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Functional messenger RNAs are produced by SP6 *in vitro* transcription of cloned cDNAs

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ABSTRACT

We describe a method for the synthesis of microgram quantities of eucaryotic messenger RNAs. Injection into the cytoplasm of frog oocytes and addition to wheat germ extracts show that these synthetic RNAs function efficiently as messenger RNAs. We confirm that a 5' cap on the mRNA is essential for translation in injected oocytes and show that most of the 3' flanking region, including the poly A tail, can be deleted without the abolition of protein synthesis. The method of mRNA synthesis involves *in vitro* transcription of cDNAs which have been cloned into SP6 vectors (described in the accompanying paper). This method enables one to produce large amounts of mRNA and consequently protein from any cDNA clone.

INTRODUCTION

In addition to serving as a template for translation, messenger RNAs are involved in several other cellular activities including transport from the nucleus (rev. in 1 and 2), attachment to the cytoskeleton (3), and localization within the cytoplasm (4). The nature of the signals within messenger RNAs which may be important for these other activities are certainly not understood. Indeed, many of these activities are themselves poorly characterized. One obvious approach to identifying and characterizing RNA signals for mRNA transport or localization is to alter the sequence of the mRNA and test its activity. In many instances this type of analysis can be performed in a coupled transcription-translation system to which altered DNA templates are added. However, it is sometimes difficult to obtain stable and efficient expression vectors for the cell type of interest. Moreover, alterations made in the DNA template may have an adverse affect on transcription or RNA processing making studies on mature mRNA a moot point. An alternative which avoids these problems is to synthesize mutant mRNAs *in vitro* and then directly test their function either *in vivo*, after microinjection, or *in vitro*.

We have previously shown that an SP6 *in vitro* transcription system (5,

accompanying paper) can be used to produce large amounts of unprocessed mRNAs and that these in vitro transcripts are biologically active. For example, unspliced globin pre-mRNAs, produced with SP6 RNA polymerase by in vitro transcription of genomic globin DNA, are correctly spliced when microinjected into the nucleus of frog oocytes (6). Similarly, SP6 RNA transcripts which extend beyond the 3' end of a histone mRNA are correctly processed in injected oocytes to yield histone mRNAs with mature 3' ends (7). In vitro transcription with SP6 RNA polymerase has also been used to obtain sufficient quantities of unspliced pre-mRNAs for use as a substrate in detailed biochemical studies of mRNA splicing reactions (8,9). In the present study, we show that in vitro transcription with SP6 RNA polymerase can be extended to the study of mRNA function. We show that SP6 in vitro transcription of cloned cDNAs can be used to produce large amounts of functional mRNAs. These synthetic mRNAs are efficiently translated in injected oocytes and in wheat germ extracts. Coupled with mutagenesis of the cDNA template this method makes it possible to analyze the significance of any structural feature of an mRNA without resorting to more conventional genetic approaches.

MATERIALS AND METHODS

Materials

Vaccinia virus guanylyltransferase was purchased from Bethesda Research Laboratories. The chloride salt of S-adenosyl-L-methionine was purchased from Sigma. ^{35}S-methionine (1020 Ci/mmol) was purchased from Amersham. Sources for all other enzymes and reagents are noted in the accompanying paper.

Plasmid constructions

pSP64-Xβm contains a Xenopus β-globin cDNA clone inserted into the SP6 cloning vector pSP64 (accompanying paper). This plasmid was constructed by the following steps such that SP6 in vitro transcription produces an RNA which closely resembles authentic β-globin mRNA. A Xenopus β-globin chromosomal gene, pXGIC3 (10), was digested with HgiA I which cuts the gene 2 bases upstream of the cap site and in the second intron. Following the addition of Hind III linkers, the HgiA I restriction fragment was inserted into the Hind III site of pSP64 giving rise to pSP64-Xβ-HgiA. A Xenopus β-globin cDNA clone, pXG8D2 (11), was digested with Nco I and Pst I. The 5' end (the Nco I end) of this fragment is the ATG found at the beginning of the protein coding region and the 3' end (the Pst I end) lies downstream of the poly A tail. The 3' end of this fragment contains all of the 3' flank-

