The EMBO Journal vol.7 no.11 pp.3559-3569, 1988

Ribosome pausing and stacking during translation of a eukaryotic mRNA

Sandra L.Wolin and Peter Walter

Department of Biochemistry and Biophysics, University of California, San Francisco, CA 94143-0448, USA

Communicated by B.Dobberstein

We have devised a sensitive assay to determine the distribution of translating ribosomes on a mRNA. Using this assay to monitor ribosome transit on bovine preprolactin mRNA, we have detected four major positions of ribosome pausing in both wheat-germ and rabbit reticulocyte extracts. Two of these rate-limiting steps represent initiation and termination. One pause occurs after ~75 amino acids have been polymerized; signal recognition particle arrests preprolactin synthesis at this position. The other internal pause occurs at 160 amino acids. In these latter two cases ribosomes stop at a GGC glycine codon; however, two other GGC codons are translated without apparent pausing. Surprisingly, we find that up to nine ribosomes are tightly stacked behind each pausing ribosome, such that the ribosome centers are only 27-29 nucleotides apart. The assay should prove useful for probing mechanisms of translational regulation.

Key words: ribosome pausing/translation/signal recognition particle

Introduction

Regulation of mRNA translation into protein is an important means of controlling gene expression. Numerous examples of translational regulation in prokaryotes have been described. Prokaryotic translation can be controlled by the activity of *trans*-acting molecules or by subtle changes in the interactions between the various components required for protein synthesis, such as mRNA, tRNA and ribosomes (reviewed by Gold, 1988). In eukaryotes, similar mechanisms appear to operate. For example, in the case of the yeast *GCN4* mRNA, which encodes a transcriptional activator of many genes involved in amino acid biosynthesis, four short upstream open reading frames act to repress translation; under conditions of amino acid starvation, two *trans*-acting factors facilitate the efficient translation of the mRNA (Mueller and Hinnebusch, 1986; Tzamarias *et al.*, 1986). Translation of the yeast *CPA1* mRNA, which encodes a subunit of an enzyme involved in arginine biosynthesis, is repressed by arginine. This repression requires the translation of an upstream ORF in the *CPA1* mRNA, which encodes a 25-amino-acid peptide (Werner *et al.*, 1987). However, even for these well-documented examples of translational regulation in eukaryotes, little is known of the molecular mechanisms involved.

For the future understanding of translational regulation, it will be increasingly important to obtain a precise picture of the dynamics of the translation process. In particular, it will be desirable to monitor ribosome activity on discrete regions of mRNA, i.e. to determine the positions of individual ribosomes during translation. For example, when multiple reading frames are involved in translational regulation (as in *GCN4* mRNA), the relative translational activities of the different reading frames may vary over time. Even within a single coding sequence, the movement of ribosomes on the mRNA is not linear with time. Rather, ribosomes are known to pause at discrete sites for unknown reasons (Protzel and Morris, 1974). Knowledge of the positions at which a ribosome stalls will allow the identification of those features of mRNA sequence or structure that result in ribosome pausing and the mechanisms by which *trans*-acting factors modulate this process. To this end, we have devised a sensitive assay to map the positions of translating ribosomes on a mRNA at steady state. We have used this assay to monitor the translation of a model mRNA (bovine preprolactin) in two *in vitro* systems. We demonstrate that we can detect and map discrete sites of ribosome pausing during preprolactin synthesis on free polysomes. Surprisingly, we find that additional ribosomes are tightly stacked behind each paused ribosome, with the centers of the ribosomes only 27-29 nt apart. Thus, translating ribosomes are not uniformly distributed along a mRNA. Because the assay can reveal the positions of translating ribosomes on an unlabeled mRNA that is part of a complex mixture, it should be possible to use this assay to detect ribosome pausing during mRNA translation *in vivo*.

Results

Experimental strategy

Our scheme for determining the steady-state distribution of ribosomes on a mRNA is diagrammed in Figure 1. Translating extracts are treated with micrococcal nuclease at a concentration sufficient to trim away portions of mRNA that are not protected by ribosomes (A in Figure 1). Ribosomes protect 30-35 nt of mRNA from ribonuclease digestion (reviewed by Steitz, 1980; Kozak, 1983). If translating ribosomes move at a constant rate along the mRNA, then all possible segments of 30-35 nt should be equally represented among the ribosome-protected fragments. However, if ribosomes pause during translation, certain fragments (that correspond to positions of pausing) will be overrepresented. The ribosome-protected fragments are purified (step B) and hybridized to the antisense cDNA strand of the mRNA (step C). The positions of the RNA fragments on the DNA are then determined by a primer extension assay. In this assay, bacteriophage T4 DNA polymerase [in conjunction with three T4 polymerase accessory proteins (genes 44/62 and 45 proteins) that increase the rate and processivity of DNA synthesis (reviewed by Nossal, 1983; Alberts, 1984)] is used to extend a labeled primer that has been annealed upstream of the protected

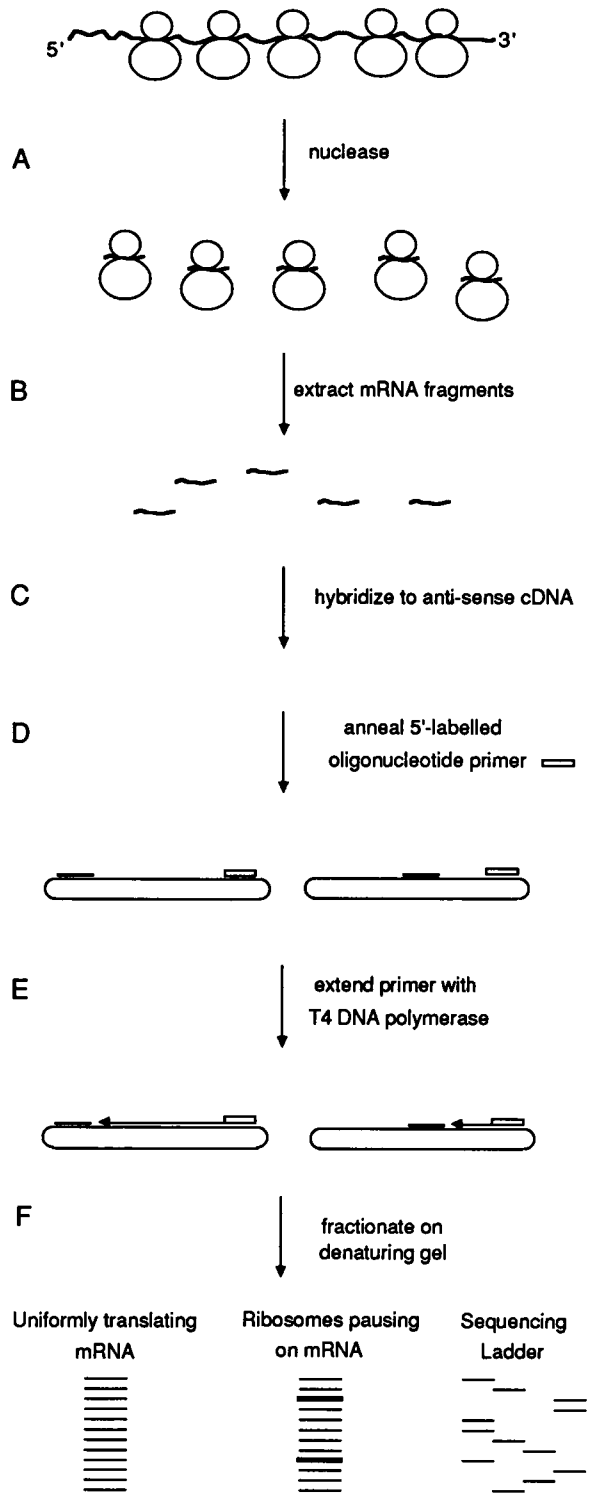

Fig. 1. Strategy for determining the positions of pausing ribosomes on a mRNA. After addition of cycloheximide to freeze ribosomes on the mRNA, translation extracts are treated with nuclease to trim away fragments of RNA that are not associated with ribosomes (**A**). The resulting ribosome-protected fragments are purified (**B**) and hybridized to a single-stranded antisense cDNA clone (**C**). A 5′-labeled oligonucleotide primer is also annealed (**D**), and is then extended with T4 DNA polymerase (**E**). Because this polymerase will not unwind the RNA–DNA hybrid, the polymerase stops at the 5′ end of the annealed ribosome-protected fragment. The products of the primer extension reaction are fractionated in sequencing gels (**F**). By using the same labeled primer in dideoxy sequencing reactions, the 5′ end of the ribosome-protected fragments can be resolved with single nucleotide precision.

fragments (steps D and E). Because this polymerase, in the absence of the T4 gene 32 protein, will not catalyze strand displacement (reviewed by Nossal, 1983; Alberts, 1984), the ribosome-protected fragments act as 'roadblocks' to the DNA synthesis reaction. [Hu and Davidson (1986) used a similar primer extension assay to determine the transcription start point of the mouse skeletal actin gene.] The lengths of the extended DNAs can be measured with single nucleotide precision by fractionating the reaction products in denaturing gels in parallel with a sequencing ladder (step F). A ladder of bands, each corresponding to the 5′ end of a ribosome-protected fragment, will be generated. If ribosomes move at a constant rate along the mRNA, this ladder will be uniform in intensity. If ribosomes pause along the mRNA, certain bands (that correspond to the trailing edges of stalled ribosomes) will be correspondingly enhanced.

