 10 ml of ethanol, dried, and the radioactivity was measured by scintillation spectrometry.

Translation in the Presence of Dog Pancreatic Microsomes: Dog pancreatic microsomes (DPM) were prepared according to the method of Shields and Blobel (25). They were stored at a concentration of 80 OD₂₈₀ U/ml in 0.25 M sucrose, 20 mM potassium HEPES (pH 7.5), and 4 mM DTT. The DPM (0.5 µl) were added to a 25-µl translation reaction. This amount of membrane decreased [³⁵S]methionine incorporation by a factor of two.

Electrophoretic Analysis of Translation Products: Proteins produced by the translation reaction were separated electrophoretically using SDS polyacrylamide gel electrophoresis. The samples were heated to 95°C for 2 min in the presence of 60 mM Tris HCl (pH 6.8), 20% (vol/vol) glycerol, 70 mM β-mercaptoethanol, 2% (wt/vol) SDS, and 0.001% (wt/vol) bromophenol blue. This solution was electrophoresed through a 10%-SDS polyacrylamide gel (26). The gel was impregnated with Enhance (New England Nuclear, Boston, MA), dried, and autoradiographed with Kodak XR-5 film.

Immunoprecipitation: Typically, 10 µl of a translation reaction was dispersed into 400 µl of 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 0.1 M NaCl, 10 mM NaPO₄ (pH 7.5), 0.01% sodium azide, and 50 mM phenylmethyl sulfonyl fluoride (buffer A). Then, 30 µl of a 10% solution (wt/vol, in buffer A) of *Staphylococcus aureus* cells (Calbiochem-Behring Corp., San Diego, CA) was added. The solution was incubated at 25°C for 15 min and centrifuged for 5 min at 16,000 g. Then, 10 µl of rabbit anti-G protein antiserum (gift of Dr. William Balch, Stanford University) was added to the supernatant. After incubating the solution for 2 h at 25°C, 20 µl of the 10% *S. aureus* solution was added for an additional 25 min. The sample was centrifuged for 5 min at 16,000 g. The pellet was resuspended in buffer A and pelleted four times (to remove loosely bound proteins). The final pellet was resuspended in SDS sample buffer, heated to 95°C, and loaded on an SDS polyacrylamide gel.

Trypsin Treatment of Microsomal Membranes: After a 25-µl translation reaction containing microsomes was performed, the sample was split into two aliquots. Then, 1 µl of a newly made solution of 10 mg/ml (in H₂O)

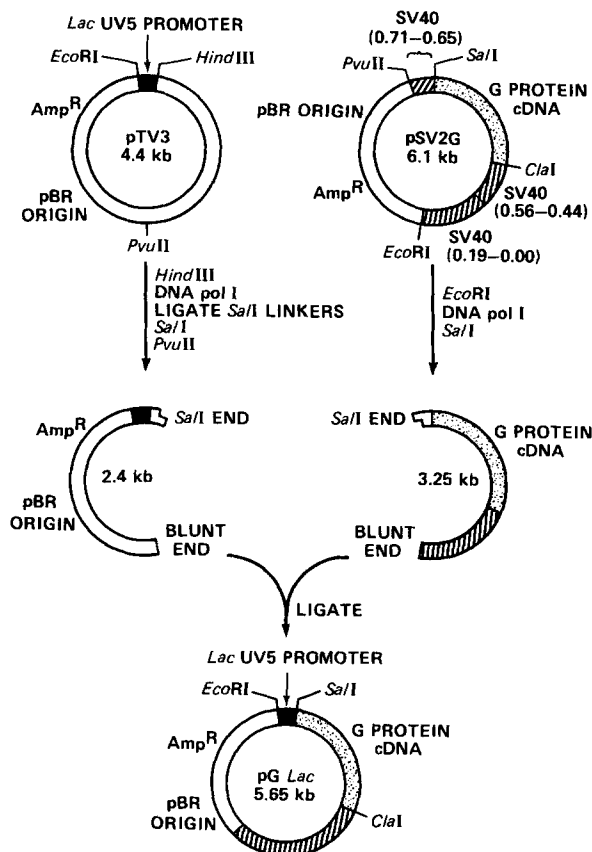


FIGURE 1 Cloning of pG *Lac*. This flow chart outlines the procedure used to construct pG *Lac*. Details can be found in Materials and Methods. The drawings do not precisely represent the actual relative sizes of the plasmids or domains within the plasmids.