ing region of the mRNA, a poly dA-dT stretch of 23 residues and about 30 dC-dG residues resulting from the GC tailing used to clone the cDNA (11). This Nco I - Pst I restriction fragment was inserted into pSP64-Xβ-HgiA, which had been previously digested with Nco I and Pst I, to give rise to pSP64-Xβm (Figure 1). This plasmid contains 12 bases of extra sequences between the start site of transcription by SP6 RNA polymerase and the cap site of the normal β-globin mRNA. Consequently, SP6 transcripts of Pst I digested pSP64-Xβm DNA are identical to mature β-globin mRNAs except that they contain 12 extra bases at their 5' end and a stretch of about 30 C residues at the 3' end after the poly A tail.

pSP64T is an SP6 cloning vector designed to provide 5' and 3' flanking regions from an mRNA which is efficiently translated (β-globin) to any cDNA which contains its own initiation codon. This 'translation' SP6 vector was constructed by digesting pSP64-Xβm with Bal I and BstE II , filling in the staggered ends with T4 DNA polymerase and adding a Bgl II linker by ligation. Bal I cuts the β-globin cDNA 2 bases upstream of the ATG (start codon) and BstE II cuts 8 bases upstream of the TAA (stop codon). There is only one Bgl II site in pSP64T so that restriction enzymes cutting in the polylinker fragment, from Pst I to EcoR I (Figure 1), can still be used to linearize the plasmid for transcription.

pSP64T-IFN contains a human interferon gene cloned into pSP64T. Human β-interferon DNA was digested with Hinc II, which cleaves the DNA 3 bases upstream from the initiation codon (12), a Bgl II linker was added and the DNA was digested with Bgl II which cleaved the linker and cuts the interferon sequence 2 bases after the termination codon. The 580 bp Bgl II fragment containing the entire protein coding sequence of the β-interferon gene was ligated into Bgl II digested pSP64T.

In vitro transcription with SP6 RNA polymerase

Linear DNA templates (100μg/ml) are transcribed in 40 mM Tris, pH 7.5, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, RNAsin (1 unit/μl), 100 μg/ml BSA, and 400 μM of each rNTP. Typically, 1 unit of SP6 RNA polymerase is added per μg of DNA template for a 1 hour synthesis at 40°. Following RNA synthesis the DNA template is removed by the addition of RNAsin and RNAse-free DNAse to final concentrations of 1 unit/μl and 20 μg/ml, respectively. After a 10 min incubation at 37° the reaction is extracted with phenol:chloroform and the RNA is purified by Sephadex G100 chromatography in 10mM Tris-HCl, pH 7, 0.1% SDS. The RNA is then recovered by addition of sodium acetate, pH 6.5, to 300mM and precipitation with 2 volumes of ethanol. SP6 transcripts stored in ethanol at -20° C are stable for several

months. Further details on SP6 RNA synthesis can be found in the accompanying paper.

Gel analyses

RNA transcripts are stored in ethanol and collected by centrifugation immediately before use. The precipitates are dissolved in diethylpyrocarbonate-treated H_2O and mixed with 3 volumes of loading buffer (67% deionized formamide, 20% formaldehyde, 13% 10X MOPS buffer). Following incubation at 60°C for 5 min the sample is electrophoresed in agarose (1%) containing 7.5% formaldehyde (v:v) in 1X MOPS buffer. Electrophoresis is carried out in 1X MOPS without formaldehyde. 10X MOPS buffer is 0.2M 3-N-morpholino-propanesulfonic acid, 0.05M sodium acetate, and 0.01M EDTA (13,14).

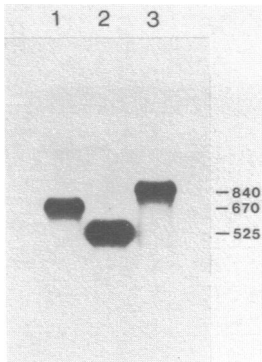
Proteins were fractionated by electrophoresis in SDS-acrylamide gels (15) using a 17 % acrylamide resolving gel with a 4% stacking gel. Gels were fixed with methanol(45%), acetic acid (10%) and prepared for fluorography (16).