Multiple translating ribosomes can stack tightly on mRNA

To begin our analysis, we prepared a radiolabeled synthetic mRNA (encoding bovine preprolactin) by transcription with SP6 RNA polymerase and directly examined the RNA fragments protected by translating ribosomes. We incubated the mRNA for 25 min in a wheat-germ extract and then added cycloheximide to block further elongation and to 'freeze' the ribosomes on the mRNA (Columbo *et al.*, 1966). Following digestion with micrococcal nuclease, we pelleted the ribosomes and compared the nuclease-resistant fragments in the ribosomal pellet (Figure 2A, lanes 2–4) with those in the supernatant (Figure 2A, lanes 8–10). At high concentrations of micrococcal nuclease, the ribosomal pellet contained RNA fragments of ~30 nt (Figure 2A, lane 4), while the supernatant contained very little undegraded RNA (Figure 2A, lane 10). When translation was prevented with the cap analogue 7-methylguanosine-5′-monophosphate (7mG)[which inhibits mRNA joining to 43S pre-initiation complexes (Both *et al.*, 1976; Hickey *et al.*, 1976)], no nuclease-resistant fragments were detected in the pellet (Figure 2B, compare lanes 1 and 2). Thus, the nuclease-resistant fragments in the ribosomal pellet represent regions of mRNA protected by translating ribosomes.

We sized the ribosome-protected fragments more precisely by fractionating them in a denaturing gel alongside a sequencing ladder. The majority of the fragments were between 24 and 32 nt in length (Figure 2C, lane 1), which agrees with the sizes of ribosome-protected fragments determined by others (Hindley and Staples, 1969; Steitz, 1969; Kozak and Shatkin, 1977a,b; Legon *et al.*, 1977).

At low concentrations of micrococcal nuclease, several larger fragments of lower abundance appeared to be protected from digestion (visible as faint bands in Figure 2A, lane 3). These larger fragments were found only in the ribosomal pellet; a different set of nuclease-resistant fragments was found in the supernatant (Figure 2A, cf. lane 3 and lane 9). Intriguingly, the fragments contained within the pellet formed a regular ladder of bands, with a periodicity of 27–30 nt. Because of the even spacing of these bands, we thought that the larger fragments might represent protection by multiple ribosomes, closely stacked together on the mRNA.

We reasoned that this tight stacking of ribosomes might be caused by the pausing of ribosomes on the mRNA. To enhance ribosome pausing, we added the signal recognition

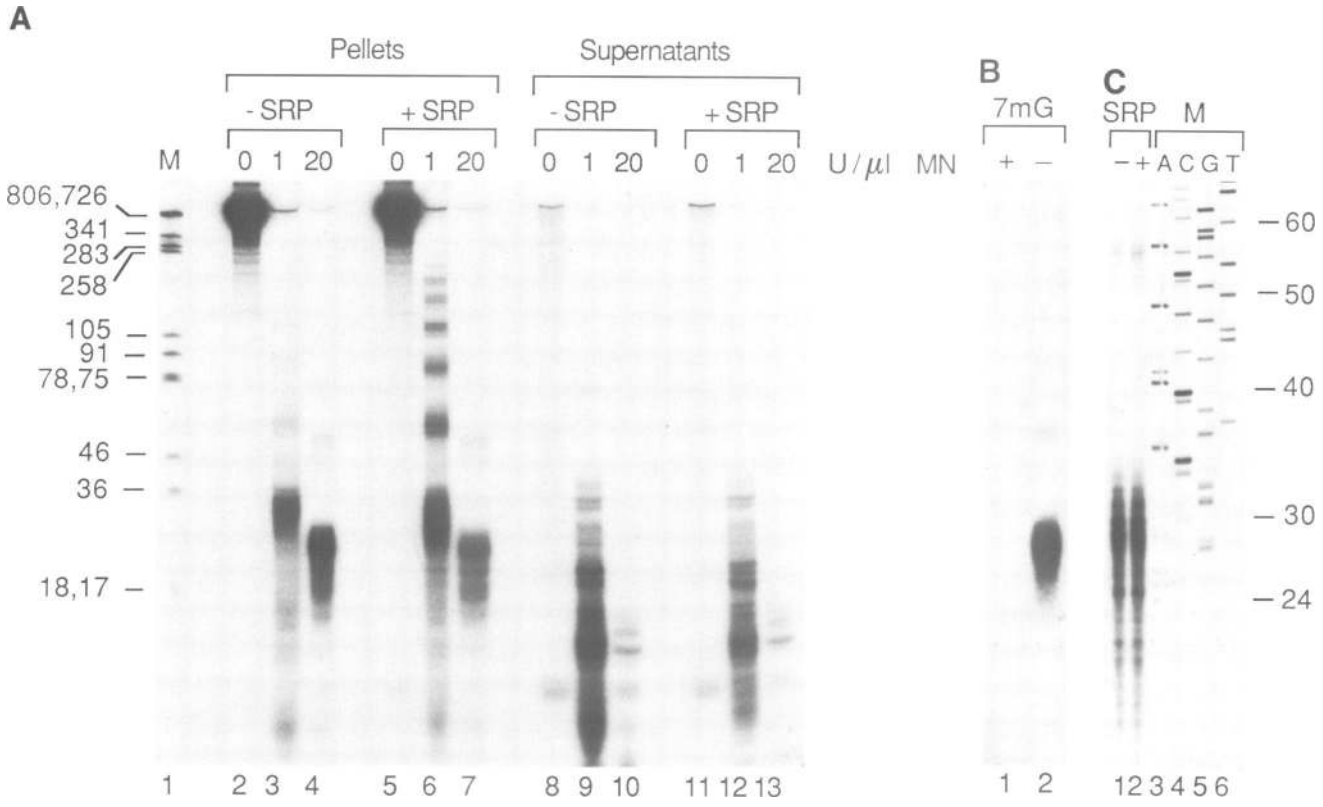

Fig. 2. RNA fragments protected by translating ribosomes from micrococcal nuclease digestion. (**A**) ^{32}P-Labeled synthetic mRNA was used to program a wheat-germ translation reaction in the absence (**lanes 2–4, 8–10**) or presence (**lanes 5–7, 11–13**) of 30 nM canine SRP. Following 25 min of translation at 26°C, cycloheximide was added to 1 mM to block further elongation and to freeze the ribosomes on the mRNA. Micrococcal nuclease was added to a final concentration of either 0 (**lanes 2, 5, 8** and **11**), 1 (**lanes 3, 6, 9** and **12**) or 20 (**lanes 4, 7, 10** and **13**) U/μl. Following 30 min of nuclease digestion at 26°C, ribosomes were pelleted as described in Materials and methods. The RNA fragments associated with the ribosomal pellets (**lanes 2–7**) and the supernatants (**lanes 8–13**) were analyzed by electrophoresis in a 8.3 M urea, 8% polyacrylamide gel. For optimal visualization, the undigested mRNA samples (**lanes 2, 5, 8** and **11**) represent 1/5 the amount of samples loaded in the other lanes. ^{32}P-Labeled mol. wt standards (**lane 1**) were provided from a *Sau*3A digest of plasmid pGEM1 (Promega) DNA. (**B**) ^{32}P-Labeled synthetic mRNA was added to a wheat-germ translation reaction in the presence (**lane 1**) or absence (**lane 2**) of 10 mM 7mG. Following a 25-min translation, cycloheximide was added as in (A), followed by the addition of micrococcal nuclease to a final concentration of 20 U/μl. Following nuclease digestion, ribosomes were pelleted and the ribosome-associated fragments extracted and analyzed as in (A). (**C**) Ribosome-protected fragments were isolated as described above from a translation reaction performed in the presence (**lane 2**) or absence (**lane 1**) of 30 nM SRP. Micrococcal nuclease was used at a final concentration of 20 U/μl. The protected fragments were fractionated in a 8.3 M urea, 8% polyacrylamide gel. To generate molecular size markers, dideoxy sequencing reactions were performed using M13mp18 as a template with [α-^{35}S]ATP as the source of label (**lanes 3–6**).

particle (SRP) to the translation reaction. SRP is a small cytoplasmic ribonucleoprotein particle that is required for translocation of nascent secretory proteins across the rough endoplasmic reticulum. SRP binds to the signal peptide as it emerges from the ribosome and, in wheat-germ extracts, tightly arrests elongation of the nascent polypeptide. The translational arrest is released upon proper targeting to the endoplasmic reticulum membrane, where SRP interacts with the SRP receptor (also called docking protein) (reviewed by Hortsch and Meyer, 1984; Walter and Lingappa, 1986). We found that, in the presence of SRP, ribosomes again protected fragments of ~30 nt from digestion by high concentrations of nuclease (Figure 2A, lane 7 and 2C, lane 2). However, at low concentrations of micrococcal nuclease, the ladder of larger fragments was considerably more prominent (Figure 2A, lane 6). Note that the addition of SRP does not affect the total amount of mRNA that associates with ribosomes. In both the presence and absence of SRP, ~95% of the input mRNA pelleted with ribosomes (Figure 2A, compare lanes 2 and 5, ribosomal pellets, with lanes 8 and 11, supernatants).

