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The signal recognition particle receptor α subunit assembles co-translationally on the endoplasmic reticulum membrane during an mRNA-encoded translation pause *in vitro*

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Many proteins, including the α subunit of the signal recognition particle receptor (SRα), are targeted within the cell by poorly defined mechanisms. A 140 residue N-terminal domain of SRα targets and anchors the polypeptide to the endoplasmic reticulum membrane by a mechanism independent of the pathway involving the signal recognition particle. To investigate the mechanism of membrane anchoring, translation pause sites on the SRα mRNA were used to examine the targeting of translation intermediates. A strong pause site at nucleotide 507 of the mRNA open reading frame corresponded with the shortest nascent SRα polypeptide able to assemble on membranes. An mRNA sequence at this pause site that resembles a class of viral –1 frameshift sequences caused translation pausing when transferred into another mRNA context. Site-directed mutagenesis of the mRNA greatly reduced translation pausing without altering the polypeptide sequence, demonstrating unambiguously a role for this mRNA sequence in translation pausing. SRα polypeptides synthesized from the non-pausing mRNA were impaired in co-translational membrane anchoring. Furthermore, co-translational membrane assembly of SRα appears to anchor polysomes translating SRα to membranes.

Keywords: endoplasmic reticulum/frameshifting/protein targeting/signal recognition particle receptor/translation pausing

Introduction

A large variety of proteins are targeted within the cytoplasm to specific sites on intracellular membranes or structures. In general, targeting requires some form of localization signal to specify the intracellular destination. This signal may be contained within the sequence of the targeted protein (reviewed in Verner and Schatz, 1988), or on the sequence of the mRNA encoding the polypeptide (reviewed in Wilhelm and Vale, 1993). However, for many proteins the mechanisms responsible for localization are poorly defined. One such protein is the α subunit of the signal recognition particle receptor (SRα). In a cell-free system, SRα is targeted to the endoplasmic reticulum (ER) membrane by a mechanism apparently unrelated to other known pathways (Andrews *et al.*, 1989). The amino acid sequences responsible for targeting and anchoring SRα to the ER membrane have recently been identified (Young *et al.*, 1995). Here, we further characterize this targeting pathway by examining the membrane assembly event *in vitro*. We demonstrate a novel mode of targeting in which nascent polypeptides are localized during an mRNA-encoded pause in translation.

SRα is essential for the translocation of nascent polypeptides across the ER membrane via a mechanism mediated by the signal recognition particle (SRP) (Gilmore *et al.*, 1982a,b). The SRα polypeptide (638 residues, 72 kDa apparent mol. wt) is bound to the cytoplasmic face of the ER membrane largely by strong interactions with the transmembrane β subunit of the receptor (SRβ) (Gilmore *et al.*, 1982b; Hortsch *et al.*, 1985; Lauffer *et al.*, 1985; Tajima *et al.*, 1986; Miller *et al.*, 1995; Young *et al.*, 1995). In a cell-free system, novel SRα polypeptides are assembled onto ER microsomes in a urea- and salt-resistant manner by an SRP-independent pathway (Andrews *et al.*, 1989). An independently folded N-terminal domain of SRα, containing ~140 residues, is necessary and sufficient for targeting and tight binding onto membranes (Young *et al.*, 1995). Furthermore, SRα translation intermediates with discrete apparent molecular weights as low as 23 kDa can anchor onto membranes while attached to ribosomes in cell-free translation reactions. Synthesis of these 23 kDa nascent polypeptides coincides with the emergence of the N-terminal anchoring domain from the ribosome, indicating that SRα might assemble on membranes co-translationally (Young *et al.*, 1995).

The discrete size of the nascent 23 kDa SRα polypeptide suggested it was produced by a translation pause. Translation pausing has been demonstrated for several different mRNAs both *in vitro* and *in vivo*, but in most cases the mechanisms and biological importance of pausing are unknown (Kim *et al.*, 1991; Doohan and Samuel, 1992; Kim and Hollingsworth, 1992; Wolin and Walter, 1993). In this paper, we have identified an mRNA element responsible for ribosome pausing at the site on the SRα mRNA corresponding to synthesis of the 23 kDa membrane-binding translation intermediate. The assay we used was employed previously to examine the translation pausing of secreted polypeptides during SRP-mediated membrane translocation (Wolin and Walter, 1988, 1989, 1993). The mRNA sequence at the SRα pause site resembles a class of viral and bacterial frameshift sites, and was sufficient to mediate pausing in an unrelated mRNA context. Moreover, site-directed mutagenesis of the putative pause sequence abolished pausing and demonstrated the importance of pausing for co-translational membrane binding of SRα. Finally, co-translational binding of SRα onto microsomes was shown to play a role in polysome targeting. From these experiments, the localization of the SRα mRNA at steady-state translation *in vitro* was determined.

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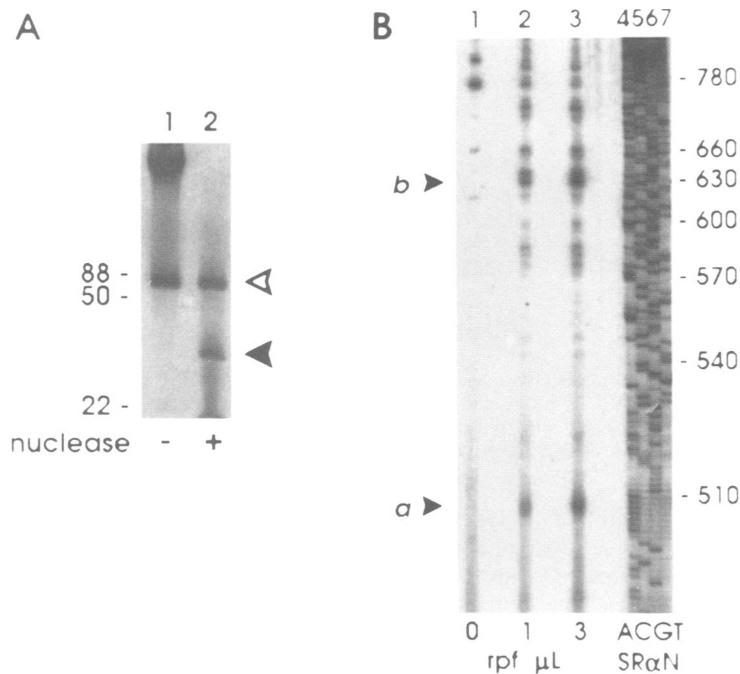

Fig. 1. Translation pause sites on the SRα mRNA. (**A**) Ribosome-bound ^{35}S-labelled mRNA was isolated from RRL translation reactions of SRαN either before (lane 1) or after (lane 2) nuclease digestion. Ribosome protected fragments (rpfs) are marked with a solid arrowhead, and an unidentified non-RNA species derived from the RRL reaction is marked with an open arrowhead. The lengths in nucleotides of RNA molecular weight standards are marked on the left. (**B**) Either 0 µl (lane 1), 1 µl (lane 2) or 3 µl (lane 3) of rpfs were annealed to a single-stranded DNA template of SRαN and mapped by primer extension with T4 DNA polymerase using a primer within the coding region of SRα. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 4–7). The two strong pause sites identified are marked *a* and *b*.