Capping reactions

A 5' terminal cap structure, the methylated cap ^{7}mGpppNp, is added to SP6 transcripts *in vitro* with guanylyltransferase using a modification of published procedures (17,18). SP6 RNAs (0.1-1µg) are mixed with guanylyltransferase (1 unit/µg RNA) in a 30 µl reaction containing 50 mM Tris-HCl, pH 7.9, 1.25 mM $MgCl_2$, 6mM KCl, 2.5 mM dithiothreitol, 100 µg/ml BSA, RNAsin (1 unit/µl), 50-100 µM S-adenosyl methionine, and 40 µM GTP. Following a 45 min incubation at 37 °C the reaction is extracted with phenol:chloroform and precipitated with ethanol.

mRNA translation

RNAs were dissolved in 0.1X MBS-H solution (19) for injection into oocytes. Injected oocytes were incubated in 1X MBS-H containing ^{35}S-methionine at 0.5-1 mCi/ml for 15 hrs at 18°C. To examine the newly synthesized intracellular proteins, oocytes were homogenized in 60 mM Tris-HCl, pH 6.8, 1 mM PMSF, 1% SDS, 100 mM β-mercaptoethanol using 50 µl/oocyte. The secreted proteins were recovered from the incubation media by the addition of carrier BSA (2 µg/ml) and precipitation with 3 volumes of acetone:ether (3:1,v:v). *In vitro* translations with wheat germ extracts were performed according to the manufacturer's (BRL) specifications.



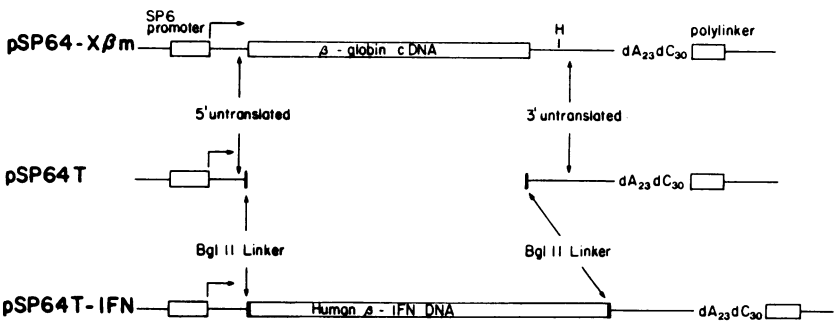


Figure 1. SP6 *in vitro* transcription of cloned cDNAs. The DNA templates shown at the bottom are those used to synthesize mRNAs *in vitro* with SP6 RNA polymerase. pSP64-Xβm can be digested with Pst I which is the first site in the remaining polylinker fragment of pSP64. SP6 transcripts (lane 1) of this DNA are about 670 bases long and contain a poly A tract at their 3' end. This SP6 transcript closely resembles authentic *Xenopus* β-globin mRNA. SP6 transcripts of Hinf I (H) digested pSP64-Xβm DNA (lane 2) are about 525 bases long and lack most of the 3' flanking region. Synthetic human β-IFN-mRNAs, about 840 bases long, are produced by SP6 transcription of Sal I digested pSP64T-IFN (lane 3). Details of each plasmid can be found in the materials and methods. ^{32}P-labelled SP6 transcripts were electrophoresed in a 1% denaturing agarose gel and dried before exposure to X-ray film. An autoradiogram of the gel is shown with the approximate lengths of transcripts indicated in bases.

RESULTS

SP6 transcripts of globin cDNA clones can function as messenger RNAs in injected oocytes

A cloned cDNA containing the entire *Xenopus* β-globin protein coding region and flanking regions was inserted into pSP64 giving rise to pSP64-Xβm (Figure 1). SP6 *in vitro* transcripts of Pst I digested pSP64-Xβm are nearly identical to authentic globin messenger RNA except that there are 12 extra bases at the 5' end and a stretch of about 30 C residues following the poly