To determine whether these larger fragments were indeed protected by multiple ribosomes, we performed sucrose gradient sedimentation. (An alternative possibility would be that these larger RNA fragments were generated from protection by a single ribosome, which interacts with the mRNA to produce several approximately equally spaced sites of nuclease sensitivity.) To one gradient, we applied a translation reaction containing SRP in which the nascent preprolactin chains were labeled with [^{35}S]methionine. Under the translation conditions used, about half of the ribosome-associated nascent polypeptides sedimented with monosomes (Figure 3A), while the remainder of the nascent chains sedimented with polysomes containing between two and seven ribosomes. When we analyzed the sedimentation of ^{32}P-labeled ribosome-protected fragments in a parallel gradient (Figure 3B), we found that the 30 nt fragments sedimented with monosomes, while the larger fragments sedimented exactly as expected for multiple ribosomes. Note that a population of fragments ~70 nt in length sediments at 48S in the gradient (Figure 3B). These fragments may represent protection by 40S ribosomal subunits, as it has previously been observed that small ribosomal subunits protect a larger stretch of RNA than do 80S complexes

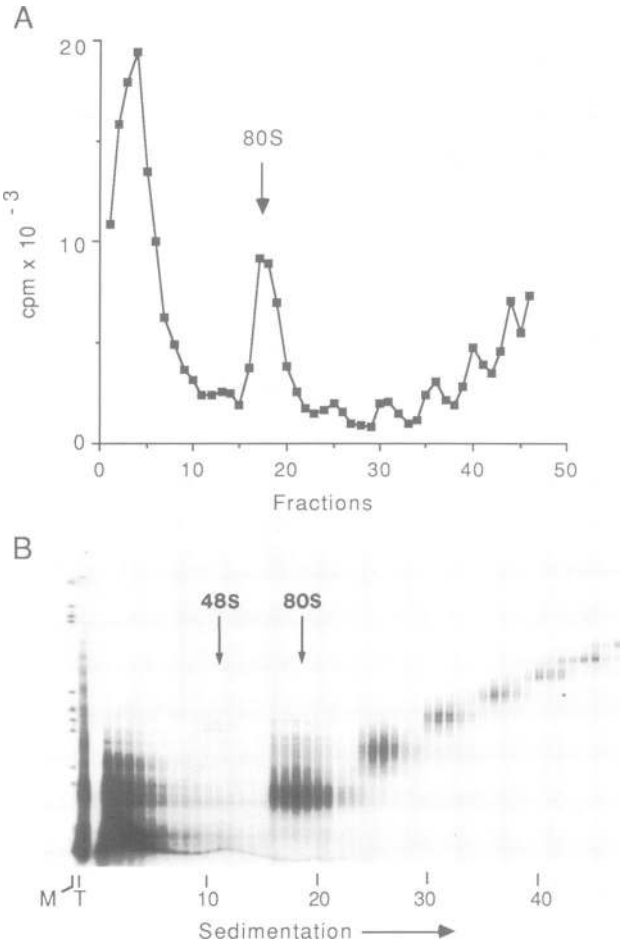

Fig. 3. Sedimentation of ribosome-protected fragments in a sucrose gradient. (**A**) 30 nM SRP was included in a 100-μl translation reaction in which the source of label was [^{35}S]methionine. Translation was terminated after 25 min by the addition of cycloheximide, and the reaction was layered on a 10–30% sucrose gradient and sedimented as described in Materials and methods. Forty-six fractions (250 μl each) were collected and analyzed by precipitation with 10% TCA, followed by deacylation of tRNA^{met} as described. (**B**) ^{32}P-Labeled mRNA was translated in a 100-μl reaction containing 30 nM SRP. After 25 min, translation was terminated by the addition of cycloheximide as in (A) and micrococcal nuclease was added to 20 U/μl. After 30 min of digestion at 0°C (which gives approximately the same amount of digestion as 1 U/μl at 26°C) the digestion was terminated as described in Materials and methods. The mixture was layered on a 10–30% sucrose gradient as in (A) and sedimented in parallel with the gradient shown in (A). Forty-seven fractions (250 μl) were collected and subjected to phenol extraction and ethanol precipitation. RNAs extracted from the fractions were separated in a 8.3 M urea, 8% polyacrylamide gel. The two leftmost lanes contain ^{32}P-labeled molecular size standards (**lane M**; sizes of bands given in Figure 2A) and an aliquot of the total digestion products (**lane T**).

(Legon, 1976; Kozak and Shatkin, 1977a,b; Lazarowitz and Robertson, 1977).

The sizes of the larger protected fragments, as measured by electrophoresis in denaturing gels, are shown in Table I. Because we determined from the sucrose gradient analysis (Figure 3) the number of ribosomes that occupied each protected fragment, we were able to calculate how many nucleotides of RNA were spanned by each ribosome. For the largest protected fragments, each ribosome occupied only 27–28 nt of RNA (Table I).

Table I. Sizes of RNA fragments protected by multiple stacked ribosomes from nuclease digestion

Number of ribosomes	Length of nucleotides	Nucleotides/ribosomes
Wheat-germ		
1	28–40	
2	58–62	29.0–31.0
3	83–87	27.7–29.0
4	108–117	27.0–29.3
5	133–141	26.6–28.2
6	160–169	26.7–28.2
7	189–197	27.0–28.1
Reticulocyte		
1	30–44	
2	58–68	29.0–34.0
3	87–102	29.0–34.0

RNA fragments protected by multiple ribosomes were prepared as described in Materials and methods. Fragments were fractionated in a 8.3 M urea, 8% polyacrylamide gel and sized by comparing the mobility of each fragment with dideoxy sequencing markers. The number of ribosomes that were bound to each fragment was determined from the sucrose gradient analysis shown in Figure 3. Note that at the concentration of micrococcal nuclease used to isolate fragments protected by multiple ribosomes (1 U/μl), the RNA fragments protected by single ribosomes from nuclease digestion are somewhat larger than those obtained following digestion with higher concentrations of nuclease (compare lanes 3 and 4, Figure 2A).

Ribosomes pause at several positions during translation of preprolactin mRNA

To examine the steady-state distribution of ribosomes during preprolactin synthesis, we mapped the origins of the ribosome-protected fragments on the mRNA using the strategy diagrammed in Figure 1. For this analysis we used high concentrations of micrococcal nuclease, so that the protected fragments were primarily 24–32 nt in length (as shown in Figure 2C). As a control, we also analyzed the ribosome-protected fragments from a translation performed in the presence of 7mG (as in Figure 2B, lane 1). The results of this analysis are shown in Figure 4A (e.g. compare lanes 1 and 3).

In our assay, if two ribosome-protected fragments hybridize to the same DNA molecule, the signal from the downstream fragment will be lost, as the polymerase will stop upon encountering the upstream fragment. It was therefore necessary to vary the amount of RNA fragments, relative to a constant amount of DNA, in the hybridization reaction. When high concentrations of protected fragments were used (Figure 4A, lanes 1–3), the signals that corresponded to ribosome pausing were very strong. However, the majority of the T4 DNA polymerase molecules stopped before reaching a region of strong secondary structure in the M13 vector downstream of the cDNA insert (arrow in Figure 4A; compare lanes 1 and 3), indicating that most of the DNA molecules in the reaction contained at least one hybridized RNA fragment. When the amount of RNA fragments in the reaction was reduced 10-fold (lanes 7–9), the bands corresponding to ribosome pausing were weaker (compare lanes 1 and 7); yet the majority of the polymerase molecules traversed the entire cDNA, indicating that DNA was present in excess in the hybridization reaction.

The first major ribosomal pause is at nucleotides −12 and −13. (In our numbering system, the first nucleotide of the coding sequence is +1.) Since the ribosome-protected