N-tosyl-L-phenylalanine chloromethyl ketone (TPCK) trypsin (Millipore Corp., Bedford, MA) was added to one of the aliquots. Both samples were incubated at 4°C for 1 h. Next, 2.5 μ l of 10 mg/ml (in 140 mM NaCl, 5 mM KCl, and 25 mM Tris HCl [pH 7.4]) soybean trypsin inhibitor (Millipore Corp.) was added to both samples. Then, 160 μ l of microsomes (400 OD₂₆₀ U/ml in 0.25 M sucrose, 20 mM potassium HEPES, [pH 7.5], and 4 mM DTT) was added to both samples as a carrier. The membranes were pelleted by centrifugation for 10 min, 4°C, at 20 pounds per square inch (psi) in the Beckman airfuge (Beckman Instruments, Inc., Fullerton, CA). The supernatants were removed, and the pellets were resuspended in 150 μ l of immunoprecipitation buffer (buffer A) containing 1 mg/ml soybean trypsin inhibitor. The G protein was immunoprecipitated and analyzed by electrophoresis through an SDS polyacrylamide gel and autoradiography.

Endoglycosylase H (Endo H) Treatment of Microsomes:

After a 25- μ l translation reaction was performed, the G protein was immunoprecipitated. The immunoprecipitate was resuspended in 50 μ l of 50 mM Tris HCl (pH 6.8), 1% (wt/vol) SDS, and 15 mM DTT, and heated to 100°C for 3 min. The *S. aureus* cells were pelleted by centrifugation at 16,000 *g* for 1 min. The supernatant was recovered and split in half. To one half, 10 μ l of 0.3 M sodium citrate (pH 5.5), 0.1% (wt/vol) SDS was added. The other half received 10 μ l of the same solution with 1.5 μ g/ μ l of Endo H (gift of Dr. P. Robbins, Massachusetts Institute of Technology, Cambridge, MA). Both samples were incubated at 37°C for 10 h, and then electrophoresed through an SDS polyacrylamide gel, and autoradiographed.

Preparation of VSV RNA: Total RNA was isolated from baby hamster kidney cells infected for 4 h with VSV at an infection multiplicity of five. The cells were lysed with guanidinium thiocyanate and sodium *N*-lauroyl sarcosine, and the RNA was purified on a CsCl step gradient (27).

RESULTS

Insertion of the VSV G Protein Gene 3' to the E. coli Lactose Promoter

Fig. 1 diagrams the construction of pG *Lac*, a plasmid in which the VSV G protein cDNA gene (kindly provided by Dr. J. K. Rose, The Salk Institute) is under the transcriptional control of the *Lac* UV5 promoter. The details of this plasmid's construction are covered in Materials and Methods.

The 95-base-pair *Lac* UV5 fragment was chosen because it contains a strong promoter (19), and because its transcript does not contain an initiation codon (20). Because of the promoter's strength and the plasmid's copy number (~20), it is possible that the bacteria are producing large quantities of G protein. Rose and Shafferman (28) found that when the amino terminus of G protein was fused to the first seven amino acids of the tryptophan E gene, the resulting protein was toxic to *E. coli*. We, however, did not observe any detrimental effects on the bacteria harboring the pG *Lac* molecule. This difference could result from the different plasmid construction used. Among possible explanations are that the G protein should not be translated efficiently from the pG *Lac* transcript because of the large distance (60 \pm 10 bases) between ribosome binding site and the initiation codon. This distance is far from optimal for translation in *E. coli* (29). Secondly, fusing the N-terminus of the G protein to seven amino acids of the tryptophan E protein may interfere with the proper processing of the membrane insertion (signal) sequence (28). This could lead to a blockage of the cellular machinery involved in biosynthesis of membrane proteins (28).

Construction of the Synthetic mRNA

We isolated a 1.75-kb fragment from pG *Lac* that contained the UV5 promoter and the G protein gene. This fragment was the template in an in vitro transcription reaction, using *E. coli* RNA polymerase. Fig. 2 is an autoradiograph of an agarose gel in which glyoxal-treated transcripts were electrophoresed. This gel demonstrates that transcription from the UV5-G fragment produces predominantly a 1.7 \pm 0.05 kb product. The