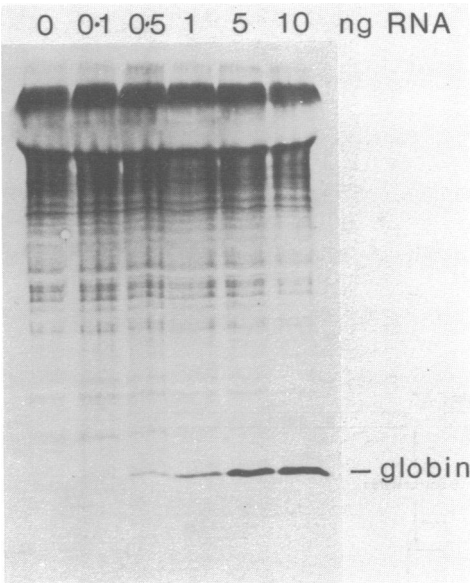


Figure 2. Globin protein is synthesized in oocytes injected with SP6-derived globin mRNAs. SP6 transcripts of Pst I digested pSP64-Xβm were synthesized and capped as described in the material and methods. The SP6 transcripts were labeled with ^{3}H-UTP during synthesis so that exact amounts of this synthetic globin mRNA could be measured for injection. Ten hours after injection of the mRNA, oocytes were incubated for a further 15 hrs in ^{35}S-methionine. Proteins were extracted from injected oocytes and fractionated on a 17% SDS-acrylamide gel and fluorographed. Groups of 20 oocytes were injected for each concentration of mRNA, though each lane contains the protein extracted from the equivalent of only 1/20th of an oocyte. The autoradiogram of the gel shows the amount of globin mRNA injected per oocyte at the top of each lane.

A tail at 3' end (see Materials and Methods for details). Synthetic globin mRNA was produced by SP6 transcription, capped *in vitro* with guanylyl-transferase and tested for activity as a translation template by injection into frog oocyte cytoplasm.

Figure 2 shows that oocytes injected with synthetic globin mRNA synthesize globin protein. The amount of newly synthesized globin protein is directly proportional to the amount of the synthetic mRNA injected, consistent with observations made using authentic mRNA (20,21). At high concentrations of injected RNA (50 ng/oocyte) as much as 40% of the newly synthesized protein in the oocyte is globin (data not shown). Using the known protein synthetic rate of Stage VI oocytes (17 ng protein/hr, ref 22) we calculate that oocytes injected with 50 ng of synthetic globin mRNA can make

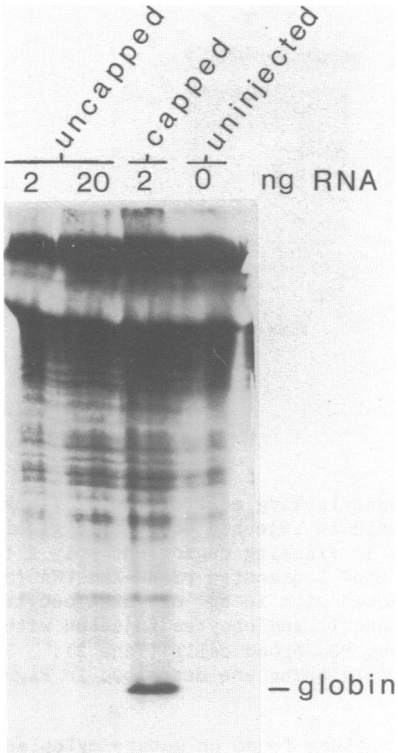


Figure 3. A 5' terminal cap on mRNA is required for functional activity in injected oocytes. Synthetic globin mRNAs were produced by transcription of Pst I digested pSP64-Xβm. These mRNAs were injected into oocytes with or without a 5' cap as indicated. The amount of mRNA injected in each case is indicated at the top of the protein gel. Gel analysis and labelling conditions are given in Figure 2.

about 5 ng of globin protein/oocyte/hr. The distinct protein band labelled globin comigrates with the protein synthesized following injection of authentic poly A+ RNA isolated from Xenopus red blood cells (see below, Figure 4).