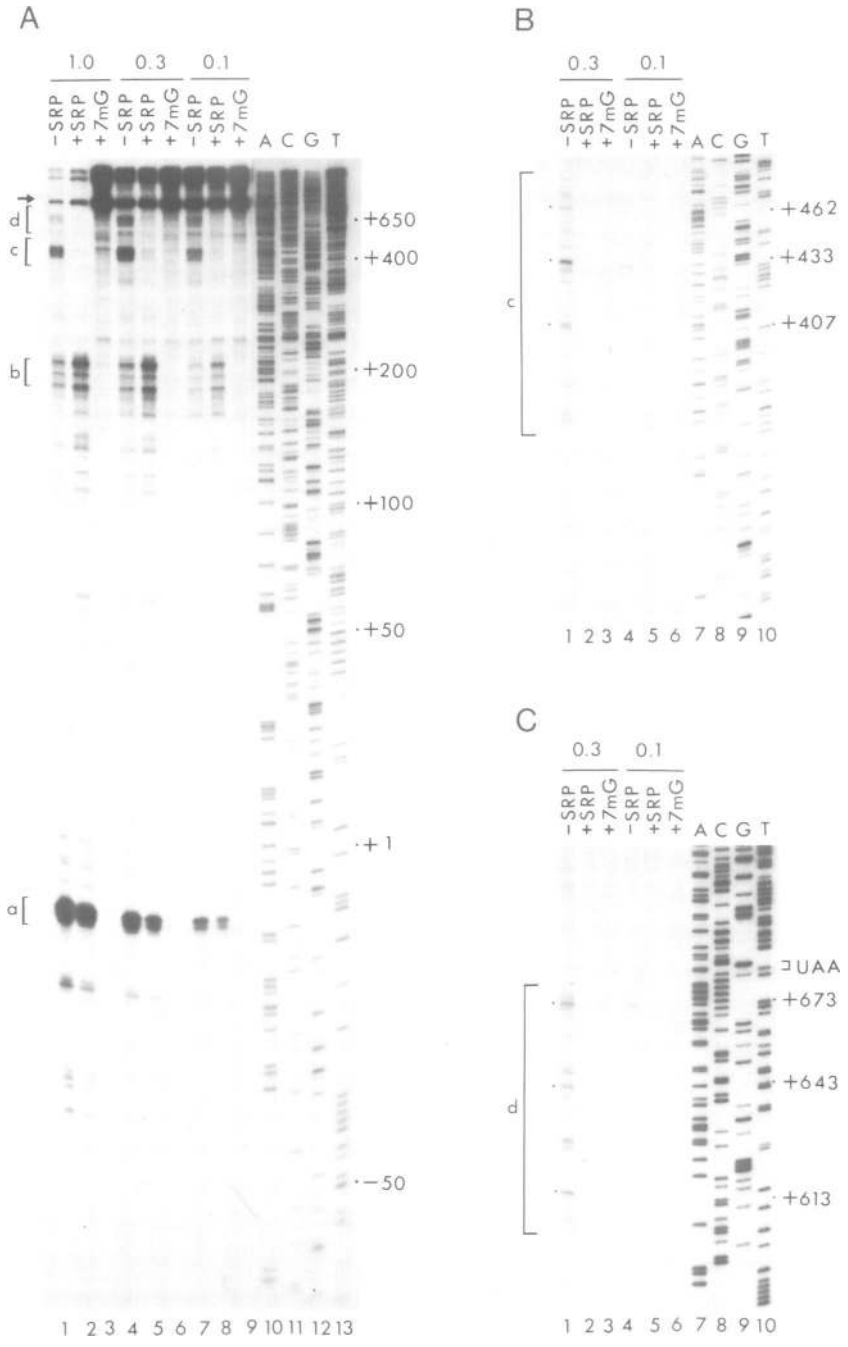

Fig. 4. Ribosome pausing during translation of preprolactin mRNA in a wheat-germ translation extract. (**A**) Ribosome-protected fragments of pSPBP4 RNA were prepared from wheat-germ translations done in the absence of SRP (**lanes 1, 4** and **7**), or in the presence of either 30 nM SRP (**lanes 2, 5** and **8**) or 10 mM 7mG (**lanes 3, 6** and **9**) as described and annealed to a single stranded antisense cDNA clone (the *Hin*dIII/*Eco*RI fragment of pSPBP4 inserted into M13mp18). Numbers at the top represent microliter amounts of ribosome-protected fragments used in each reaction. A 5′-labeled oligonucleotide primer (the M13 −40 primer GTTTTCCCAGTCACGAC) was also annealed to the cDNA clone. The primer was extended using T4 DNA polymerase and the genes 44/62 and gene 45 accessory proteins. The primer extension products were fractionated in a 8.3 M urea, 5% polyacrylamide gel. To generate molecular size markers, the labeled M13 −40 primer was also used in dideoxy sequencing reactions with reverse transcriptase (**lanes 10−13**). The band marked with an arrow is generated by a strong T4 DNA polymerase stop in the M13 vector. The two heavily labeled bands above this strong stop have not been analyzed. They probably represent extension of the primer completely around the circular single-stranded DNA (which should stop when the primer is encountered). (**B**) Ribosome-protected fragments were extracted, annealed and analyzed by primer extension as in (A), except that the primer consisted of the oligonucleotide GCTGCCATACCTCCTCC which spans nucleotides 260−276 in the pSPBP4 sequence. **Lanes 1** and **4**, analysis of ribosome-protected fragments in the absence of SRP. **Lanes 2** and **5**, ribosome-protected fragments in the presence of 30 nM SRP. **Lanes 3** and **6**, ribosome-protected fragments obtained from translation in the presence of 7mG. Numbers at the top represent microliter amounts of ribosome protected fragments. **Lanes 7−10**, dideoxy sequencing reactions using the above oligonucleotide primer. (**C**) Ribosome-protected fragments were obtained and analyzed as in (A), except that the primer consisted of the sequence GCCAAAGAGACTGAGCC which spans nucleotides 511−527 of the pSPBP4 sequence. **Lanes 1** and **4**, analysis of ribosome-protected fragments from translation of pSPBP4 RNA in the absence of SRP. **Lanes 2** and **5**, analysis of ribosome-protected fragments derived from translation of pSPBP4 RNA in the presence of 30 nM SRP. **Lanes 3** and **6**, ribosome-protected fragments from translation in the presence of 7mG. Numbers at the top represent microliter amounts of ribosome-protected fragments. **Lanes 7−10**, dideoxy sequencing reactions using the labeled oligonucleotide and reverse transcriptase.

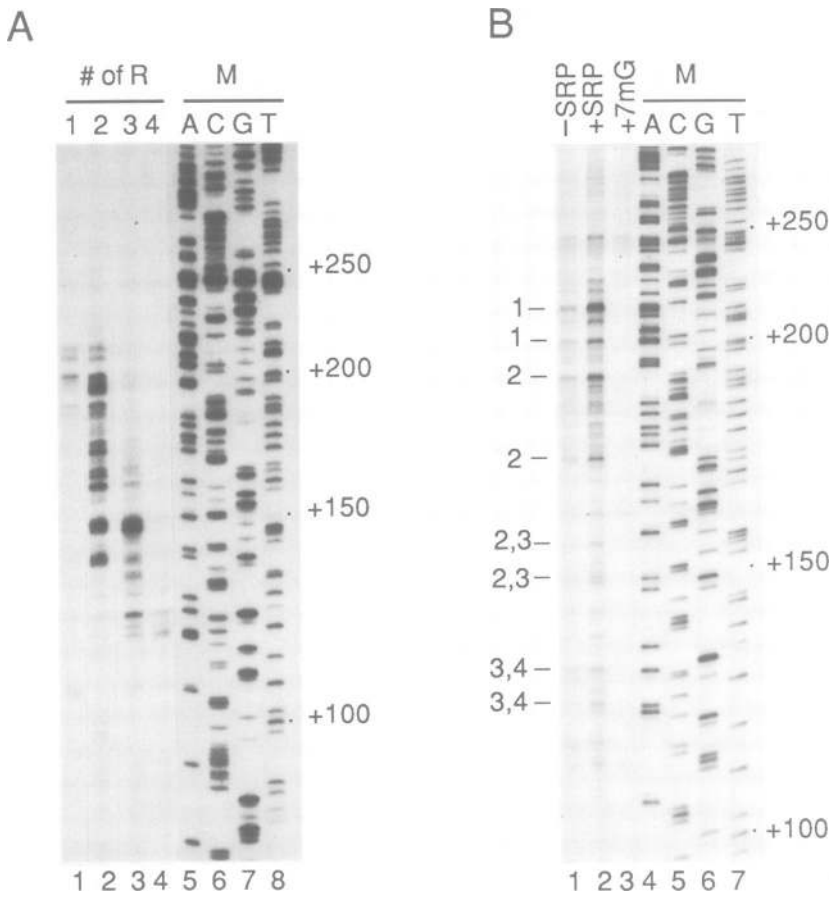

Fig. 5. 5′ end analysis of RNA fragments protected by multiple ribosomes from micrococcal nuclease digestion. **(A)** ^{32}P-Labeled pSPBP4 RNA was translated in a 150 μl reaction containing wheat-germ extract as described in Materials and methods. The reaction was treated with micrococcal nuclease (1 U/μl final concentration for 30 min at 26°C) and the ribosome-protected RNA fragments isolated as described. The protected fragments were fractionated in a 8.3 M urea, 8% polyacrylamide gel. RNA fragments protected by 1–4 ribosomes were excised and eluted from the gel, annealed to the anti-sense cDNA and analyzed by primer extension as described. The primer used was the M13 −40 primer. **Lanes 1–4**, analyses of fragments protected by 1–4 ribosomes respectively. **Lanes 5–8**, dideoxy sequencing reactions using the labeled oligonucleotide primer and reverse transcriptase. **(B)** Total ribosome-protected fragments were isolated from translations containing either no SRP (**lane 1**), 30 nM SRP (**lane 2**) or 10 mM 7mG (**lane 3**) and analyzed by annealing to the antisense cDNA and primer extension with T4 DNA polymerase as described. The primer consisted of the oligonucleotide GTCCCGCCTGCTCCTGC, which spans nucleotides 30–46 of the pSPBP4 RNA sequence. Positions of ribosome stalling that can be assigned to stacking of ribosomes are indicated on the left, with the numbers corresponding to the number of stacked ribosomes at each position.