Results

Translation pause sites within the SRα mRNA

SRαN, a Leu2→Val point mutant of SRα, is translated at higher levels in the rabbit reticulocyte lysate (RRL) cell-free system than wild-type SRα, without affecting the targeting or translocation activity of the protein (Falcone and Andrews, 1991; Young *et al.*, 1995). Nascent SRαN polypeptides that have discrete molecular weights and that can bind to ER microsomes are observed in RRL translation reactions (Young *et al.*, 1995). To identify specific translation pause sites on the SRαN mRNA and investigate the possible relationships of these pause sites to membrane targeting, a previously developed assay (Wolin and Walter, 1988, 1989) was used to map the distribution of ribosomes on *in vitro* transcribed mRNAs encoding SRαN. Cell-free translation reactions of [α-^{35}S]UTP-labelled SRαN mRNA were terminated with the elongation inhibitor cycloheximide during steady-state synthesis. The reactions were digested with staphylococcal nuclease to produce monosomes, and mRNA fragments protected from the nuclease by the ribosomes were isolated. These ribosome protected fragments (rpfs) were analysed by electrophoresis and as expected are ~30 nucleotides in length (Figure 1A, lane 2, solid arrowhead) (Wolin and Walter, 1989). The isolated rpfs were distinct from the undigested mRNA (Figure 1A, lane 1) and an unidentified species derived from the RRL reaction (Figure 1A, open arrowhead, and data not shown). The rpfs were then annealed onto a single-stranded SRα DNA template and their positions 'toeprinted' using a primer extension reaction with T4 DNA polymerase (Wolin and Walter, 1988) and a primer within the SRα coding region (nucleotides 325–346).

The positions of the rpfs revealed a series of putative translation pause sites on the mRNA (Figure 1B, lanes 2 and 3). Two exceptionally strong and consistent pause sites were identified, site *a* around nucleotide 507 (Figure 1B, marked *a*, and Figure 3A, insert) and site *b* around nucleotide 627 (Figure 1B, marked *b*), using a dideoxy sequencing ladder from the same primer to provide size markers (Figure 1B, lanes 4–7). Few toeprint bands could be attributed to aberrant termination of the T4 polymerase on the single-stranded template (Figure 1B, lane 1). As expected, when rpfs obtained from translation reactions of an unrelated mRNA (chimpanzee α-globin) were added to the SRαN single-stranded DNA as a control, no toeprint bands were observed other than those produced in the absence of rpfs (data not shown). Moreover, although there is a possibility of mRNA secondary structure near pause site *a* (discussed below), the toeprint band at site *a* is not due to incompletely digested base-paired mRNA, since supplementing the staphylococcal nuclease with the double-strand RNA-specific nuclease V1 (Wolin and Walter, 1993) did not affect this toeprint band (data not shown).

An mRNA pause sequence resembling a frameshift site

Ribosomes paused at site *a* (nucleotide 507) will have synthesized ~170 amino acids of SRα. This polypeptide chain likely corresponds to a 23 kDa translation intermediate previously estimated to contain ~180 amino acid residues (Young *et al.*, 1995). The 23 kDa translation

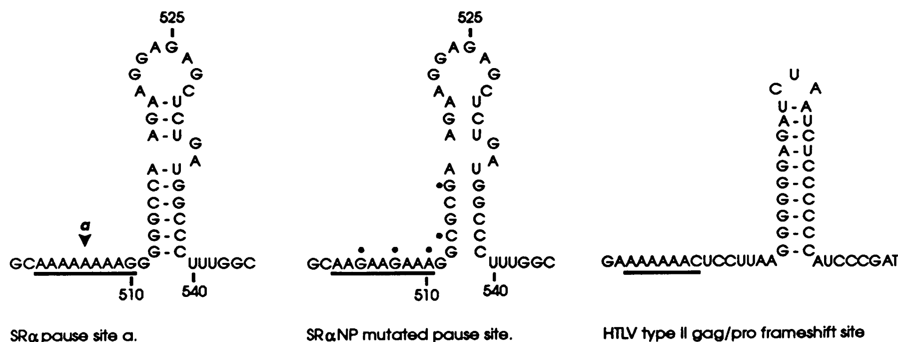

Fig. 2. The mRNA at pause site *a* resembles a frameshift sequence. The predicted secondary structure of the SRα mRNA at ribosome pause site *a* is compared with the mutated pause site of the SRαNP mRNA and a frameshift signal in the HTLV type II *gag/pro* sequence (adapted from Falk *et al.*, 1993). The position of the pause site *a* toeprint band is marked with an arrowhead, the frameshift heptamer and the heptamer-like sequences are underlined, and mutated residues on the SRαNP mRNA are marked with dots.

intermediate is the minimum length of polypeptide required to bind to microsomes in a urea-resistant manner while still attached to ribosomes treated with cycloheximide (Young *et al.*, 1995). Pause site *a* therefore appears to be closely correlated with the membrane anchoring of nascent SRα, and the predicted sequence of the surrounding mRNA (Lauffer *et al.*, 1985) was examined for structures that may cause the translation pause.