A 5' cap structure on synthetic mRNA is required for its stability in oocytes

The results presented in Figure 3 show that synthetic globin mRNA that lacks a 5' terminal cap is unable to direct globin protein synthesis in injected oocytes. Even a 10 fold increase in the amount of uncapped SP64-Xβm RNA relative to capped mRNA is not sufficient to allow for a detectable amount of globin protein synthesis. Previous studies have shown shown that

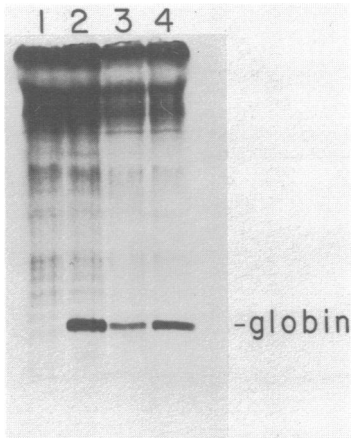


Figure 4. Synthetic mRNAs lacking most of the 3' flanking region can still direct protein synthesis in injected oocytes. β-globin mRNAs with (lane 4) or without (lane 3) the 3' flanking region and poly A tail were synthesized from either Pst I or Hinf I digested pSP64-Xβm DNA (see Figure 1). Groups of 20 oocytes were injected with 10 ng of mRNA/oocyte. Controls include uninjected oocytes (lane 1) and oocytes injected with 10 ng of poly A+ RNA isolated from anemic frog red blood cells (lane 2). Protein gel analysis and labelling with ^{35}S-methionine are described in Figure 2.

the 5' terminal cap structure found on mature cytoplasmic messenger RNAs is essential for their stability in injected oocytes (23,24). Moreover, the 5' cap is required for the stability of SP6 transcripts injected into oocyte nuclei (6). In the light of these previous reports it is expected that uncapped synthetic mRNAs would be unable to direct protein synthesis because of mRNA instability. Indeed, direct analysis of RNA stability by Northern blots shows that the uncapped RNA is degraded within 15 min whereas the capped synthetic mRNA is stable for at least 2 days (data not shown).

The 3' flanking region, including the AAUAAA sequence and poly A tail, is not absolutely required for protein synthesis

To test whether the 3' flanking region of the synthetic globin mRNA contains sequences important for mRNA stability and/or translation, an mRNA lacking this region was synthesized and injected into oocytes. SP6 transcription of Hinf I digested pSP64-Xβm DNA produces mRNAs with only 28 bases downstream of the termination codon, UAA (see Figure 1). The Hinf I derived SP6 RNAs lack about 165 bases of 3' flanking sequence; the AAUAAA sequence and poly A tail are missing.

The results presented in Figure 4 show that a synthetic mRNA lacking

most of the normal 3' flanking region is nevertheless able to direct globin protein synthesis in oocytes. In this experiment equal amounts of poly A+ mRNA (transcripts of Pst I digested pSP64-Xβm) and poly A- mRNA (transcripts of Hinf I digested pSP64-Xβm), were injected into oocytes. Both synthetic β-globin mRNAs direct the synthesis of a β-globin protein which comigrates with the globin proteins synthesized when natural globin mRNA isolated from red blood cells is injected (Figure 4). The poly A+ mRNAs from red blood cells direct the synthesis of α and β globins which are not separated in this gel system. The amount of β-globin protein synthesized by injection of 10 ng of synthetic β-globin poly A+ mRNA (lane 4) is about equal to the combined amounts of α and β globins synthesized following injection of 10 ng of red blood cell poly A+ mRNA. We therefore conclude that the rate of protein synthesis directed by injection of the synthetic poly A+ mRNA is not significantly different from that of natural mRNA.

The amount of globin protein produced in a 15 hr labelling period following injection of the mRNA lacking the 3' flanking region (lane 3) is reduced compared to the protein synthesized from poly A+ mRNAs (lanes 2 and 4). This observation is consistent with the view that the poly A tail is important for mRNA stability. Northern blot analyses of the injected RNAs show that Xenopus red blood cell globin mRNA and the poly A+ synthetic mRNA are equally stable in oocytes over a two day period, whereas the poly A- mRNA is somewhat less stable (data not shown). We conclude that the 3' flanking region has a slight effect on the stability of an injected mRNA during a 24 hr incubation in oocytes and this results in a corresponding decrease in the amount of protein synthesized. However, unlike the 5' cap, it is not essential that a synthetic mRNA possess a perfect 3' flanking region in order to direct the synthesis of a detectable amount of protein in injected oocytes.