fragments are largely between 24 and 32 nt in length (and our assay detects the 5′ end of protected fragments), the first major ribosomal pause (labeled a in Figure 4A) is directly over the initiating AUG codon. This first pause presumably represents ribosomes in the process of initiation. After this first pause there are several additional regions of strong pausing further into the coding sequence (labeled b–d in Figure 4A). Each of these regions was mapped more precisely with oligonucleotide primers that anneal closer to these regions. The second region of ribosome pausing (region b in Figure 4A; expanded in Figure 5B) is between nucleotides 175 and 216 (which corresponds to nascent polypeptide chains of 63–77 amino acids). As will be demonstrated below (Figure 5), ribosome stalling at a primary pause site is accompanied by the stacking of other ribosomes behind the paused ribosome. In the primer extension analysis, these secondary pauses give rise to additional bands 5′ of the ones arising from the primary pause. This (and the minor length heterogeneity of the RNA fragments) accounts for most of the complexity of the banding pattern seen in each region. The third and fourth major regions of pausing (c and d in Figure 4A) are shown in greater detail in Figure 4B and C respectively. As shown in Figure 4B (lanes 1 and 4), pause region c is roughly between nucleotides 380 and 480 (amino acids 131–165). Interestingly, the last ribosomal pause (region d in Figure 4A) is at the end of mRNA coding sequence (Figure 4C, lanes 1 and 4). The trailing edge of the stalled ribosome is at nucleotide 673. Since the termination codon, UAA, is at nucleotides 685–687, this last point of ribosome stalling is directly over this codon, and is likely to represent ribosomes in the process of terminating. Note that the bands corresponding to the trailing edges of the stalled ribosomes are precisely 12–13 nt from the AUG (region a) and the UAA (region d) codons respectively.

Because we used cycloheximide to stop protein synthesis, we wondered if any of these pauses were due to the addition of this inhibitor. We therefore compared the pauses shown in Figure 4A to those seen when cycloheximide was omitted. (In this case, the micrococcal nuclease digestion will halt ribosome movement). In the absence of cycloheximide, the fragments protected by ribosomes from nuclease digestion were shorter (20–24 nt; not shown). When these RNA fragments were analyzed in the primer extension assay, the

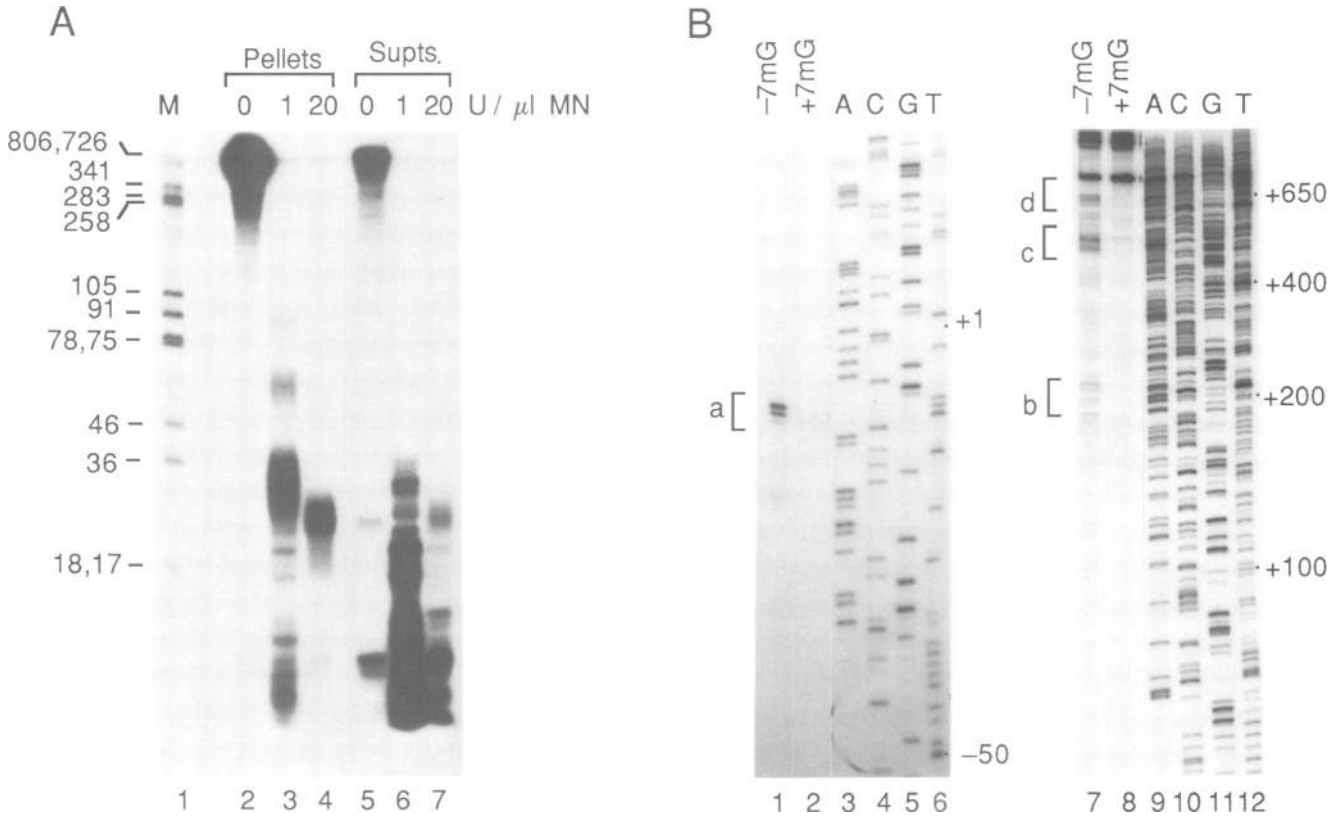

Fig. 6. Ribosome pausing during translation of pSPBP4 RNA in rabbit reticulocyte lysate. (**A**) ^{32}P-Labeled pSPBP4 mRNA was translated for 25 min at 26°C in a rabbit reticulocyte extract. Cycloheximide was then added to 1 mM, and micrococcal nuclease was added to a final concentration of either 0 (**lanes 2** and **5**), 1 (**lanes 3** and **6**) or 20 (**lanes 4** and **7**) U/μl. Following 30 min of digestion at 26°C, ribosomes were pelleted, and the RNA fragments associated with the ribosomal pellets (**lanes 2–4**) and supernatants (**lanes 5–7**) were analyzed by electrophoresis in a 8.3 M urea, 8% polyacrylamide gel. The amount of samples loaded in **lanes 2** and **5** represents 1/5 the amount of samples loaded in **lanes 3, 4, 6** and **7**. Lane 1, ^{32}P-labeled molecular size markers. (**B**) Ribosome-protected fragments of pSPBP4 RNA (resistant to 20 U/μl micrococcal nuclease) were isolated as described in (A). The protected fragments were annealed to the single stranded antisense cDNA clone and analyzed by primer extension with T4 DNA polymerase exactly as described in the legend to Figure 4A. The results of the analysis are shown in **lanes 1** and **7**. As a control, ribosome-protected fragments obtained from translation of pSPBP4 RNA in the presence of 7mG were also analyzed (**lanes 2** and **8**). Molecular size markers (**lanes 3–6** and **lanes 9–12**) were generated by dideoxy sequencing as described. **Lanes 7–12** represent the top half of the gel shown in **lanes 1–6**. Each of the major regions of ribosome pausing was also mapped more precisely using the oligonucleotides described in the legends to Figure 4B and C. The major regions of pausing (labeled b, c and d) were found to be identical to the pauses detected during translation of preprolactin in wheat-germ extracts (data not shown).

same four pauses were detected (data not shown). Because the trailing edges of the paused ribosomes (the 5′ ends of the protected fragments) mapped to the identical positions, the fragments protected in the absence of cycloheximide must be shorter at their 3′ ends.

We compared the distribution of ribosomes during translation of preprolactin mRNA with that found when translation was arrested by SRP. In the presence of SRP, only the pauses in region a and b were detected, and the bands in region b were enhanced ~3-fold in the presence of SRP (Figure 4A, compare lanes 2, 5 and 8 with 1, 4 and 7). SRP has been shown to arrest translation when the nascent polypeptide chain is ~70 amino acids long (Walter and Blobel, 1981; Meyer *et al.*, 1982). Since 30–40 amino acids of the growing polypeptide chain are sequestered within the ribosome, this corresponds to the length at which the signal sequence has emerged completely from the ribosome. Thus, enhanced ribosome stalling at the second pause site (and the lack of detectable ribosome activity beyond this point) is consistent with the known mechanism of elongation arrest by SRP. It is also evident from this data that the SRP-mediated translational arrest occurs at a natural pause site in the synthesis of preprolactin; no additional pauses occur in the presence of SRP.

As noted above, each of the major pauses did not appear to occur at a single point on the mRNA. Rather, each strong ribosomal pause was represented by a group of closely spaced bands. Intriguingly, several of the prominent bands in each region of ribosome pausing occurred at 26- to 30-nt intervals (e.g. bands marked with dots in Figure 4B and C). We reasoned that these groups of bands might represent the stacking of ribosomes observed when we directly examined ribosome-protected fragments (Figure 2A, lanes 3 and 6). That is, the most 3′ band in each group would represent primary pausing by the leading ribosome, and the other prominent bands would correspond to secondary pausing by the upstream ribosomes as their progress became impeded upon encountering the stalled ribosome.