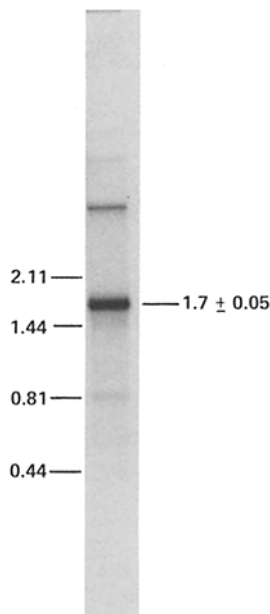


FIGURE 2 RNA transcripts from the UV5-G template. This figure is an autoradiograph of an agarose gel in which glyoxal-treated RNA was electrophoresed. On the left of the gel lane are the positions and sizes (in kilobases) of marker molecules. On the right of the gel lane is the calculated size (in kilobases) of the predominant transcript.

transcript's length is consistent with its 5' end being at the UV5 transcription initiation site, and its 3' end being at the 3' end of the G gene (the *Clal* site). There are also less abundant transcripts (4 \pm 0.5, 1.5, 1.1, 0.82, 0.56, and 0.45 kb) that have not been characterized.

Adding the cap structure proved to be a critical step in producing an RNA capable of translation in the wheat germ cell-free system. The commercial preparation of the vaccinia guanylyltransferase was efficient at capping RNA (as judged by incorporating an α -³²P-GTP onto >50% of the molecules in a reaction). However, the enzyme preparation was contaminated with ribonuclease. Fortunately, the placental ribonuclease inhibitor virtually eliminated the RNA degradation during the capping reaction.

Translation of the Synthetic mRNA in Wheat Germ Extracts

The synthetic mRNA was added to a cell-free wheat germ translation reaction (23). The incubation was split into three aliquots after translation. One was incubated with rabbit anti-G protein antiserum, the second was incubated with the rabbit preimmune serum, and no antiserum was added to the final aliquot. After the first two samples were immunoprecipitated, all three samples were electrophoresed through an SDS polyacrylamide gel. Fig. 3, which is an autoradiograph of this gel, demonstrates that the synthetic mRNA directs the translation of a single protein, whose size is identical to that of G protein. Furthermore, this protein binds to the anti-G protein antibodies but does not interact with the antibodies from the preimmune serum. Taken together, these observations indicate that the synthetic RNA is a functional template for eucaryotic ribosomes.

Table I compares the translation efficiency of the synthetic mRNA with authentic mRNA. The synthetic RNA is, within a factor of two, as efficient as is poly A-containing VSV RNA. Furthermore, Table I points out that capping the synthetic RNA increases the incorporation of [³⁵S]methionine by a factor of 25. Although the uncapped RNA does slightly stimulate the incorporation of [³⁵S]methionine into acid-precipitable material, it does not produce full-length G protein (data not shown).

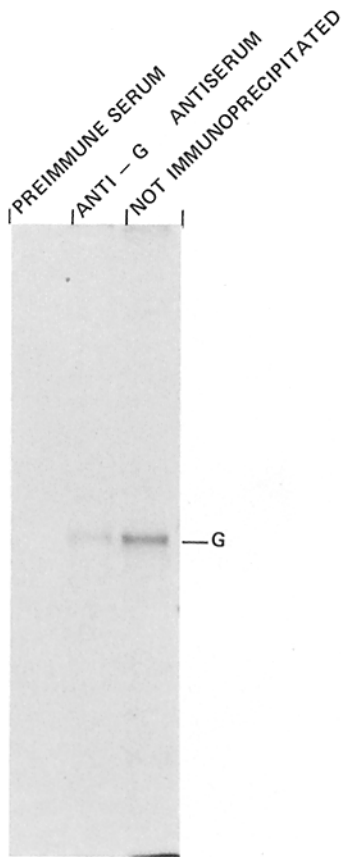


FIGURE 3 The synthetic mRNA produces a polypeptide of the correct size and antigenicity. This figure is an autoradiograph of an SDS polyacrylamide gel. In the lane labeled "Not Immunoprecipitated," a complete in vitro translation reaction, using the synthetic mRNA as a template, was electrophoresed. In the lanes labeled "Anti-G Antiserum" and "Preimmune Serum," only the immunoprecipitates from the same translation reaction were electrophoresed. The line, labeled G, marks the position at which authentic G protein migrates.

TABLE I
Translation Efficiency: Synthetic vs. Authentic mRNA

	[³⁵ S]methionine incorporation into protein cpm/ng of mRNA
VSV poly A RNA*	10,000 ± 5,000
Synthetic RNA capped	5,000 ± 2,500
Synthetic RNA uncapped	200 ± 100

* Total RNA was used in the translation reactions. The poly A RNA comprises ~5% (by weight) of total RNA (as judged by purification of poly A RNA using oligo dT cellulose [Collaborative Research, Inc.]).