Synthetic human interferon mRNA is translated in oocytes and interferon protein is secreted into the media

As a test for whether synthetic heterologous mRNAs can also serve as templates for translation, we inserted a human β-interferon DNA sequence into pSP64T (Figure 1). Previous studies have shown that oocytes injected with poly A+ mRNA containing interferon message will synthesize and secrete interferon protein (25,26). SP6 transcripts of Sal I digested pSP64T-IFN are hybrid mRNAs containing the 5' and 3' flanking regions of Xenopus β-globin mRNA and the protein coding region of the human β-interferon gene (Figure 1). When synthetic IFN-mRNA is capped and injected into oocytes it

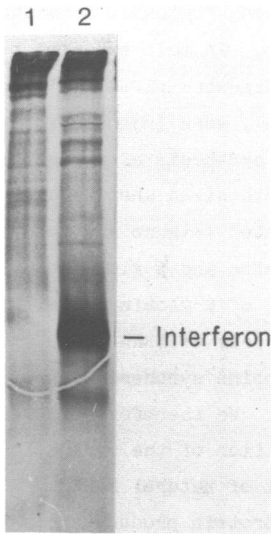


Figure 5. Interferon is secreted from oocytes injected with synthetic interferon mRNA. pSP64T-IFN DNA (Figure 1) was digested with Sal I and transcribed with SP6 RNA polymerase. The RNA transcripts were capped and injected into oocytes (2ng/oocyte). After a 10 hr labelling period, the media from 5 oocytes was precipitated and fractionated by gel electrophoresis as described in Figure 2. Lane 1, uninjected oocytes; lane 2, oocytes injected with pSP64T-IFN mRNA.

directs the synthesis of substantial amounts of interferon protein which is secreted into the media (Figure 5). There is no direct positive control in this experiment because it is not possible to obtain sufficient quantities of natural interferon mRNA for comparison by this type of analysis. However, separate injection experiments with the synthetic IFN-mRNA show that the media containing the secreted protein has 2 X 10^4 units of IFN/oocyte as measured by anti-viral activity.

These results show that hybrid mRNAs produced with the SP64T vector are translated in injected oocytes. This establishes a general method for producing large amounts of any messenger RNA if the protein coding region of a gene has been cloned. It is important to note that it is not necessary to have a full length cDNA clone because the pSP64T provides functional 5' and 3' flanking regions for the mRNA.

SP6 transcripts of cDNA clones are functional mRNAs in wheat germ translation extracts

The data presented in Figure 6 show that both the globin and IFN syn-

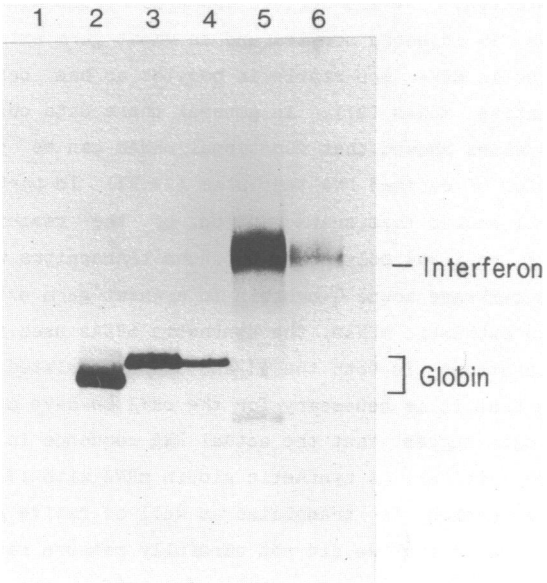


Figure 6. Translation of synthetic mRNAs in wheat germ extracts. 100 ng of the mRNA was added to wheat germ extracts and proteins were labelled with ^{35}S-methionine during a 1 hr reaction. The proteins were fractionated by gel electrophoresis as described in Figure 2. Lane 1, control without added RNA. Lane 2, control of rabbit red blood cell poly A+ RNA. Capped (lane 3) and uncapped (lane 4) synthetic Xenopus globin mRNAs transcribed from Pst I digested pSP64-Xβm DNA. Note that the rabbit globin protein migrates farther in this gel than does the Xenopus β-globin protein. Capped (lane 5) and uncapped (lane 6) synthetic interferon mRNAs transcribed from Sal I digested pSP64T-IFN DNA. See Figure 1 for plasmid diagrams.

thetic mRNAs direct the synthesis of the appropriate protein in wheat germ extracts. In contrast to the situation in injected oocytes, a 5' terminal cap is not absolutely required for translation in this in vitro system. Uncapped synthetic mRNAs are translated in the wheat germ extract, though there is a significant reduction in the amount of protein synthesized relative to that observed using capped mRNAs (Figure 6). The different translation efficiencies observed for capped and uncapped synthetic mRNAs is consistent with results obtained from previous studies on various natural messages in wheat germ extracts (27-29).