The primer extension band at pause site *a* lies within a series of three lysine codons with the sequence AAA-AAA-AAG, starting at nucleotide 502. A potential stem–loop structure is located immediately 3′ on the mRNA of the lysine codons (Figure 2). Interestingly, this mRNA sequence closely resembles a class of ribosomal frameshift structures. These structures, typified by the human T-cell leukaemia virus (HTLV) type I *gag/pro* −1 ribosomal frameshift and conserved in the HTLV type II, bovine leukaemia virus and equine infectious anaemia virus frameshifting mRNAs, consist of a heptamer sequence of A-AAA-AAC followed by a stem–loop structure of varying size and distance from the heptamer (Figure 2) (reviewed in Hatfield *et al.*, 1992). Both the heptamer sequence and the stem–loop structure have been shown to be necessary and sufficient for frameshifting on the HTLV type II mRNA *in vitro* (Falk *et al.*, 1993). A similar group of −1 frameshift structures is found in the *Escherichia coli* mRNA coding for the *dnaX*, IS150 and IS911 proteins, and consists of the heptamer sequence A-AAA-AAG followed by a stem–loop (Blinkowa and Walker, 1990; Flower and McHenry, 1990; Tsuchihashi and Kornberg, 1990; Polard *et al.*, 1991; Vogele *et al.*, 1991).

The thermodynamic stability of the predicted RNA stem–loop at the SRα pause site is calculated to have a ΔG of −10.3 kcal (Freier *et al.*, 1986), comparable with the stability of the viral frameshift structures (between −19.0 and −7.9 kcal) (Hatfield *et al.*, 1992). However, unlike the viral frameshift structures, the SRα heptamer-like sequence has a G in the final position and as predicted (Brierley and Jenner, 1992; Chamorro *et al.*, 1992; Garcia *et al.*, 1993), frameshifted premature termination products are not prominently observed in optimized RRL translation reactions of SRαN (data not shown). The *gag/pro* −1 frameshift structure of the mouse mammary tumour virus containing an A-AAA-AAC heptamer has been shown to require a pseudoknot for efficient frameshifting (Chen *et al.*, 1995) and there is no predicted pseudoknot structure at the SRα pause site.

To determine whether the signal for translation pausing at site *a* resides on the SRα mRNA and not on the encoded polypeptide, point mutations were introduced into the plasmid encoding SRαN that do not change the encoded amino acid sequence. In the *in vitro* transcribed mutant mRNA (referred to as encoding SRαNP), the frameshift-like structure is disrupted by changing the heptamer-like sequence from AAA-AAA-AAG to AAG-AAG-AAA, and interrupting the putative stem–loop with G→C and C→G substitutions (Figure 2). The remainder of the coding region and both the 5′ and 3′ untranslated regions are identical to that of SRαN. Rpfs were isolated from RRL translation reactions of SRαN and SRαNP in parallel and mapped by primer extension to assay the level of translation pausing at the mutated site relative to the wild-type sequence.

Rpfs isolated from translation reactions of SRαN produced toeprint bands at the two strong pause sites, as expected (Figure 3A, lane 1, marked *a* and *b*). However, rpfs from SRαNP translation reactions revealed a considerably fainter toeprint band near the mutated pause site compared with the SRαN toeprint band at site *a*, while the toeprint band at pause site *b* remained prominent on the SRαNP mRNA (Figure 3A, compare lane 3 with lane 1). The toeprint band near the mutated site of SRαNP was close in intensity to the surrounding background pause sites and, moreover, was shifted by ~3 nucleotides 3′ of the original position (Figure 3A, lane 3, and inserts). A dideoxy sequencing ladder of SRαNP is shown (Figure 3A, lanes 4–7) as size markers. The changes in intensity and position of the primer extension band suggest that the mutations in SRαNP have abolished the strong translation pause on the mRNA. Densitometry of the autoradiograms from three different experiments revealed that the toeprint band near the mutated site was reduced to below 35% of the original intensity at site *a* in the SRαN mRNA. As expected, few primer extension bands were due to the SRαNP single-stranded DNA (Figure 3A, lane 2). Therefore, these results suggest that the specific sequence of the SRα mRNA at this site, possibly including the frameshift-like structure, is required for the strong translation pause.

To determine whether the isolated SRα sequence is sufficient for translation pausing, this sequence was