The cap's stimulation of translation is consistent with many other studies (30). Bergmann and Lodish (31) reported that lowering the ionic strength of the wheat germ translation reaction significantly decreased the requirement of the cap for efficient translation. We did not find that these lower ionic strength conditions helped the translation of the uncapped RNA. Furthermore, adding *S*-adenosylmethionine to the translation reaction did not significantly increase the translation efficiency of the uncapped RNA (30).

The Newly Synthesized G Protein is Inserted into Microsomes

To further confirm that the polypeptide produced from the synthetic mRNA was indistinguishable from G protein produced from VSV mRNA, we tested the protein's ability to be properly inserted into microsomal membranes and be glycosylated. This experiment was performed by adding DPM to the translation reaction. The products from the reaction were analyzed by electrophoresis on an SDS polyacrylamide gel.

Fig. 4, which is an autoradiograph of this gel, demonstrates that the polypeptide produced from the synthetic mRNA has the same decrease in mobility as G protein produced from VSV mRNA. The G protein, when inserted into microsomes in vivo, has its signal sequence removed and has two high mannose oligosaccharides attached (32). The net result of these modifications is slower mobility on the SDS polyacrylamide gel (33). Not all of the G protein is inserted into the membranes, but the efficiency is similar for authentic and synthetic mRNAs.

Next, we checked whether the polypeptide was inserted with the proper topology. Authentic G protein (511 amino acids) has about 450 of its amino terminal amino acids in the lumen of the microsome, some 20 hydrophobic amino acids spanning the lipid bilayer, and about 30 carboxyl terminal amino acids remain on the exterior of the microsome (18). We tested the polypeptide's topology by digesting the G protein containing microsomes with trypsin, to cleave off the exposed carboxyl terminus of G protein (33). The products of this incubation were analyzed by electrophoresis through an SDS polyacrylamide gel. Fig. 5, which is an autoradiograph of this gel, demonstrates that the trypsin-digested G proteins produced

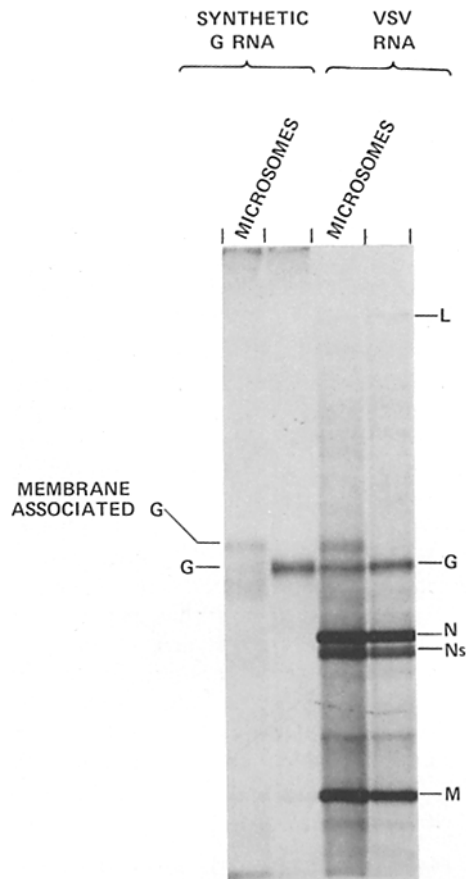


FIGURE 4 The synthetic mRNA produces a polypeptide that inserts into membranes. This figure is an autoradiograph of an SDS polyacrylamide gel. Synthetic and VSV mRNAs were translated with and without microsomes, and the entire reaction was analyzed by electrophoresis. When microsomes were added, ~75% of the G protein was converted to a slightly larger molecule. The labels L, N, Ns, and M correspond to the other proteins produced by the VSV. In the samples containing microsomes, there is a protein that is slightly smaller than the G molecule. This protein, which does not react with anti-G antibodies, is derived from an mRNA in the microsome preparation.