To determine if this was indeed the case, we mapped the location on the mRNA of some of the fragments protected by multiple ribosomes in the presence of SRP. Each of these larger fragments was excised from a gel (such as that shown in Figure 2A, lane 6) and analyzed with the assay shown in Figure 1. We found that several of these bands were

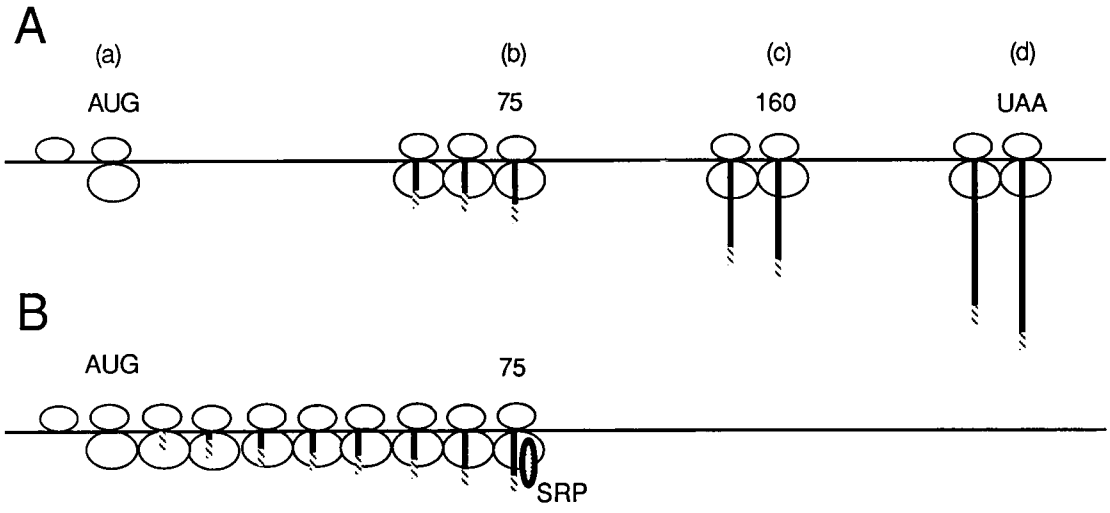

Fig. 7. Cartoon of ribosome distribution during translation of pSPBP4 RNA. (**A**) The four major positions of ribosome pausing during translation of pSPBP4 RNA, labeled a–d, are indicated. Behind each stalled ribosome, other ribosomes stack. (**B**) Signal recognition particle arrests translation at pause region b. Behind the arrested ribosome, other ribosomes stack. This stacking of ribosomes can extend to the 5′ terminus of the mRNA.

actually a mixture of fragments of similar lengths (Figure 5A, lanes 1–4). This is partly due to the fact that the fragments are derived from partial digestion with nuclease, so from seven ribosomes packed together on a single mRNA there are several ways to generate fragments protected by two ribosomes. Each of the endpoints of these larger fragments, however, corresponded to pauses seen when the total set of ribosome-protected fragments was analyzed in the mapping assay (Figure 5B). Thus, we were able to assign many of the bands that occur 5′ to the position at which SRP arrests translation (Figure 5B, lane 2) to protection of RNA by either two, three or four tightly packed ribosomes. Note that each of the bands corresponding to tightly stacked ribosomes is also seen in the absence of SRP (Figure 5B, lane 1). Thus, it appears that ribosomes stack when the leading ribosome pauses, and this stacking is enhanced when SRP arrests translation.

To exclude the possibility that the observed phenomena were peculiar to the wheat-germ translation system, we also examined ribosomal movement during translation of preprolactin mRNA in a rabbit reticulocyte lysate. Again, we found that ribosomes protected fragments of ~30 nt from digestion by micrococcal nuclease (Figure 6A, lane 4). The reticulocyte ribosome-protected fragments appeared to be slightly larger than those protected by wheat-germ ribosomes, averaging between 29 and 35 nt in length (data not shown). At low concentrations of nuclease, we again saw protection by multiples of ribosomes (Figure 6A, lane 3), although these protected fragments were also somewhat larger than those found during translation in wheat-germ extracts (Table I) and less abundant. When the positions of these fragments were mapped as diagrammed in Figure 1, we saw the same four primary pauses (Figure 6B, lanes 1 and 7) that we had previously identified in the wheat-germ extract.

Discussion

We have devised a sensitive assay to determine the steady-state distribution of translating ribosomes on mRNA. Using this assay to monitor ribosome transit on a model mRNA (bovine preprolactin), we have identified four major positions of ribosome stalling (shown diagrammatically in Figure 7A). One pause is found at the initiation site, another after ~75 amino acids have been polymerized, a third pause after ~160 amino acids have been synthesized and the final pause at the termination site. These pauses are not unique to the wheat-germ system, but also occur when preprolactin mRNA is translated by mammalian ribosomes.

Surprisingly, we found that additional ribosomes are stacked behind each paused ribosome. This results in a very tight stacking of the ribosomes, with individual ribosomes oocupying only 27–29 nt of RNA. Thus, a mRNA that is being translated does not have a uniform distribution of ribosomes across its length; instead, densely stacked clusters of ribosomes (caused by the pausing of the leading ribosome) alternate with mRNA regions that are at steady state only sparsely populated by ribosomes (Figure 7A).

Pausing of ribosomes

It is very likely that two of the pauses we detect correspond to initiation and termination, as the leading ribosome in each case is directly over the relevant codon. It is not surprising that initiation and termination are slow steps in protein synthesis. In most kinetic models of protein synthesis, initiation is a rate-determining step under normal physiological conditions (von Heijne *et al.*, 1978; Bergmann and Lodish, 1979; Heinrich and Rapoport, 1980). Also, for the synthesis of globin in a reticulocyte lysate, the rate of termination at 25°C was determined to be one-tenth the rate of elongation (Lodish and Jacobsen, 1972).

As the trailing edge of the first paused ribosome (pause a in Figure 4A) is 12–13 nt before the initiation codon, the first pause is likely to involve 80S ribosomes, rather than 40S ribosomal subunits. Monosomes have been described to protect 11–13 nt 5′ to the AUG from digestion by T1 or pancreatic ribonuclease, whereas 40S complexes protect additional nucleotides on the 5′-terminal side (Legon, 1976; Kozak and Shatkin, 1977a,b; Lazarowitz and Robertson, 1977). Although the larger protected fragments of RNA that sedimented at 48S in the sucrose gradient (Figure 3B) probably resulted from protection by the small ribosomal

subunit, they constituted only a very small percentage of the total yield of ribosome-protected fragments. Protection of the AUG by 80S ribosomes cannot result from association of the 60S subunit during the nuclease digestion, as the addition of edeine [which inhibits this step in initiation (Legon *et al.*, 1976; Kozak and Shatkin, 1978; Safer *et al.*, 1978)] prior to micrococcal nuclease treatment did not affect either the sizes of the protected fragments or the position of the pausing ribosome (data not shown). Thus, it appears that after the 40S subunit selects the initiation site and is joined by the 60S subunit, the completely assembled monosome pauses, leading to a major rate-limiting step in the elongation reaction.

Ribosome pausing has been suggested to occur at positions of rare codons. In virtually all species examined, codon usage in natural mRNAs is strongly correlated with the relative abundance of their respective tRNA species (Ikemura and Ozeki, 1982). Thus, codons that correspond to rare tRNA species may slow down translation of a protein at particular points in elongation (Varenne *et al.*, 1984). However, ribosome pausing at the initiation site cannot be due to a limited supply of the cognate aminoacyl tRNA, as the initiator $tRNA^{met}$ is bound at the earlier stage of pre-initiation complex formation (reviewed by Moldave, 1985).

Since at the initiation pause (region a) and the termination pause (region d), the trailing edge of the ribosome mapped precisely 12–13 nt from the AUG and UAA codons respectively, we can interpret the ribosome positions at the two internal pause sites in an analogous manner. In both regions b and c, the leading ribosome is stalled directly over a GGC (glycine) codon [gly 77 (nucleotides 229–231); gly 159 (nucleotide 475–477)]. However, this same codon is used elsewhere in the protein [gly 39 (nucleotides 115–117) and gly 165 (nucleotides 493–495)], yet is not associated with ribosome pausing. It is possible that we might have missed pausing at these latter points, if the hybridization of ribosome-protected fragments from this region to the antisense cDNA clone was very inefficient (due to secondary structure in either the protected fragments or the cDNA clone). Alternatively, codon context may influence the efficiency with which a particular codon is translated (see Yarus and Folley, 1985, and references therein). The fact that the major pauses are so similar in both the wheat-germ and reticulocyte extracts suggests that a feature common to both systems must be responsible. Although both translation systems were supplemented with calf liver tRNA, each extract also contains a large amount of endogenous tRNA.

Secondary structure in the mRNA can also result in ribosome pausing. During frameshifting in the *gag-pol* gene of Rous sarcoma virus, ribosomes pause (T.Jacks, H.Madhani and H.Varmus, personal communication). This pausing (and subsequent frameshifting) has recently been shown to require a stem–loop structure that is located just 3′ to the frameshift site. Mutations that disrupt the stem inhibit frameshifting; compensatory changes that restore the stem return frameshifting to near normal levels (T.Jacks, H. Madhani and H.Varmus, personal communcation). Our scrutiny of the sequences that are immediately 3′ to the leading ribosome in pauses b and c (Figure 4A) has revealed weak potential stem–loop structures with predicted ΔG of −9.7 and −15.2 kcal/mol at 25°C respectively (calculated as described by Cech *et al.*, 1983). More experiments, utilizing mutations that disrupt these structures (or change the glycine GGC codons) will be required to determine why ribosomes pause at these two internal positions.