DISCUSSION

The data presented in this paper show that synthetic messenger RNAs produced by SP6 in vitro transcription of cDNA clones are effective tem-

plates for translation. The SP6 derived mRNAs are translated as efficiently as native mRNAs in injected oocytes and in wheat germ extracts. Furthermore, these synthetic mRNAs are stable in oocytes as has been demonstrated for injected native mRNAs (27). In general these data confirm results of previous studies which showed that functional mRNAs can be produced by *in vitro* transcription of defined DNA templates (28-31) In particular, Rubenstein and Chappell showed that transcription of the vesicular stomatitis virus G gene by *E. coli* RNA polymerase produces transcripts which direct the synthesis of the membrane bound G-protein in a wheat germ extract (32).

Compared to authentic mRNAs, the synthetic mRNAs used in the present study have alterations in both the 5' and 3' untranslated regions. While our results show that it is necessary for the mRNA to have a 5' cap for mRNA stability, the data suggest that the actual RNA sequence in the 5' flanking region may not be critical. A synthetic globin mRNA with 12 extra bases in the 5' flanking region is translated as well as native globin mRNA. It should be noted however that we did not carefully measure rates of protein synthesis, only accumulation of protein over a 15 hr period. We therefore conclude that the sequence of the 5' untranslated region can be altered without the abolition of mRNA function.

Similarly, in the case of the 3' flanking region, the data presented here suggest that an extra 30 C residues following the poly A tail at the 3' end of an mRNA does not significantly affect translation. Moreover, most of the 3' flanking region can be removed without destroying the translational activity of the message. This latter result confirms findings obtained with rabbit β-globin mRNAs in wheat germ extracts (30). Again, provisos about possible effects of 3' flanking sequences on the rate of protein synthesis must be considered. A large number of carefully controlled experiments have shown that the poly A tail is essential for the functional stability of mRNAs in injected oocytes, a result apparently contradicting our conclusions about the 3' flanking region. The experiments which showed that unadenylated or deadenylated mRNAs are unstable in injected oocytes (33-36) all measured mRNA stability over a period of days. In contrast, our experiments test the functional stability of mRNAs only during the first day. It is therefore reasonable to conclude that a poly A tail is required for long term mRNA stability, but not for experiments in which protein synthesis is assayed in shorter periods.

The advantages of using SP6 RNA polymerase for *in vitro* transcription are documented in the accompanying paper. In short, this transcription system produces large amounts (micrograms) of mRNAs in a simple enzymatic reac-

tion and does so in the absence of other unwanted transcriptional events (such as transcribing both strands of the DNA template). We describe here a convenient vector, pSP64T, into which any protein coding cDNA clone can be inserted for the purpose of synthesizing functional mRNA. SP6 transcription of the recombinant pSP64T plasmids will produce synthetic mRNAs that contain 5' and 3' flanking regions, including a poly A tail. Our results show that these 5' and 3' flanking regions, which are derived from globin mRNA, allow for the efficient translation of the inserted protein coding region both in injected oocytes and in wheat germ extracts (Figs 5,6). These experiments suggest that this procedure can be used to identify and synthesize the protein encoded by any cloned cDNA. This may therefore be an attractive alternative to so called hybrid selected translation assays when the mRNA of interest is rare. In addition, it may be possible to use this method to produce sufficient amounts of mRNA and subsequently protein in order to generate antibodies against the products of cloned cDNAs. Finally, as an alternative to DNA expression vectors, it should be possible to inject synthetic SP6 mRNAs into cells in order to direct the synthesis of specific proteins and mutants thereof.

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