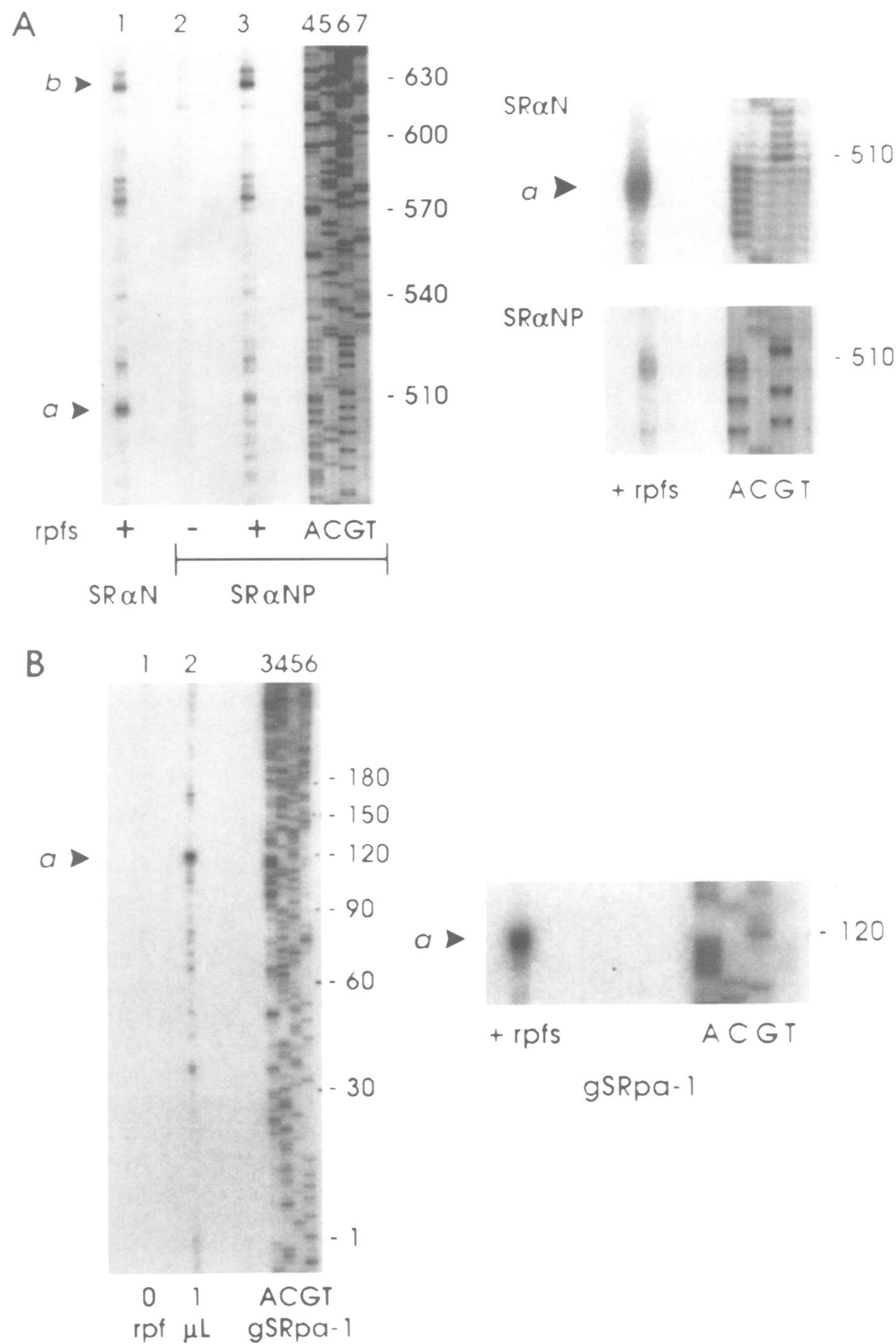

Fig. 3. An mRNA sequence necessary and sufficient for translation pausing. (**A**) Rpfs were isolated from RRL translation reactions of SRαN and the pause site mutant SRαNP. Primer extension assays were performed in the presence of rpfs from SRαN reactions (lane 1), or in the absence (lane 2) or presence (lane 3) of rpfs from SRαNP reactions. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 4–7). Pause sites *a* and *b* of SRα are marked. Inserts on right: magnification of primer extension assays at pause site *a* for SRαN (from Figure 1B) and SRαNP (from left panel). (**B**) Rpfs were isolated from RRL translation reactions of the fusion polypeptide gSRpa-1 containing the sequence of SRα at pause site *a* between protein sequences derived from chimpanzee α-globin and *S.aureus* protein A. Primer extension assays were performed in the absence (lane 1) or presence (lane 2) of these rpfs. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 3–6). A pause site similar to SRα pause site *a* is marked. Insert on right: magnification of primer extension assay at the SRα sequence of gSRpa-1 (from left panel). Nucleotide 120 of the gSRpa-1 sequence, the first G residue following eight A residues (reading upwards), corresponds to nucleotide 510 of the SRα sequence.

inserted into an mRNA encoding a fusion protein containing a fragment of chimpanzee α-globin and the Fc-binding region of *Staphylococcus aureus* protein A (gPA) (Janiak *et al.*, 1994). The plasmid vector was constructed to code for the globin fragment, the short segment of SRα and the protein A domain in one continuous open reading frame (gSRpa-1). The sequence derived from SRα (nucleotides 478–530) included the mRNA heptamer-like sequence and putative stem–loop of pause site *a*. When rpfs were isolated from RRL translations of this construct

and mapped as above, a strong toeprint band was observed at about nucleotide 120 of the fusion protein coding region (Figure 3B, lane 2, marked *a*). This pause site is within the section of mRNA derived from SRα. The primer extension band is close to the position in the sequence corresponding to SRα pause site *a*, although shifted 3′ by about two nucleotides (Figure 3B, insert). The strong toeprint band was not observed in pausing assays using cell-free translation reactions of gPA alone (data not shown). These results suggest that the SRα site *a* translation pause has been transferred into gSRpa-1, and the 3′ shift in position may be due to the different mRNA context. Therefore, the site *a* mRNA sequence containing a frameshift-like structure is both necessary and sufficient for a strong translation pause in RRL translation reactions. Experiments to determine the exact sequence requirements for translation pausing at site *a* are currently under way.

Translation pausing facilitates co-translational membrane binding of SRα

Translation pausing at site *a* on the SRα mRNA is closely correlated with membrane anchoring of nascent SRα (Young *et al.*, 1995). By isolating cytoplasmic and membrane-bound ribosomes from RRL translation reactions of SRαN supplemented with ER microsomes (Wolin and Walter, 1993), we used the pause sites at nucleotides 507 and 627 as markers to confirm that nascent SRα polypeptides can be targeted to the ER membrane during translation. As expected, the pause sites observed were similar to those in Figure 1B including the two strong toeprints noted above (Figure 4, lanes 1 and 2, marked *a* and *b*). Furthermore, toeprint bands 5′ of pause site *a* were mostly produced by rpfs from free ribosomes (Figure 4, lane 1), whereas toeprint bands 3′ of pause site *b* were primarily due to rpfs from bound ribosomes (Figure 4, lane 2). Minor toeprint bands representing rpfs that mapped to the region between the two strong pause sites were distributed between the free and bound ribosome fractions (Figure 4, lanes 1 and 2). These results indicate that ribosomes translating the SRα polypeptide become bound to the membrane during the translation of the region of the mRNA containing the two strong ribosome pause sites. This more precisely locates ribosomes that have synthesized the minimum N-terminal sequence necessary for assembly on the membrane and is in general agreement with earlier evidence (Young *et al.*, 1995).

To determine whether translation pausing at site *a* contributes to the co-translational targeting of SRα, we repeated this experiment using translation reactions of the non-pausing SRαNP mRNA supplemented with microsomes. As predicted, the pause sites observed matched those in Figure 3A, including a prominent toeprint band at site *b* and a fainter band near the mutated sequence of site *a* (Figure 4, lanes 3 and 4). However, rpfs isolated from membrane-bound ribosomes on the SRαNP mRNA produced significant toeprint bands only 3′ of pause site *b* (Figure 4, lane 4) compared with 3′ of pause site *a* on the SRαN mRNA (Figure 4, lane 2). Also, rpfs from cytoplasmic ribosomes on the SRαNP mRNA produced a considerably stronger toeprint band at pause site *b* compared with rpfs from membrane-bound ribosomes (Figure 4, compare lanes 3 and 4). In contrast, free and membrane-bound ribosomes on the SRαN mRNA