Stacking of ribosomes

We have observed that ribosomes can pack tightly during translation, with each ribosome occupying as little as 27 nt of RNA. The diameter of the ribosome is ~25 nm. If we assume a RNA extension length of 6–7 Å per nucleotide (Saenger, 1984), then these ribosomes must be so close as to contact each other. Stacking of ribosomes was proposed to occur in a kinetic model of protein synthesis as rates of termination decreased (Bergmann and Lodish, 1979). A similar phenomenon was previously observed in prokaryotic systems when translation was artificially perturbed. When bacteria were grown in the presence of certain elongation inhibitors, polysomes were found to have increased electrophoretic mobility in agarose–acrylamide gels and a reduced sensitivity to ribonuclease (Dahlberg *et al.*, 1973). These observations were explained by a model in which a sequential stacking of the ribosomes on the mRNA occurred. Transient stacking of ribosomes (with two ribosomes whose centers were between 33 and 42 nt apart) was also observed during translation reactions performed in the presence of oligonucleotides complementary to the mRNA (Haeuptle *et al.*, 1986). In contrast to these artificial conditions, we have observed stacking of ribosomes during ongoing protein synthesis.

When we assayed ribosome transit in the presence of SRP, we found that the extent of ribosome stacking increased significantly (shown diagrammatically in Figure 7B). We routinely observed up to seven ribosomes stacked behind the leading ribosome and have occasionally seen up to nine (making a total of 10 ribosomes). We were unable to obtain enough of the RNA fragments corresponding to protection by this number of ribosomes to analyze; however, if we extrapolate the endpoints of these fragments from the data we did obtain, it appears that ribosomes can pile up all the way to the initiating AUG codon (for a total of nine stacked ribosomes). The fragment corresponding to protection by 10 ribosomes would then presumably represent protection by nine ribosomes and a 40S subunit. Because the 5′ untranslated sequence of the synthetic preprolactin mRNA is relatively short (63 nt), the protection by nine monosomes and one 40S subunit would represent the maximum number of ribosomes that could fit on this length of RNA.

Our results also indicate that, at least in the case of preprolactin, the translational arrest mediated by SRP does not result in ribosome pausing at a new position, but rather leads to a significant enhancement of ribosome stalling at the pause site that occurs after ~75 amino acids have been polymerized. This is consistent with the data of Siegel and Walter (1988b), who examined the size distribution of nascent polypeptide chains during preprolactin synthesis. They observed that the polypeptide fragment produced during an SRP arrest of translation was similar in molecular size to a nascent chain synthesized in the absence of SRP. Also, Rapoport *et al.* (1987), who described a mathematical model of the effects of SRP on translation, predicted that the major sites of interaction between SRP and ribosomes would be at the natural pause sites of ribosomes. It will, however, be necessary to monitor ribosome pausing on other secretory and membrane proteins to determine if this is a general phenomenon.

Although we do not yet know if ribosome stacking is physiologically important, this phenomenon could serve several functions. As the pause at 75 amino acids occurs just after the signal peptide is exposed on the surface of the ribosome for binding by SRP, this pause site in preprolactin mRNA could serve to load this mRNA with a defined number of ribosomes before it is targeted to the membrane. It is interesting in this regard that electron microscopy of rat pituitary mammatrophs (which are cells specialized to produce and secrete prolactin) shows that the majority of membrane-bound polysomes in these cells contain between six and seven ribosomes (Christensen *et al.*, 1987). It is furthermore intriguing to note that, in these membrane-bound polysomes, the ribosomes appear to be evenly spaced along the mRNA. Thus, the distribution of ribosomes on the mRNA may change upon membrane engagement, possibly as a result of ribosome binding to receptor proteins on the membrane.

Ribosome stacking could also function in translational control. An arrest of translation at the level of elongation (such as that mediated by SRP) would rapidly turn off or diminish gene expression as well as load the mRNA with ribosomes to give a quick burst of protein synthesis when the arrest was released. Since the absolute number of ribosomes engaged on the mRNA would remain about equal, this form of translational regulation would not have been detected with traditional assays (such as polysome gradients).

The method we have devised can be used to map the positions of translating ribosomes on any mRNA for which a full-length cDNA clone is available. We are now in a position to evaluate the effects that codon selection, codon context and RNA structure have on mRNA translation. Because the assay utilizes unlabeled RNA fragments that can be part of complex mixtures, it can potentially be used to probe ribosome transit *in vivo* as well as *in vitro*. Thus, our ability to monitor the dynamics of ribosome movement will enable us to probe mechanisms of translational control, and should continue to give new insights into the general process of translation.

Materials and methods

Reagents

Wheat-germ extract and reticulocyte lysate were prepared as described (Erickson and Blobel, 1983; Jackson and Hunt, 1983). SRP was prepared as described by Walter and Blobel (1983), frozen in liquid nitrogen after the DEAE−Sepharose step and stored at −80°C. T4 DNA polymerase, gene 45 protein and 44/62 proteins were the kind gifts of Jack Barry and Bruce Alberts (University of California, San Francisco).

In vitro transcription

The construction of plasmid pSPBP4 has been described in detail (Siegel and Walter, 1988a). In this construction in pSP64 (Promega), the 5′ untranslated region of preprolactin is replaced by the 5′ untranslated region of *Xenopus* β-globin; however, the entire coding sequence of bovine preprolactin, as well as the 3′ untranslated region and poly A and G tails, is present (Sasavage *et al.*, 1982). pSPBP4 was linearized with *Eco*RI and transcribed with SP6 RNA polymerase (Promega) for 1 h at 40°C in 25 μl as described (Melton *et al.*, 1984) except that each reaction contained 0.5 mM each of ATP, CTP, UTP and the dinucleotide G(5′)ppp(5′)G and 0.1 mM GTP. For labeled RNA, 50 μCi[α-^{32}P]UTP (>400 mCi/mmol; Amersham) was added to the reaction. For translations in rabbit reticulocyte lysate, 7mG(5′)ppp(5′)G was used in the transcription reaction. [Although transcripts capped with G(5′)ppp(5′)G were efficiently translated in reticulocyte lysate, we found that the methylated cap was necessary to be able to inhibit translation efficiently with the cap analogue 7mG.] Following synthesis, the RNA was extracted with phenol/chloroform/isoamyl alcohol (50:50:1), precipitated with ethanol, resuspended in 40 μl of distilled water and stored at −80°C until use.

Isolation of ribosome-protected fragments

Translations using wheat-germ extract were performed as described (Erickson and Blobel, 1983) with the following modifications. A 25-μl translation reaction contained 5 μl of the RNA described above, 6 μl of wheat-germ extract, 0.1 U/μl RNase inhibitor, 150 mM potassium acetate and 3.5 mM magnesium acetate. If desired, 7mG or SRP was added to concentrations of 10 mM or 30 nM respectively. Following a 25-min incubation at 26°C (at which point incorporation of amino acids was still linear with time), the reaction tube was placed in ice, and cycloheximide was added to a final concentration of 1 mM to 'freeze' the ribosomes on the mRNA (Columbo *et al.*, 1966). Micrococcal nuclease (Boehringer Mannheim; diluted in 5 mM $CaCl_2$, 50 mM glycine, pH 9.2) was added to the desired concentration, and the volume of the reaction adjusted to 40 μl. If necessary, additional magnesium acetate was added to maintain a final concentration of 3.5 mM. Following a 3-min incubation on ice, the mixture was digested for 30 min at 26°C. The nuclease digestion was terminated by the addition of 60 μl of 20 mM Hepes, 150 mM potassium acetate, 10 mM magnesium acetate, 5 mM EGTA, 2 mM dithiothreitol (buffer T). The 100-μl reaction was overlayed on a 60-μl cushion of 0.25 M sucrose in buffer T and pelleted at 30 p.s.i. for 30 min in an A-110 rotor in a Beckman airfuge. Following sedimentation, the top 120 μl was removed (the 'ribosomal supernatant'). To the bottom 40 μl (the 'ribosomal pellet'), 100 μl of 50 mM sodium chloride, 50 mM Tris, pH 7.5, 5 mM EDTA, 0.5% SDS, 200 μg/ml proteinase K was added and the mixture was incubated at 37°C for 30 min. The resuspended pellet was then removed, extracted with phenol/chloroform/isoamyl alcohol, and precipitated with ethanol in the presence of 20 μg *E.coli* tRNA. The resulting pellet was resuspended in 10 μl of distilled water and stored at −80°C.

Translations using reticulocyte lysate contained 12.5 μl lysate, 150 mM potassium acetate, 2 mM magnesium acetate and 2.5 μl of the transcribed RNA per 25 μl reaction. Following a 25-min incubation at 26°C, isolation of ribosome-protected fragments was performed exactly as described above.