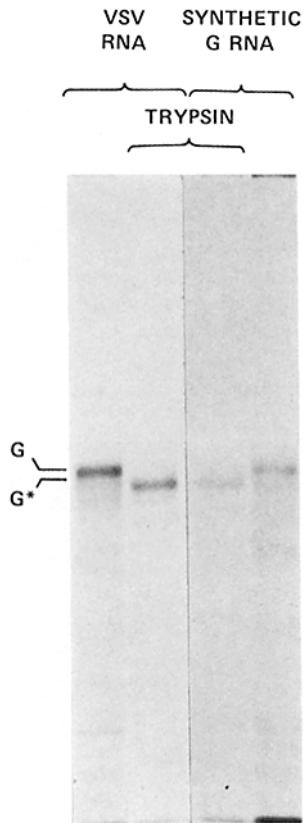


FIGURE 5 Trypsin digestion of the membrane-associated, in vitro translated G protein. This figure is an autoradiograph of an SDS polyacrylamide gel. Synthetic and VSV mRNAs were translated in the presence of microsomes. One-half of each reaction was treated with trypsin (see Materials and Methods for the details). All four samples were immunoprecipitated with anti-G protein antiserum and then electrophoresed. Full-length G protein is designated by the letter G, and the protease-cleaved G protein is designated by the letter G*.

from the synthetic and the authentic mRNAs have the same apparent molecular weights. This result further strengthens the evidence that the synthetic mRNA codes for an authentic G protein.

Finally, we tested directly for the glycosylation of the polypeptide synthesized in the presence of microsomes. The enzyme endoglycosidase H (Endo H) cleaves after the first *N*-acetylglucosamine (GlcNAc) residue of the high mannose oligosaccharide (32), removing all but a single GlcNAc residue attached to the asparagine. We incubated the translation products (made in the presence of microsomes) with Endo H and then electrophoresed the products through an SDS polyacrylamide gel. An autoradiograph of this gel (Fig. 6) shows that Endo H-sensitive forms of G have been synthesized in vitro, and that the products of the Endo H digestion are the same for the polypeptide translated from the synthetic mRNA as for the G protein translated from the VSV mRNA. Taken together, all of these results confirm that the synthetic mRNA codes for a polypeptide that is indistinguishable from authentic G protein.

DISCUSSION

We have adapted a technique (30, 34) in which a synthetic mRNA directs the translation of an integral membrane protein. Specifically, we fused the *Lac* UV5 promoter to the VSV G protein gene, transcribed the gene in vitro using *E. coli* RNA polymerase, and added a "cap" to the RNA product. This synthetic mRNA was translated by a wheat germ extract containing pancreatic microsomes, producing a polypeptide that was indistinguishable from the authentic G protein (Figs. 3-6). Therefore, this technique can be used to synthesize normal or mutant proteins in vitro, merely by inserting the wild type or modified gene next to the *Lac* promoter.

The construction of a well-characterized mRNA in vitro has

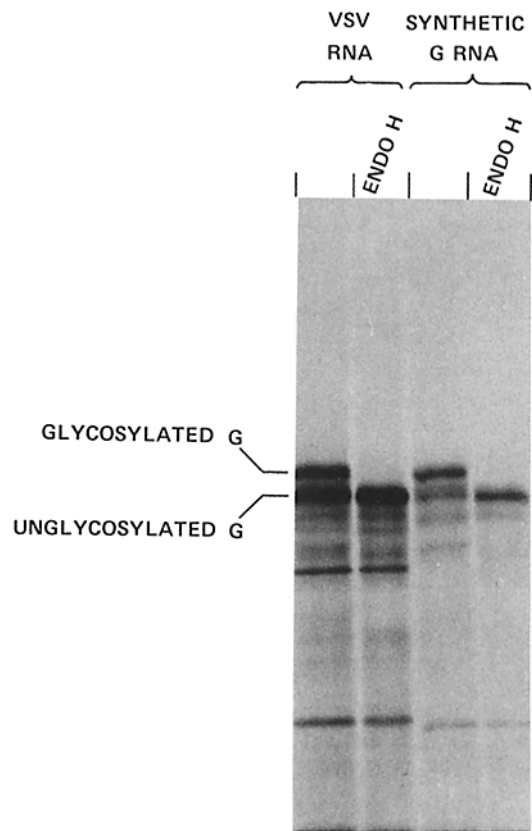


FIGURE 6 Endo H digestion of the membrane-associated, in vitro translated, G protein. This figure is an autoradiograph of an SDS polyacrylamide gel. Synthetic and VSV mRNAs were translated in the presence of microsomes, and the G protein was immunoprecipitated. One-half of each precipitate was digested with Endo H (see Materials and Methods for details), and then the samples were analyzed by electrophoresis. The samples, which were not treated with Endo H, display two species of G protein on the gel. The larger molecule is the species that has been processed by the microsomes (glycosylated and signal sequence removed). The smaller molecule is the G protein that is unprocessed by the microsomes (primary translation product). The Endo H-treated samples display one species of G protein on the gel. Actually, in this case, there are two types of G proteins that co-migrate. The two molecules are the primary translation product and the Endo H-digested, membrane-associated G protein.