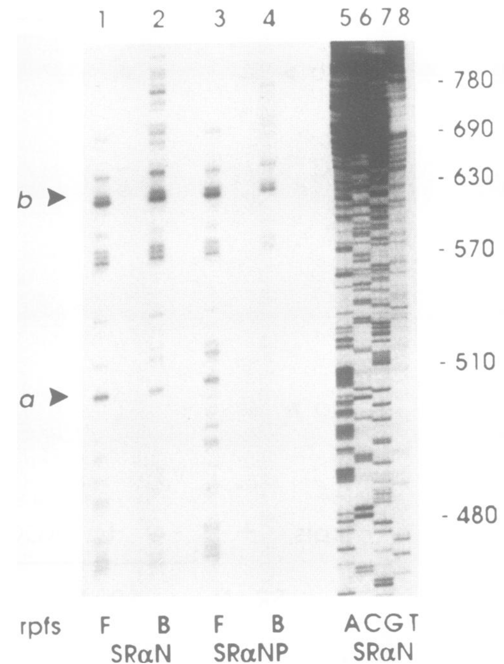

Fig. 4. Pausing contributes to targeting of SRα nascent chains. Rpfs from free cytoplasmic and membrane-bound ribosomes were isolated from RRL translation reactions of SRαN (lanes 1 and 2) or SRαNP (lanes 3 and 4) supplemented with CRMs. Primer extension assays were performed in the presence of rpfs from the free (lanes 1 and 3, marked F) and membrane-bound (lanes 2 and 4, marked B) ribosome fractions. A dideoxy sequencing ladder of SRαN using the same primer was used as size markers (lanes 5–8). Pause sites *a* and *b* on the SRαN mRNA are marked.

produced roughly equal bands at pause site *b* (Figure 4, compare lanes 1 and 2). Therefore, ribosomes translating the non-pausing SRαNP mRNA bind to membranes after they are 3′ of pause site *b*, while ribosomes translating SRαN mRNA bind to membranes 3′ of pause site *a*. This suggests that a significantly longer nascent polypeptide is required to bind ribosomes translating SRαNP mRNA onto the membrane than for ribosomes translating SRαN mRNA. Therefore, nascent polypeptides synthesized from the non-pausing SRαNP mRNA are temporally impaired in co-translational membrane assembly. Since the polypeptide sequence encoded by the SRαNP and SRαN mRNAs is identical, these results suggest that the translation pause at site *a* directly contributes to co-translational membrane binding of SRα.

Membrane binding of polysomes translating SRα

Polysomes bound to the membrane by nascent SRα polypeptides should contain ribosomes at various stages of translation. As shown in Figure 4, many ribosomes 5′ of the pause sites are not directly associated with the membrane. Therefore, it was predicted that some of these ribosomes would be tethered to the ER membrane via the mRNA attached to membrane-bound ribosomes at later stages of translation. However, the relatively small population of such tethered ribosomes is difficult to detect in unfractionated translation reactions. To identify these ribosomes, RRL translation reactions of SRαN supplemented with microsomes were terminated at steady state with cycloheximide and the membranes were isolated.

Membrane-bound polysomes were then digested with nuclease, fractionated by centrifugation as above, and the rpfs from both membrane-bound and nuclease-released monosomes were isolated and mapped by primer extension.

Toeprint bands in the region bounded by the two strong pause sites (Figure 5A, marked *a* and *b*) were examined. Consistent with the prediction that some ribosomes are tethered to the membrane by the mRNA, a number of toeprint bands were produced by rpfs from nuclease-released ribosomes (Figure 5A, lane 1). Moreover, toeprint bands produced by rpfs from membrane-bound ribosomes increased in intensity 3′ of pause site *a* (Figure 5A, lane 2). At pause site *b*, the primer extension bands revealed roughly equal populations of nuclease-released and membrane-bound ribosomes, and 3′ of pause site *b* comparatively more membrane-bound ribosomes were detected (Figure 5A, compare lanes 1 and 2). Thus, as expected, the distribution of paused ribosomes is similar to that observed in Figure 4. Furthermore, the selective nuclease release of ribosomes 5′ of the strong pause sites suggested that entire polysomes are bound to membranes by targeted nascent SRα polypeptides.

To investigate this possibility, polysomes translating SRαN were assayed for binding onto microsomal membranes. If polysomes are bound to the membrane primarily by the nascent polypeptides, then the impaired membrane binding of SRαNP polypeptides should result in reduced membrane binding of polysomes translating the non-pausing SRαNP mRNA. In addition, preventing new synthesis of SRαN with the initiation inhibitors 7-methylguanosine-5′-phosphate (7mG) and aurintricarboxylic acid (ATA) should prevent membrane binding of polysomes. Cytoplasmic and membrane-bound polysomes were isolated from RRL translation reactions of SRαN or SRαNP terminated at steady state with cycloheximide or incubated prior to initiation with 7mG and ATA. To reveal the location of the ribosomes, the polysomal [α-^{35}S]UTP-labelled mRNA was isolated and analysed by electrophoresis.

As expected, after adding cycloheximide a large proportion, 56%, of the SRαN polysomal mRNA was recovered from the membrane-bound polysome fraction (Figure 5B). Also as predicted, the proportion of membrane-bound non-pausing SRαNP mRNA in cycloheximide-terminated translation reactions, 28%, was significantly lower (Figure 5B). Furthermore, the proportion of SRαN mRNA in the membrane-bound fraction was reduced to 31% when 7mG and ATA were used to prevent translation initiation (Figure 5B). In control assays without membranes, 21% of the SRαN mRNA was recovered after the free cytoplasmic fraction was removed (Figure 5B), and may result from uncharacterized interactions with components of the translation system. In additional control assays using microsome-supplemented translation reactions of chimpanzee α-globin, a protein not expected to interact with membranes, 24% of the mRNA was recovered in the membrane-bound fraction (Figure 5B). Therefore, this amount of mRNA represents the membrane-independent background in this assay. These results confirm that the association of the nascent SRα polypeptides with the ribosomes and with the ER membrane is largely responsible for the membrane binding of the polysomes. In addition, the delay