Mapping the positions of ribosome-protected fragments

The fragments were first annealed to a single-stranded DNA (the *Hin*dIII/*Eco*RI insert of pSPBP4 inserted into M13mp18). Each annealing reaction contained 20 ng of the M13 construct, 0.05−0.1 ng of the 5′-labeled oligonucleotide primer, the desired amount of ribosome-protected fragments, 33 mM Tris acetate, pH 7.7 and 67 mM potassium acetate in a total volume of 9 μl. The annealing reaction was heated to 65°C for 5 min, and then placed at 37°C for 3 h. After annealing the reaction was mixed with 10 μl of 33 mM Tris acetate, pH 7.7, 67 mM potassium acetate, 20 mM magnesium acetate, 1 mM DTT, 1 mM ATP, 0.334 mM dATP, dCTP, dGTP and dTTP. An enzyme cocktail was added sufficient to bring the final concentration in the reaction of T4 DNA polymerase to 2 μg/ml, 44/62 proteins to 25 μg/ml, and T4 gene 45 protein to 2.5 μg/ml. (When the polymerase accessory proteins are omitted from the primer extension reaction, the polymerization rate is slower and more background bands are generated by premature stopping of the polymerase). After a 15-min incubation at 37°C, the primer extension products were extracted with phenol/chloroform/isoamyl alcohol, precipitated with ethanol, resuspended in sequencing dyes (95% formamide, 10 mM Na_2EDTA, 0.1% bromophenol blue, 0.1% xylene cyanol), heated to 65° for 4 min and fractionated in a 8.3 M urea, 5% polyacrylamide gel. To generate markers, the labeled oligonucleotide primer was used in dideoxynucleotide sequencing reactions using AMV reverse transcriptase (Life Sciences).

Sucrose gradient analysis

A marker gradient was generated in the following manner. One hundred microliters of ^{35}S-labeled translation reaction containing 30 nM SRP was allowed to incubate for 25 min, at which time cycloheximide was added to 1 mM. The mixture was layered on a 13-ml 10−30% sucrose gradient in buffer T and spun for 2 h at 39 000 r.p.m. in a Beckman SW40 rotor as described by Siegel and Walter (1988a). Forty-seven fractions (250 μl each) were collected by underlaying with 60% sucrose using an Isco gradient fractionator. A 100-μl aliquot of each fraction was analyzed by precipitation with 10% trichloroacetic acid (TCA), followed by hydrolysis of the initiator $tRNA^{met}$ by boiling the samples in 5% TCA for 15 min. A separate 50-μl aliquot was analyzed without RNA hydrolysis to determine the positions of 43S and 48S preinitiation complexes.

To analyze the sedimentation of ribosome-protected fragments, a parallel 100-μl reaction containing ^{32}P-labeled mRNA was allowed to translate for 25 min, and cycloheximide was then added as above. Micrococcal nuclease

was added to 20 U/μl as described above, and the mixture was digested for 30 min at 0°C. Following addition of buffer T to 400 μl, the mixture was layered on a 10–30% sucrose gradient, sedimented and fractionated as described. Fractions were subjected to phenol extraction and ethanol precipitation, and analyzed by electrophoresis in 8.3 M urea, 8% polyacrylamide gels.

Acknowledgements

We are grateful to Jack Barry and Bruce Alberts for generously providing T4 DNA polymerase, accessory proteins and advice. We thank Vivian Siegel and Pablo Garcia for many helpful suggestions, and Mark Poritz for suggesting the use of the T4 polymerase accessory proteins. We are also grateful to Carl Hashimoto, David Brow, Mark Solomon, Katharina Strub and Harris Bernstein for critical reading of the manuscript. This work was supported by grants from the National Institutes of Health (GM-32384) and the Juvenile Diabetes Foundation (187668). S.L.W. is a fellow of the Helen Hay Whitney Foundation.

References

Alberts,B.M. (1984) *Cold Spring Harbor Symp. Quant. Biol.*, **49**, 1–12.
Bergmann,J.E. and Lodish,H. (1979) *J. Biol. Chem.*, **254**, 11927–11937.
Both,G.W., Furuichi,Y., Muthukrishnan,S. and Shatkin,A.J. (1976) *J. Mol. Biol.*, **104**, 637–658.
Cech,T.R., Tanner,N.K., Tinoco,I., Weir,B.R., Zuker,M. and Perlman,P.S. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3903–3907.
Christensen,A.K., Kahn,L.E. and Bourne,C.M. (1987) *Am. J. Anat.*, **178**, 1–10.
Columbo,B., Felicetti,L. and Baglioni,C. (1966) *Biochim. Biophys. Acta*, **119**, 109–119.
Dahlberg,A.E., Lund,E. and Kjeldgaard,N.O. (1973) *J. Mol. Biol.*, **78**, 627–636.
Erickson,A.H. and Blobel,G. (1983) *Methods Enzymol.*, **96**, 38–50.
Gold,L. (1988) *Annu. Rev. Biochem.*, **57**, 199–233.
Haeuptle,M,-T., Frank,R. and Dobberstein,B. (1986) *Nucleic Acids Res.*, **14**, 1427–1448.
Heinrich,R. and Rapoport,T.A. (1980) *J. Theor. Biol.*, **86**, 279–313.
Hickey,E.D., Weber,L.A. and Baglioni,C. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 19–23.
Hindley,J. and Staples,D.H. (1969) *Nature*, **224**, 964–967.
Hortsch,M. and Meyer,D.I. (1984) *Biol. Cell.*, **52**, 1–8.
Hu,M.C.-T. and Davidson,N. (1986) *Gene*, **42**, 21–29.
Ikemura,T. and Ozeki,H. (1982) *Cold Spring Harbor Symp. Quant. Biol.*, **47**, 1087–1097.
Jackson,R.J. and Hunt,T. (1983) *Methods Enzymol.*, **96**, 50–74.
Kozak,M. (1983) *Microbiol. Rev.*, **47**, 1–45.
Kozak,M. and Shatkin,A.J. (1977a) *J. Mol. Biol.*, **112**, 75–96.
Kozak,M. and Shatkin,A.J. (1977b) *J. Biol. Chem.*, **252**, 6895–6908.
Kozak,M. and Shatkin,A.J. (1978) *J. Biol. Chem.*, **253**, 6568–6577.
Lazarowitz,S.G. and Robertson,H.D. (1977) *J. Biol. Chem.*, **252**, 7842–7847.
Legon,S. (1976) *J. Mol. Biol.*, **106**, 37–53.
Legon,S., Model,P. and Robertson,H.D. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 2692–2696.
Legon,S., Robertson,H.D. and Prensky,W. (1976) *J. Mol. Biol.*, **106**, 23–26.
Lodish,H.F. and Jacobsen,M. (1972) *J. Biol. Chem.*, **247**, 3622–3629.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Meyer,D.I., Krause,E. and Dobberstein,B. (1982) *Nature*, **297**, 647–650.
Moldave,K. (1985) *Annu. Rev. Biochem.*, **54**, 1109–1149.
Mueller,P.P. and Hinnebusch,A.G. (1986) *Cell*, **45**, 201–207.
Nossal,N.G. (1983) *Annu. Rev. Biochem.*, **52**, 581–615.
Protzel,A. and Morris,A.J. (1974) *J. Biol. Chem.*, **249**, 4594–4600.
Rapoport,T.A., Heinrich,R., Walter,P. and Schulmeister,T. (1987) *J. Mol. Biol.*, **195**, 621–636.
Saenger,W. (1984) *Principles of Nucleic Acid Structure*. Springer-Verlag, New York.
Safer,B., Kemper,W. and Jagus,R. (1978) *J. Biol. Chem.*, **253**, 3384–3386.
Sasavage,N.L., Nilson,J.H., Horowitz,S. and Rottman,F.M. (1982) *J. Biol. Chem.*, **257**, 678–681.
Siegel,V. and Walter,P. (1988a) *Cell*, **52**, 39–49.
Siegel,V. and Walter,P. (1988b) *EMBO J.*, **7**, 1769–1775.
Steitz,J.A. (1969) *Nature*, **224**, 957–964.
Steitz,J.A. (1980) In Chambliss,G., Craven,G.R., Davies,J., Davis,K., Kahan,L. and Nomura,M. (eds), *Ribosomes: Structure, Function and Genetics*. University Park Press, Baltimore, pp. 479–495.
Tzamarias,D., Alexandraki,D. and Thireos,G. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 4849–4853.
Varenne,S., Buc,J., Lloubes,R. and Lazdunski,C. (1984) *J. Mol. Biol.*, **180**, 549–576.
von Heijne,G., Nilsson,L. and Blomberg,C. (1978) *Eur. J. Biochem.*, **92**, 397–402.
Walter,P. and Blobel,G. (1981) *J. Cell Biol.*, **91**, 557–561.
Walter,P. and Blobel,G. (1983) *Methods Enzymol.*, **96**, 682–691.
Walter,P. and Lingappa,V.R. (1986) *Annu. Rev. Cell Biol.*, **2**, 499–516.
Werner,M., Feller,A., Messenguy,F. and Pierard,A. (1987) *Cell*, **49**, 805–813.
Yarus,M. and Folley,L.S. (1985) *J. Mol. Biol.*, **182**, 529–540.

Received on June 28, 1988; revised on July 29, 1988