some clear advantages over in vivo transcription of transplanted eucaryotic genes. These advantages follow from the fact that mRNA is the immediate precursor of protein. Producing large quantities of a mRNA in vivo requires that a gene must be under the control of a strong promoter. Presently, a number of transcription initiation sequences have been used for expressing cloned genes in vivo (7, 8). However, all promoters are not equally functional in all cell types (35). Therefore, it is a gamble to expect adequate transcription of a gene that is transplanted into a foreign cell. Another problem results because eucaryotic genes have various numbers of introns. Very little is known about their function or the rules of the splicing process (36). Therefore, it is possible that proper RNA processing will not occur when introducing a gene into a new cell or a modified gene into the same cell. The production of synthetic mRNA bypasses both of these problems.

This technique may have a wide variety of applications, of which those involving cell-free translation are the most imme-

diately feasible. For instance, to study the amino acid sequences of G protein needed for membrane insertion, glycosylation, and membrane attachment, mutations could be made in the pertinent parts of the G protein gene in vitro and tested for their effects. Similar experiments could be performed to help identify the signals on cytoplasmic precursor proteins destined for import into mitochondria, chloroplasts, and peroxisomes. Finally, this technique could also be extended to study the effect of RNA structure on translation efficiency. The effect of various lengths and sequence arrangements in the untranslated leader sections of the RNA can be examined.

We believe that synthetic mRNA (possibly with the addition of a 3' poly A tail) will also be useful for in vivo experiments. For instance, when gene expression in a foreign cell is not possible because of an inability to produce a stable mRNA, microinjection of the synthetic mRNA into the cytoplasm will allow the production of the desired protein (37, 38).

Furthermore, because the techniques can generate microgram quantities of translatable RNA, experiments involving many cells on a scale suitable for biochemical analysis should be possible. We produce 1 μ g of a specific RNA (Fig. 2) from 1 μ g of DNA template. 1 μ g of the 1.7-kb RNA is $\sim 10^{12}$ molecules. This is equivalent to the number of G protein mRNA molecules found in $\sim 2 \times 10^8$ VSV-infected cells.¹

For this to be possible, a bulk method to introduce the mRNA into cells will be needed. It is reasonable to anticipate that in the near future liposomes (39) will be nearly as efficient as membrane-enveloped viruses at delivering their contents into cells. For example, assume that the efficiency of liposomal delivery of mRNA to the cell's cytoplasm is 10%. Then, if 1 μ g of mRNA is packaged into liposomes and applied to $\sim 10^7$ cells, 100 ng of mRNA will be available for translation. Based upon the previous calculation, this is equivalent to the amount of G mRNA produced in the same number of cells during a VSV infection.

We thank David Peabody for introducing us to the idea of making the synthetic RNA, and Thomas Kadesch for providing the materials that made the transcription experiments possible. We thank William Braell for his invaluable aid with the wheat germ translations, and for his help in the preparation of the dog pancreatic microsomes. We are grateful to James E. Rothman for his enthusiastic support of this work and for his careful reading of this manuscript. We thank Paul Berg for opening his facilities to us. Finally, we thank Debra Forseth and Karen Martin for their excellent work on this manuscript.

This work was supported by American Cancer Society grant No. BC-394 to James Rothman. John L. R. Rubenstein is a recipient of a National Institutes of Health Medical Scientist Training Program predoctoral fellowship, and Thomas G. Chappell is a recipient of a National Science Foundation predoctoral fellowship.

Received for publication 13 September 1982, and in revised form 24 January 1983.

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¹ The number of G protein mRNA molecules found in 2×10^8 cells was calculated as follows: we isolate 5 mg of total RNA from 10^9 VSV-infected baby hamster kidney cells. From the 5 mg of RNA, we isolate 100 μ g of poly A RNA. We estimate that roughly 5% of poly A RNA is G protein mRNA. Therefore, there is $\sim 5 \mu$ g of G mRNA/ 10^9 cells, or $\sim 5 \times 10^{12}$ molecules of G mRNA/ 10^9 cells (VSV G mRNA is ~ 1.7 kb). Hence, 1 μ g of synthetic G RNA is equivalent to the number of G mRNA molecules found in 2×10^8 cells.

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