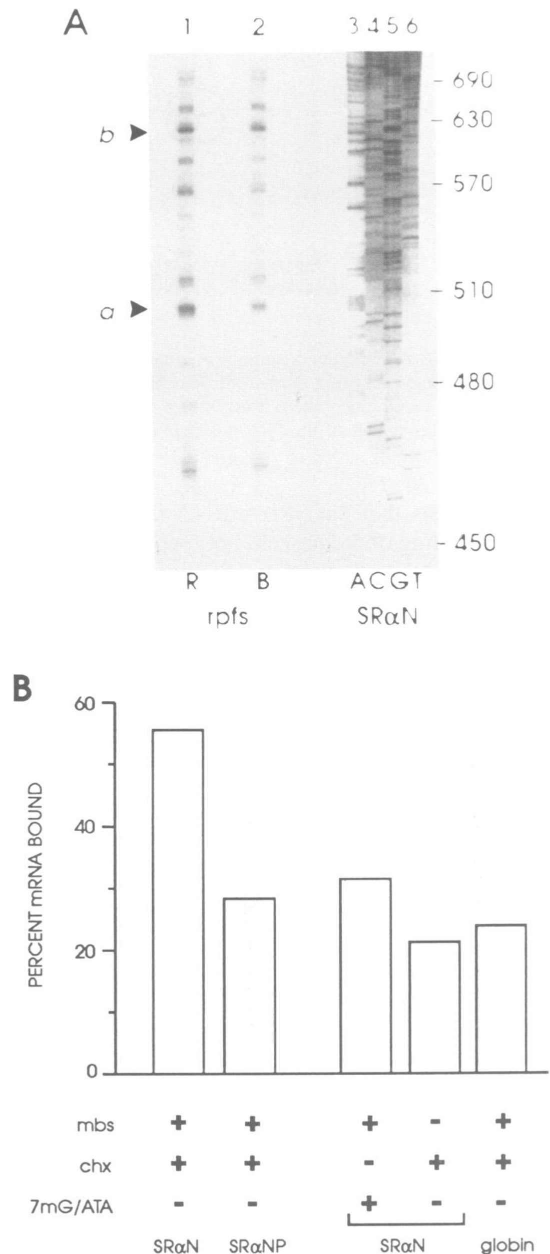

Fig. 5. Anchoring of polysomes via nascent SRα chains. (**A**) Membrane-bound polysomes were digested with nuclease, and rpfs were isolated from monosomes released from membranes by the nuclease and from membrane-bound monosomes. Primer extension assays were performed in the presence of rpfs from the nuclease-released (lane 1, marked R) and membrane-bound (lane 2, marked B) monosome fractions. A dideoxy sequencing ladder using the same primer was used as size markers (lanes 3–6). Pause sites *a* and *b* are marked. (**B**) [α-^{35}S]UTP-labelled mRNA from cytoplasmic and membrane-bound polysomes was isolated from cycloheximide (marked chx) terminated RRL translation reactions of SRαN, SRαNP or globin supplemented with CRMs (marked mbs). One set of translation reactions of SRαN were incubated with 7mG and ATA, and another set of translation reactions were performed in the absence of microsomes. Isolated mRNA was analysed by electrophoresis and quantified using a PhosphorImager. The percentage of membrane-bound mRNA was calculated using the sum of free and bound populations as the total.

observed in membrane binding of ribosomes translating SRαNP mRNA is reflected in the total membrane binding of the polysomes. Taken together with the results of Figure

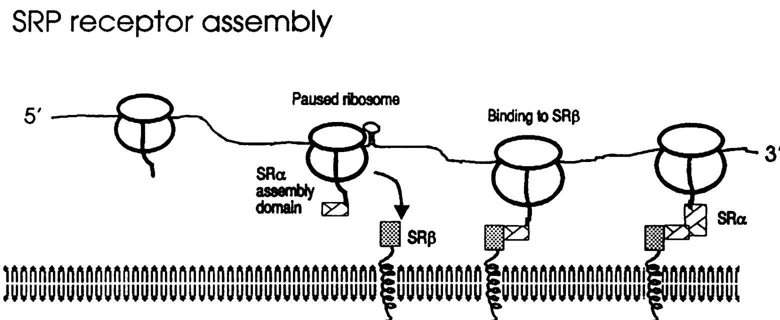

Fig. 6. Model of SRα membrane assembly. A ribosome synthesizing SRα reaches a translation pause site on the mRNA. At this point, the N-terminal membrane-anchoring domain of SRα has been synthesized and has emerged from the ribosome. The anchoring domain folds and is targeted to the ER membrane, where it assembles onto SRβ. Other proteins may participate in the assembly reaction, but are omitted for clarity. Translation of the remainder of the SRα polypeptide resumes. Some ribosomes translating sequences 5′ of the pause sites are tethered to the membrane by the mRNA.

5A, this suggests that the SRα mRNA is targeted by the membrane binding of the nascent polypeptides synthesized on ribosomes rather than by a specific mRNA targeting mechanism.

Discussion

The model of SRα targeting suggested by our data is depicted in Figure 6. As the N-terminal anchoring domain of SRα emerges from the ribosome, translation is paused at site *a* on the mRNA (Figure 1B). At this stage, membrane targeting of nascent SRα is initiated (Figure 4), most likely by folding of the nascent polypeptide into a conformation capable of assembling onto the SRβ subunit on the ER membrane (Young *et al.*, 1995). While our data do not rule out interactions between the nascent SRα polypeptide and other membrane proteins, our previous data suggest that the interaction between SRα and SRβ is sufficient for membrane binding of SRα (Young *et al.*, 1995). As synthesis of the SRα polypeptide continues beyond the pause site, the nascent chains remain membrane bound and thereby attach the polysome to the membrane surface (Figure 5A and B). The novel aspect of this model is the connection between the translation pause and the membrane assembly event, suggested by the impaired membrane binding of the nascent polypeptide (Figure 4) and of polysomes (Figure 5B) when the level of translation pausing was reduced (using SRαNP mRNA). Previously, ribosome pausing has been detected in connection with other regulatory events during translation, including initiation, termination, recognition of signal sequences by SRP and frameshifting (Wolin and Walter, 1988; Somogyi *et al.*, 1993). Our data suggest a functional role for the regulation of protein synthesis by translation pausing, in folding and/or targeting of nascent proteins.

The resemblance between pause site *a* and a class of frameshift sites suggests that the mechanism of frameshifting on the viral mRNAs is related to or even adapted from a general mechanism for translation pausing. However, since the primer extension toeprint band lies within the heptamer-like sequence on the SRα mRNA (Figure 1B and Figure 3A, insert), ribosomes paused at this site appear to have already passed over at least part of the heptamer-like sequence. On the other hand, the presence of an mRNA stem–loop structure alone has been shown not to affect the rate of polypeptide elongation (Lingelbach and Dobberstein, 1988). Therefore, the exact mechanism of pausing at this site remains to be determined.

How does ribosome pausing facilitate the co-translational targeting of SRα? Evidence has been published that a nascent polypeptide can acquire secondary and even tertiary structure as it emerges from the ribosome (Krashenninikov *et al.*, 1991; Crombie *et al.*, 1992), in some cases with the aid of heat shock proteins [Frydman *et al.*, 1994; reviewed in Gething and Sambrook (1992) and Hartl *et al.* (1994)]. Since membrane assembly of SRα appears to require an independently folded domain (Young *et al.*, 1995), the co-translational targeting of the molecule demonstrated here indicates that the membrane-anchoring domain folds soon after synthesis. Therefore, the delayed co-translational targeting of non-pausing SRαNP polypeptides may be due to a reduction in the efficiency of protein folding. For example, in the absence of a translation pause at site *a* on the SRα mRNA, polypeptide sequences C-terminal to the anchoring domain may interfere with folding. However, misfolded polypeptides synthesized from the non-pausing mRNA would probably be folded into the proper final conformation by chaperone proteins over time and would then assemble onto membranes later in translation, as observed in Figure 4, or post-translationally. We therefore hypothesize that translation pausing facilitates or complements the action of protein chaperones in the co-translational folding of SRα and perhaps of other proteins. Similarly, SRP-mediated translation pausing increases the efficiency of, but is not essential for, the transition from free to membrane-bound ribosomes (Siegel and Walter, 1986).

Although ribosome pausing may contribute in general to polypeptide folding, we predict a more specific role for pausing in co-translational targeting events. In addition to secretory proteins, certain cytoskeletal proteins have been reported to target co-translationally. Myosin heavy chain, titin and in certain cases vimentin can be assembled onto the appropriate cytoskeletal networks during translation *in vivo* (Isaacs and Fulton, 1987; Isaacs *et al.*, 1989a,b). The appearance of nascent polypeptides of discrete length during the synthesis of these cytoskeletal proteins (Isaacs and Fulton, 1987; Isaacs *et al.*, 1989a) suggests that translation pausing may be occurring at specific sites on the mRNAs encoding these polypeptides. The co-ordination between ribosome pausing and co-translational assembly we have demonstrated for SRα may also con-

tribute to the targeting of one or more of these proteins. Indeed, translation pausing may be a general mechanism for regulating the folding and/or targeting of a variety of nascent polypeptides.

Materials and methods

Materials and general methods

General chemical reagents were obtained from either Fisher Scientific, Sigma Chemicals or Gibco BRL. SURE™ *E.coli* cells used for plasmid construction and the preparation of single-stranded DNA were purchased from Stratagene. Unless otherwise stated, restriction enzymes and other molecular biology enzymes and reagents were from New England Biolabs. ^{35}S-Labelled methionine and UTP were from Dupont-NEN. SP6 polymerase was purchased from Epicentre Technologies. Creatine kinase, cycloheximide and staphylococcal nuclease were from Boehringer Mannheim. RNAguard (an RNase inhibitor) and V1 nuclease were from Pharmacia.

Plasmid construction and sequencing, electrophoresis of RNA and single-stranded DNA preparation using M13 R408 helper phage were accomplished using standard techniques (Sambrook *et al.*, 1989). Transcription reactions with SP6 polymerase were performed as previously described (Gurevich *et al.*, 1991). To radiolabel mRNA, the reactions were supplemented with [α-^{35}S]UTP at 12.5 μCi/10 μl reaction. RRL was prepared and transcription-linked cell-free translation reactions were performed as published previously (Jackson and Hunt, 1983; Perara and Lingappa, 1985). Canine pancreatic rough microsomes were prepared as described and further purified by Sepharose CL-2B gel exclusion chromatography (CRMs) or extracted with 0.5 M KOAc (KRMs) (Walter and Blobel, 1983). The RNAfold program (Scientific & Educational Software, State Line, PA), based on the methods of Freier *et al.* (1986), was used for predictions of RNA secondary structure.

Plasmids

Plasmid pMAC191 has been previously reported, and encodes a point mutant of SRα termed SRαN (Falcone and Andrews, 1991). The C→G substitution at nucleotide 4 of the coding region increases the translation efficiency of the polypeptide in the cell-free system, but the resulting Leu2→Val mutation does not affect the membrane targeting, translocation activity or translation pausing of SRαN compared with wild-type SRα [data not shown and Young *et al.* (1995)]. All nucleotides within the SRα coding region are numbered relative to the initial AUG codon rather than the start of the reported cDNA sequence (Lauffer *et al.*, 1985).

Plasmid pMAC623 encodes a globin–protein A fusion protein (Janiak *et al.*, 1994) with a pause site from SRα inserted between the chimpanzee α-globin and *S.aureus* protein A domains (gSRpa-1). To assemble this plasmid, a synthetic oligonucleotide linker containing *Pst*I and *Sac*I sites was ligated between the *Bgl*II and *Nco*I sites 5′ of the coding region of the protein A Fc-binding domain. DNA coding for a fragment of globin was then inserted at the *Bgl*II site. This allowed a synthetic oligonucleotide linker coding for amino acids 160–183 of SRα (nucleotides 478–530) to be inserted between the *Pst*I and *Sac*I sites without altering the reading frame of the fusion protein. The coding region of gSRpa-1 followed an SP6 RNA polymerase promoter and the pSPUTK 5′ untranslated sequence (Falcone and Andrews, 1991).

Plasmid pMAC714 contains the sequence encoding SRαN with point mutations at nucleotides 504 (A→G), 507 (A→G), 510 (G→A), 513 (G→C) and 516 (C→G) of the coding region. The mutations were designed to alter the mRNA structure at a translation pause site without changing the polypeptide sequence. For clarity, the protein encoded by the mutant mRNA is termed SRαNP. To construct plasmid pMAC714, a fragment of DNA was amplified from plasmid pMAC191 by the polymerase chain reaction using an oligonucleotide primer with the sequence 5′-GCCATCAGAGCTCTCCTTCTTCTCGCCTTTCTTCTT-GCTG and another primer complementary to the SP6 promoter. The amplified fragment was cleaved at a *Hin*dIII site 3′ of the SP6 promoter and at a *Sac*I site 3′ of the introduced mutations. The fragment was inserted into the corresponding *Hin*dIII and *Sac*I sites of plasmid pMAC191.

To obtain single-stranded DNA for primer extension assays, sequences between the *Nhe*I and *Eco*RI sites of plasmid pMAC191 and pMAC623 were ligated into the *Xba*I and *Eco*RI sites of the plasmid pGC1 containing an M13 origin of replication (Myers *et al.*, 1985). The resulting plasmids, pMAC573 and pMAC646, contain the SP6 RNA polymerase promoter, the 5′ untranslated sequence and the complete coding regions of SRαN and gSRpa-1, respectively.

The plasmid vector pSPUTKf(–) was constructed by adding an f1 origin of replication to the plasmid pSPUTK (Falcone and Andrews, 1991). To assemble this plasmid, an *Nde*I linker was inserted into the *Hpa*I site of pSPUTK, so that the T7 promoter was flanked by the *Nde*I site and an *Eco*RI site in the polylinker. A DNA fragment containing an f1 origin and T7 promoter was excised from pGEM3Zf(–) (Promega) between the *Nde*I and *Eco*RI sites, and ligated into the corresponding sites in the modified pSPUTK to produce pSPUTKf(–). To insert the coding region of SRαNP into pSPUTKf(–), the DNA sequence of pMAC714 between the *Nco*I sites (at nucleotides –1 and 1505) was inserted into the *Nco*I site of pSPUTKf(–). This plasmid was cleaved at *Sac*I sites 3′ of the silent mutations and in the polylinker 3′ of the stop codon, and the corresponding region of plasmid pMAC191 (encoding SRαN) digested at similarly positioned *Sac*I sites was inserted. The resulting plasmid pMAC745 contains the 5′ untranslated region and coding sequence of SRαNP in pSPUTKf(–).

Plasmid pMAC35 coding for chimpanzee α-globin was as published (Andrews *et al.*, 1989).

Isolation and mapping of rpfs

A published procedure for identifying ribosomal pause sites (Wolin and Walter, 1989) was adapted as follows. To obtain monosomes from 25 μl RRL cell-free translation reactions terminated at steady state (25 min for SRαN and SRαNP, 15 min for gSRpa-1), reactions were digested with staphylococcal nuclease at a final enzyme concentration of 0.75 U/μl. Digestion was terminated by adding EGTA to 5 mM and ribonucleoside–vanadyl complex to 10 mM. Total rpfs from translations in the absence of microsomes were obtained as published (Wolin and Walter, 1989). To obtain membrane-bound rpfs, 25 μl translations containing 2.5 equivalents of CRMs were digested with nuclease as above. The reactions were adjusted to 150 mM KOAc and cytoplasmic monosomes were isolated as published (Wolin and Walter, 1993), or by centrifugation in a TLA-100 rotor at 100 000 r.p.m. (436 000 *g*) for 90 min. The microsomal fraction (120 μl supernatant) was solubilized by the addition of 6 μl of 20% CHAPS (final concentration 1% detergent). Solubilized monosomes were pelleted through a 60 μl 1.8 M sucrose step containing 1% CHAPS, and rpfs were isolated from both monosome pellets as described (Wolin and Walter, 1993). Cytoplasmic and membrane-bound polysomal mRNA were obtained by the same procedure, but with the omission of the nuclease digestion. To abolish translation, the RRL translation reactions were pre-incubated with 4 mM 7mG and 0.1 mM ATA. Isolated rpfs and polysomal mRNA were resuspended in 10 μl of sterile water following precipitation with ethanol.

To analyse the size of the rpfs, radiolabelled transcripts were added to the cell-free translation reactions and rpfs were isolated as above. One microlitre of rpfs was added to 9 μl of deionized formamide and analysed by electrophoresis on denaturing 8 M urea–10% acrylamide gels in TBE buffer (89 mM Tris, 89 mM boric acid and 2 mM EDTA) and visualized by autoradiography. To determine the distribution of rpfs on the original mRNA, aliquots of rpfs were annealed to a single-stranded DNA template and mapped by primer extension using a modification of the published procedure (Wolin and Walter, 1989). A mixture containing 2 μl of 10× T4 polymerase buffer (New England Biolabs), 2 μg acetylated bovine serum albumin, 50 ng of single-stranded DNA, 0.5 ng of a 5′-^{32}P-end-labelled primer, 1 μl of rpfs (unless otherwise indicated) and water for a final volume of 17 μl was heated to 65°C for 5 min and allowed to cool to 42°C on the benchtop. Two microlitres of a solution containing 5 mM each of dATP, dCTP, dGTP and dTTP were added followed by 3 U (1 μl) of T4 DNA polymerase (New England Biolabs). The reaction was incubated at 37°C for 30 min, nucleic acids were precipitated with ethanol and the primer extension products analysed on sequencing gels as previously described (Wolin and Walter, 1989). In comparisons of pausing on the SRαN and SRαNP mRNA, approximately equal amounts of rpfs (determined by electrophoresis) were used in the primer extension assays. The toeprint band at pause site *b* was used to control for differences in loading or labelling of the primer extension reactions.

Preparation of rpfs from nuclease-released monosomes

A 100 μl translation reaction supplemented with 10 equivalents of KRMs was terminated by adding cycloheximide to 1 mM and then mixed with 25 μl of 2.5 M KOAc, 20 mM HEPES–KOH (pH 7.5), 10 mM $MgOAc_2$ and 2 mM dithiothreitol (DTT). This was layered onto 75 μl of BK [500 mM KOAc, 20 mM HEPES–KOH (pH 7.5), 10 mM $MgOAc_2$ and 2 mM DTT] containing 1.8 M sucrose and fractionated by centrifugation

in a Beckman Instruments TLA-100 rotor at 100 000 r.p.m. (436 000 *g*) for 90 min. The top 160 μl (containing membrane-bound polysomes) were removed and divided into 35 μl aliquots. An aliquot was mixed with 5 μl of BK containing 25 mM $CaCl_2$, and digested with staphylococcal nuclease at 0.5 U/μl for 30 min at 24°C. Digestion was terminated by adding ribonucleoside–vanadyl complex to 10 mM and 10 μl of BK containing 25 mM EGTA. The mixture was layered onto a 100 μl step gradient of BK containing 1.8 M sucrose and 5 mM EGTA, and fractionated by centrifugation in a TLA-100 rotor at 100 000 r.p.m. (436 000 *g*) for 90 min. The top 120 μl (containing membrane-bound monosomes) were removed and solubilized with CHAPS as above. Nuclease-released monosomes in the pellet were resuspended in the remaining 30 μl of the cushion and rpfs were extracted as above. The membrane-bound monosomes were then pelleted and the rpfs isolated as above.

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